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ORIGINAL ARTICLE

Diversity and spatial distribution of *amoA*-encoding archaea in the deep-sea sediments of the tropical West Pacific Continental Margin

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Abstract

Aims: The ecological characteristics of the deep-sea *amoA*-encoding archaea (AEA) are largely unsolved. Our aim was to study the diversity, structure and distribution of the AEA community in the sediments of the tropical West Pacific Continental Margin, to develop a general view of the AEA biogeography in the deep-sea extreme environment.

Methods and Results: Archaeal *amoA* clone libraries were constructed. Diverse and novel *amoA* sequences were identified, with the Bohol Sea, Bashi Strait and Sibuyan Sea harbouring the highest and the Bicol Shelf the lowest AEA diversity. Phylogenetic and statistical analyses illustrate a heterogeneous distribution of the AEA community, probably caused by the differential distribution of the terrestrial or estuarine AEA in the various sampling sites.

Conclusions: The deep-sea sedimentary environments potentially harbour diverse and novel AEA in the tropical West Pacific Continental Margin. The stations in the Philippine inland seas (including station 3043) may represent AEA assemblages with various terrestrial influences and the stations connected directly to the open Philippine Sea may represent marine environment-dominant AEA assemblages.

Significance and Impact of Study: Our study indicates the potential importance of geological and climatic events in the transport of terrestrial micro-organisms to the deep-sea sedimentary environments, almost totally neglected previously.

Introduction

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 N cycle. Over geological time scales, the N cycle is thought to have affected the C cycle and hence the climate (Falkowski 1997; Gruber and Galloway 2008). Microbial nitrification may be responsible for the formation of the large deep-sea nitrate reservoir (Lam *et al.* 2007). The coupling of nitrification to denitrification or the anammox (anaerobic oxidation of ammonium) processes may contribute to bioremediation of eutrophication in estuarine and coastal systems (Seitzinger 1988; Coolen *et al.* 2007; Lam *et al.* 2007), where high input of anthropogenic active N compounds is often experienced (Francis *et al.* 2005; Caffrey *et al.* 2007). Microbial nitrifiers may also co-oxidize a variety of xenobiotic compounds (Kowalchuk and Stephen 2001; Arp and Stein 2003), thereby providing a detoxification process of excess ammonia and other toxic compounds in environment.

Similar to ammonia-oxidizing bacteria (AOB), ammonia-oxidizing archaea (AOA) in the mesophilic *Crenarchaea* group catalyse aerobic oxidation of ammonia (Könneke *et al.* 2005), the first and likely rate-limiting step of nitrification. The archaeal ammonia monooxygenase gene, *amoA*, seems to be abundant in the oceans (Francis *et al.* 2005; Beman and Francis 2006; Beman *et al.* 2007, 2008; Lam *et al.* 2007; Nakagawa *et al.* 2007;

Dang *et al.* 2008b; Park *et al.* 2008; Sahan and Muyzer 2008; Santoro *et al.* 2008). In addition, these '*amoA*-encoding archaea' (AEA) have been suggested to contribute to N cycling in a variety of environments including soils, hot springs, subsurface, caves, estuaries, seawater, sediments, hydrothermal vents, biofiltrations systems and wastewater bioreactors (Nicol and Schleper 2006; Cavicchioli *et al.* 2007; Francis *et al.* 2007; and references herein). Quantitatively, AEA appear to be more abundant than AOB (Wuchter *et al.* 2006; Mincer *et al.* 2007; Nakagawa *et al.* 2007), further suggesting their ecological importance.

Most of the oceanic sedimentary mineralization occurs over the continental margin, one of the most important boundaries on Earth (Derry and Murray 2004). Although it occupies a relatively small fraction of the ocean floor, the continental margin is very important in marine biogeochemistry (Walsh 1991), affecting the ocean chemistry, productivity and climate. The deep-sea sediments of the continental margin may provide an extreme environment due to its permanent low temperature (approx. 2°C) and high hydraulic pressure. However, most of the marine N cycling studies focused only on the estuarine and coastal areas. Data are not currently available for most of the deep marginal sea sedimentary environments about the newly discovered AEA communities (Nakagawa *et al.* 2007). The composition and transformation rate of the deep-sea nitrogenous compounds may be different from those of the estuarine and coastal areas, probably caused by the change of the nitrogen sources, the influence of the prevailing extreme environment and the shift of the microbial community. A recent study showed that the deep-sea cold seep pelagic brown sediments at the northeastern Japan Sea harboured distinct AEA not seen in other environments (Nakagawa *et al.* 2007). It is not known whether this unique sedimentary AEA assemblage is a local feature of the studied cold seep environment or a common feature of the rarely explored whole deep-sea environment.

Philippine Sea is a marginal sea of the West Pacific, where Kuroshio Current, the largest western boundary current of the Pacific Ocean originates. This strong, warm and saline current may have a significant impact on the regional marine ecosystem, fishery and climate (Lee *et al.* 2004). The bordering Philippine archipelago is located on an active convergent plate boundary between the Eurasian and Philippine Sea plates. It is also a part of the 'Pacific Ring of Fire' and the 'Pacific Seismic Rim', characterized by frequent active volcanoes and earthquakes (Ozawa *et al.* 2004). Astride the typhoon belt, the Philippine archipelago is affected frequently by heavy rains, tropical storms and typhoons (Lansigan *et al.* 2000). These tectonic and climatic factors may further trigger other disasters, such as tsunamis, floods and landslides (Liu *et al.* 2007). Most of these events may deliver tremendous amount of terrestrial materials into the ocean. We hypothesize that these events may also intensify the transport of terrestrial microbes into the deep-sea environment.

The current paper used the functional marker gene *amoA* to study the diversity, structure and distribution of the *amoA*-encoding archaea community in the marine sediments collected from the tropical West Pacific Continental Margin, to test our hypothesis and to develop a general view of the biogeography of the deep-sea AEA community.

Materials and methods

Samples collection and environmental factors measurement

Deep-sea sediments were sampled using CASQ core samplers during the Chinese-French joint MD155/MARCO POLO 2/IMAGES XIV cruise of the R/V Marion-Dufresne in the tropical western Pacific from June 11 to July 6, 2006. The sampling sites included several featured marginal seas and other geographical locations as detailed in Table 1 and Fig. S1.

The CASQ box core sampler has a 25×25 cm^2 section area and a maximum sediment retrieval depth of 12 m below the seafloor. On board, the core was opened from the side and sub-samples for microbial study were taken from the center of the core under sterile conditions and stored in airtight sterile plastic bags in a −80°C freezer. Usually, the topmost sediment was disturbed inside the core sampler, so intact sediments at depth 5 or 10 cm were collected instead of the very surface sediments. The choice of this 'surface' layer may also avoid microbial contamination from the overlaying seawater above the seafloor.

Sediment total organic carbon (OrgC) and nitrogen (OrgN) contents were measured with a PE 2400 Series II CHNS/O elemental analyzer (Perkin Elmer, Norwalk, CT, USA). A Cilas 940L laser granulometer (Company Industrielle des Lasers, Orléans, France) was used for sediment grain size analysis and sorting coefficient, median grain size, mean grain size, skewness and kurtosis were calculated (Table 1).

DNA extraction and archaeal *amoA* clone library analyses

Sediment DNA extractions followed our previous method (Dang *et al.* 2008b). Archaeal *amoA* fragments (approx. 635 bp) were amplified with primers Arch-amoAF and

Table 1 Sampling stations and environmental parameters of the deep-sea sediments collected from the tropical West Pacific Continental Margin

	Station							
Environmental factor	3043	3047	3048	3053	3057	3059	3063	3064
Site location	Bashi Strait	Benham Rise	Benham Rise	Bicol Shelf	Sibuyan Sea	Cagayan Ridge	Bohol Sea	Mindanao
Latitude (N)	19°05·50′	17°00·44′	16°34·82′	14°30·27′	13°00·15′	9°40·57′	8°28·60′	6°28·23′
Longitude (E)	122°24·21′	124°47·93′	124°39·20′	124°19·23′	122°22·22′	121°38·95′	124°37·31′	126°27·82′
Water depth (m)	2135	2510	2495	2065	1666	3520	1811	1390
CASQ core designation	MD06-3043	MD06-3047	MD06-3048	MD06-3053	MD06-3057	MD06-3059	MD06-3063	MD06-3064
Sediment								
OrgC (total organic C, %)	0·829	0·266	0·321	1·936	3·129	0·815	2·062	0·922
OrgN (total organic N, %)	0·056	0·026	0·031	0·206	0·317	0·077	0·128	0·071
OrgC/OrgN	14·804	10·231	10·355	9·398	9·871	10·584	16·109	12·986
Sand (%)	0·08	0	0	0	0	0	0	0
Silt (%)	67·76	56·40	55·75	62·55	49·53	51·01	61·03	61·09
Clay (%)	32·15	43·60	44·25	37·45	50·47	48·99	38·97	38·91
Median grain size (ø)	6·705	7·566	7·610	7·071	8·025	7·940	7·275	7·198
Mean grain size (ø)	7·032	7·649	7·659	7·299	7·886	7·819	7·498	7·386
Sorting coefficient	1·749	1·585	1·595	1·704	1·527	1·568	1·573	1·677
Skewness	1·146	−0·158	−0·528	0·908	−0·970	−0·913	0·826	0·782
Kurtosis	2·054	1·855	1·871	1·976	1·841	1·863	1·849	1·950
Sampling depth (cmbsf*)	10	10	10	5	5	5	5	5

*cmbsf, centimeters below the sea floor.

Arch-amoAR (Francis *et al.* 2005). PCR products cloning followed our previous procedure (Dang *et al.* 2008b). A miniprep method was used for recombinant plasmid preparations (Dang and Lovell 2000). Cloned *amoA* fragments were reamplified using the vector primers M13-D and RV-M (Dang *et al.* 2008a). Amplicons with correct size were digested with the restriction enzymes *Hha* I, *Msp* I and *Taq* I (Fermentas, Glen Burnie, MD, USA) respectively. Restriction fragments were resolved by 4% agarose gel electrophoreses in 0·5× TBE, and digitally photographed with an ImageMaster VDS imaging system (Pharmacia Biotech, Piscataway, NJ, USA). Restriction fragment length polymorphism (RFLP) analysis was used to identify redundant clones.

Clone vector primer RV-M was used for sequencing with an ABI 3770 sequencer (Applied BioSystems, Foster City, CA, USA). The resultant DNA sequences were grouped into operational taxonomic units (OTUs) based on a 5% sequence distance cutoff calculated with the DOTUR program (Schloss and Handelsman 2005), to facilitate comparison with other studies (Francis *et al.* 2005; Beman and Francis 2006; Park *et al.* 2006; Beman *et al.* 2007; Dang *et al.* 2008b). The putative *amoA* sequences were translated into conceptual protein sequences and the BLAST program was used for retrieval of the closest match GenBank sequences (Altschul *et al.* 1997). The AmoA protein sequences were aligned using the CLUSTALX program (Larkin *et al.* 2007) and a phylogenetic tree was constructed using the PHYLIP PROTDIST and NEIGHBOR programs (version 3.67) (Felsenstein 1989).

Statistical analyses

The coverage of each clone library was calculated as $C = [1 - (n_1/N)] \times 100$, where, n_1 is the number of unique OTUs and N the total number of clones in a library (Dang *et al.* 2008b). Indices of *amoA* gene diversity (Shannon–Wiener H and Simpson D) and evenness (J) were calculated using the OTU data (Dang *et al.* 2008b). Rarefaction analysis and two nonparametric richness estimators, the abundance-based coverage estimator (S_{ACE}) and the bias-corrected Chao1 (S_{Chao1}), were calculated using DOTUR (Schloss and Handelsman 2005). These diversity indices and richness estimators are useful for comparing the relative complexity of communities and for estimating the completeness of sampling.

Community classification of the AEA assemblages was determined with UNIFRAC environmental clustering and principal coordinates analyses (PCoA) (Lozupone *et al.* 2007). The UNIFRAC program (http://bmf.colorado.edu/unifrac/index.psp, last access: 24 July 2008) takes the molecular evolutionary distances of the sequences for community similarity analyses, particularly suitable for sequence data. Correlations between the AEA assemblages and environmental factors were analysed with canonical correspondence analysis (CCA) following previous

procedures using the software CANOCO (ver. 4.5, Microcomputer Power, Ithaca, NY, USA) (ter Braak and Šmilauer 2002; Dang *et al.* 2008b). Automatic forward selection with significance tests of Monte Carlo permutations were used to build the optimal models of the microbe–environment relationship. These multivariate statistical methods cope with major issues in microbial ecology, such as the distribution and classification of the microbial community in respect to environmental variables or their gradients (Lozupone *et al.* 2007; Ramette 2007; Magalhães *et al.* 2008).

Nucleotide sequence accession numbers

The archaeal *amoA* gene sequences determined in this study have been deposited in GenBank under accession numbers EU885546 to EU885712.

Results

Diversity of the deep-sea archaeal *amoA* genes

Of the eight libraries constructed, 735 clones were identified to contain a valid archaeal *amoA* gene fragment, resulting in a final of 167 unique sequences and 83 OTUs (Table 2). The values of the library coverage (*C*) ranged from 38·7% to 75·0% (Table 2), together with rarefaction analysis (Fig. S2), indicating that most of the libraries had quite high *amoA* gene diversity. The sampling stations 3063, 3043 and 3057 had the highest and the station 3053 had the lowest *amoA* diversity, based on the majority of the diversity indices (*H*, 1/*D* and *J*) and the S_{ACE} and S_{Chao1} richness estimators (Table 2). These data indicate a heterogeneous distribution of the deep-sea sedimentary AEA community in the tropical West Pacific Continental Margin, with the Bohol Sea, Bashi Strait and Sibuyan Sea sediments harbouring the highest and the Bicol Shelf sediments harbouring the lowest AEA diversity.

Phylogeny of the archaeal AmoA sequences

The obtained 167 distinct archaeal *amoA* sequences had 67·6–99·8% identities among each other and 83·8–99·5% identities to the closest match GenBank sequences. After translation, the corresponding AmoA sequences had 75·4–100·0% identities among each other and 93·8–100·0% identities to the closest match GenBank sequences retrieved from a variety of terrestrial, estuarine, coastal and marine environments. A majority (71·9%) of our archaeal AmoA sequences matched top hit GenBank sequences that were originally retrieved from marine or related environments including the Changjiang Estuary and its adjacent East China Sea sediments (Dang *et al.* 2008b), the Northeastern Japan Sea Hokkaido deep-sea water column or sediments (Nakagawa *et al.* 2007), the Hawaii coral reef sediments, the Southern Okinawa Trough Yonaguni Knoll IV hydrothermal sediments, the sulfides at the Clambed vent of Juan de Fuca field, the Australia Orpheus Island and Bare Island marine sponges (Steger *et al.* 2008), the Black Sea seawater (Lam *et al.* 2007) and the marine aquarium biofiltration systems (Urakawa *et al.* 2008). The remaining sequences were mainly related to terrestrial environments, including soils from China, Germany or Australia (Treusch *et al.* 2005; He *et al.* 2007), sediments of the China saline Qinghai Lake, hot spring in China Tengchong (Zhang *et al.* 2008) and anoxic granular sludge for anaerobic ammonium oxidation. Nearly one third (31·1%) of the top hit GenBank sequences were originally retrieved from the deep-sea hydrothermal vent or terrestrial hot spring environments.

Table 2 Diversity and predicted richness of the sedimentary archaea *amoA* sequences recovered from the sampling stations of the tropical West Pacific Continental Margin

Station	No. of clones	No. of *amoA* sequences*	No. of OTUs†	*C* (%)	*H*	1/*D*	*J*	S_{ACE}	S_{Chao1}	Cluster II sequences‡ (%)	Cluster II clones (%)
3043	94	39	33	54·55	4·51	19·96	0·89	51·4	44·7	65·8	51·1
3047	94	28	19	57·89	2·90	3·89	0·68	29·2	24·6	7·1	4·3
3048	94	35	23	56·52	3·55	7·31	0·78	37·3	30·5	5·7	3·2
3053	89	16	8	75·00	2·11	3·44	0·70	9·4	8·5	0·0	0·0
3057	94	34	31	38·71	4·01	10·56	0·81	67·6	59·5	54·6	26·6
3059	88	30	25	56·00	3·90	11·06	0·84	40·4	34·2	46·7	21·6
3063	92	50	37	40·54	4·57	18·28	0·88	80·7	75·5	73·5	68·5
3064	90	26	20	50·00	3·10	5·37	0·72	35·0	29·0	26·9	11·1

*Unique *amoA* sequences were determined via RFLP analysis.

†Unique OTUs of the *amoA* sequences were determined using the DOTUR program. The coverage (*C*), Shannon–Weiner (*H*), Simpson (*D*) and evenness (*J*) indices, and S_{ACE} and S_{Chao1} richness estimators were calculated using the OTUs data.

‡Cluster II sequences were determined by sequences phylogeny as the putative terrestrial or estuarine AmoA-related sequences depicted in Fig. 1.

The phylogenetic tree constructed using the 154 distinct AmoA sequences showed that two sequence clusters, with 20% between-cluster distance determined via DOTUR, could be identified (Fig. 1). The Cluster I AmoA sequences were mainly associated with marine sediment, seawater or the related environments, such as the Northeastern Japan Sea Hokkaido deep-sea water column or sediments, Southern Okinawa Trough Yonaguni Knoll IV hydrothermal sediments, Hawaii coral reef sediments, East China Sea and occasionally the Changjiang Estuary sediments, Juan de Fuca Clambed deep-sea hydrothermal vent sulfide, Black Sea seawater, Australia Bare Island and Orpheus Island marine sponges and marine aquarium biofiltration systems. The Cluster II AmoA sequences were mainly related to terrestrial or estuarine environments, such as soils from China, Germany and Australia, China saline Qinghai Lake sediments, China Tengchong hot spring, Changjiang Estuary and occasionally East China Sea sediments and anoxic granular sludge. Cluster I and II included almost equal number of AmoA sequences (50·6% *vs* 49·4%). However, Cluster I had much more AmoA clones than Cluster II (76·6% *vs* 23·4%). Most AmoA sequences of the stations 3047, 3048 and 3053 (92·9%, 94·3% and 100·0%, respectively) were affiliated within Cluster I and they accounted for 95·7%, 96·8% and 100·0% of the clones in each corresponding library. Most AmoA sequences of the stations 3063, 3043 and 3057 (73·5%, 65·8% and 54·6%, respectively) were affiliated within Cluster II and they accounted for 68·5%, 51·1% and 26·6% of the clones in each corresponding library (Table 2). Our data indicate that the Benham Rise and Bicol Shelf sampling sites may represent marine environment-dominated AEA assemblages, while the Bohol Sea, Bashi Strait and Sibuyan Sea sampling sites may represent continental margin AEA assemblages with various degrees of terrestrial impact.

Four AmoA sequences, 3043-A-01, 3043-A-02, 3043-A-03 and 3047-A-01, which were related to the clones retrieved from the East China Sea sediments (Dang *et al.* 2008b), marine aquarium biofiltration systems (Urakawa *et al.* 2008) and Northeastern Japan Sea Hokkaido deep-sea sediments and water column (Nakagawa *et al.* 2007), occurred in all the sampling stations (Fig. 1). Seven other sequences, 3043-A-04, 3043-A-05, 3043-A-06, 3047-A-02, 3047-A-03, 3059-A-01 and 3064-A-01, occurred in four to six of the stations. These common sequences were also the dominant ones (50·2% of the 735 clones screened) in the respective libraries. However, most AmoA sequences (71·4%) occurred only in one of the sampling sites, indicating their heterogeneous distribution in the tropical West Pacific Continental Margin.

Our archaeal AmoA sequences can be classified into several groups based on the prevailing environmental characteristics, such as the Tropical West Pacific Continental Margin Deep-sea Sediment Group, the Deep Marine Group (Water Column B) (Nakagawa *et al.* 2007), the Deep-sea Hydrothermal Vent and Sediment Group, the Coastal and Marginal Sea Sediment Group, the Japan Sea Deep-sea Sediment A Group (Nakagawa *et al.* 2007), the China Red Soil A Group, and the Soil, Lake and Estuarine Sediment Group (Fig. 1). There are several groups or subgroups of sequences with limited similarity to known GenBank AmoA sequences, indicating uniqueness of the AEA assemblage in the studied deep-sea sedimentary environment, such as the Tropical West Pacific Continental Margin Deep-sea Sediment Group and several subgroups in the Deep-sea Hydrothermal Vent Group, the Coastal and Marginal Sea Sediment Group, the China Red Soil A Group and the Soil, Lake and Estuarine Sediment Group (Fig. 1). The environmental specificity of these sequences indicates that the tropical West Pacific Continental Margin sediments might harbour AEA without close relatives detected in other marine environments.

AEA community classification

Significant difference of the AmoA assemblages was detected via the all environments UNIFRAC significance ($P < 0{\cdot}001$) and the P test significance ($P < 0{\cdot}001$) analyses (Lozupone *et al.* 2007). Both pairwise UNIFRAC and P test significance analyses indicated that the difference between the AEA assemblage of station 3043 or 3063 and that of station 3047, 3048, 3053 or 3064 was at least marginally significant ($P \leq 0{\cdot}028$) (Figs. S3 and S4).

The weighted UNIFRAC environmental clustering analysis indicated that the AmoA assemblages of stations 3043 and 3063 were different from all the other stations (Fig. 2). The weighted UNIFRAC PCoA analysis further confirmed this difference (Fig. 3a). Clearly, the putative terrestrial or estuarine AmoA-dominated (in the sense of

Figure 1 Phylogenetic tree constructed with distance and neighbour-joining method of the archaeal AmoA sequences recovered from the sediments of the tropical West Pacific Continental Margin. Partial archaeal AmoA sequences of 196 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 *Nitrosomonas europaea*, *Nitrosospira briensis* and *Nitrosococcus oceani* were used as the outgroup. Bootstrap values greater than 70% of 100 resamplings are shown near nodes. The archaeal AmoA sequences obtained in this study are shown in bold, along with their distribution and clone abundance in each library as depicted in the parentheses. The * symbol indicates that the AmoA sequence groups were originally designated by Nakagawa *et al.* (2007).

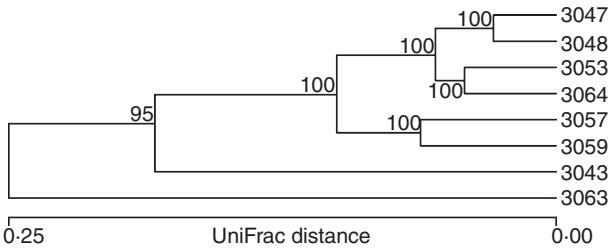

Figure 2 Dendrogram of the hierarchical clustering analysis of the tropical West Pacific Continental Margin deep-sea sedimentary AmoA assemblages constructed using the UniFrac weighted Jackknife Environment Clusters statistical method. The percentage supports of the classification tested with sequence jackknifing resamplings are shown near the corresponding nodes.

both sequences and clones) stations 3043 and 3063 could be separated from the marine AmoA-dominated stations 3047, 3048 and 3053 along the first principal coordinate (P1), which explained 81·8% of the total AEA community variability among all the sampling stations (Fig. 3a). The unweighted PCoA analysis (omitting clone abundance for each AmoA sequence) of the AmoA assemblages showed a slightly different classification schema (Fig. 3b). The stations (3063, 3043, 3057 and 3059) with the highest percentage of the putative terrestrial or estuarine AmoA sequences (Cluster II in Fig. 1 and Table 2) were grouped together. It appears that both terrestrial and estuarine microbial inputs may have a significant impact on the composition and structure of the sediment AEA community in the continental margin deep-sea environment.

Spatial distribution of the AEA community

For the weighted CCA analysis (Fig. 4a), the first two CCA axes (CCA1 and CCA2) explained 48·4% of the total variance in the AEA composition and 52·3% of the cumulative variance of the AEA–environment relationship. The CCA1 distinguished the AEA assemblages of stations 3043 and 3063 from those of the other stations and explained 31·6% of the cumulative variance of the AEA–environment relationship. The sediment OrgC/OrgN contributed the most to this distinction and was the only environmental parameter that contributed significantly ($P = 0{\cdot}005$, 1000 Monte Carlo permutations) to the AEA–environment relationship. Although none of the other environmental factors contributed significantly ($P > 0{\cdot}180$) to the AEA–environment relationship, the combination of these environmental factors provided additionally 68·3% of the total CCA explanatory power.

For the unweighted CCA analysis (omitting clone abundance for each AmoA sequence) (Fig. 4b), the first two CCA axes explained 43·4% of the total variance in the AEA composition and 48·5% of the cumulative variance of the AEA–environment relationship. The AEA assemblages of stations 4043, 4057 and 4063 were

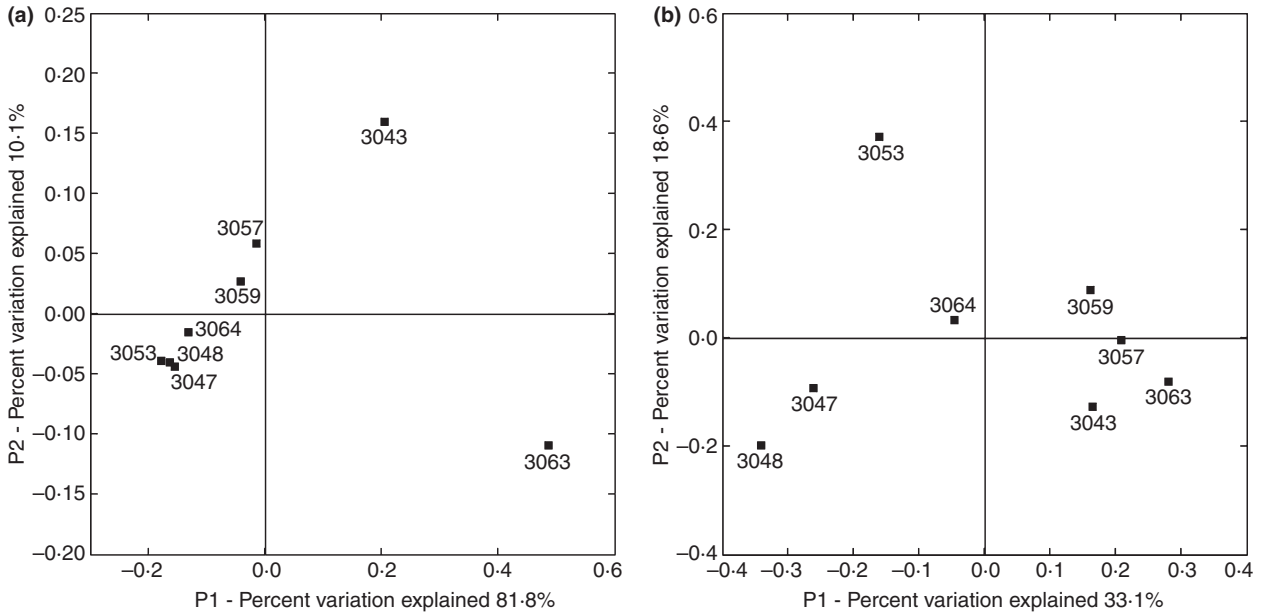

Figure 3 Ordination diagrams of the sedimentary AmoA assemblages calculated with weighted (a) and unweighted (b) UniFrac PCoA analyses using the archaeal AmoA protein sequences recovered from the deep-sea sediments of the tropical West Pacific Continental Margin. Shown are the plots of the first two principal coordinate axes (P1 and P2) for PCoA and the distributions of the AEA assemblages (designated with the sampling station names) in response to these axes.

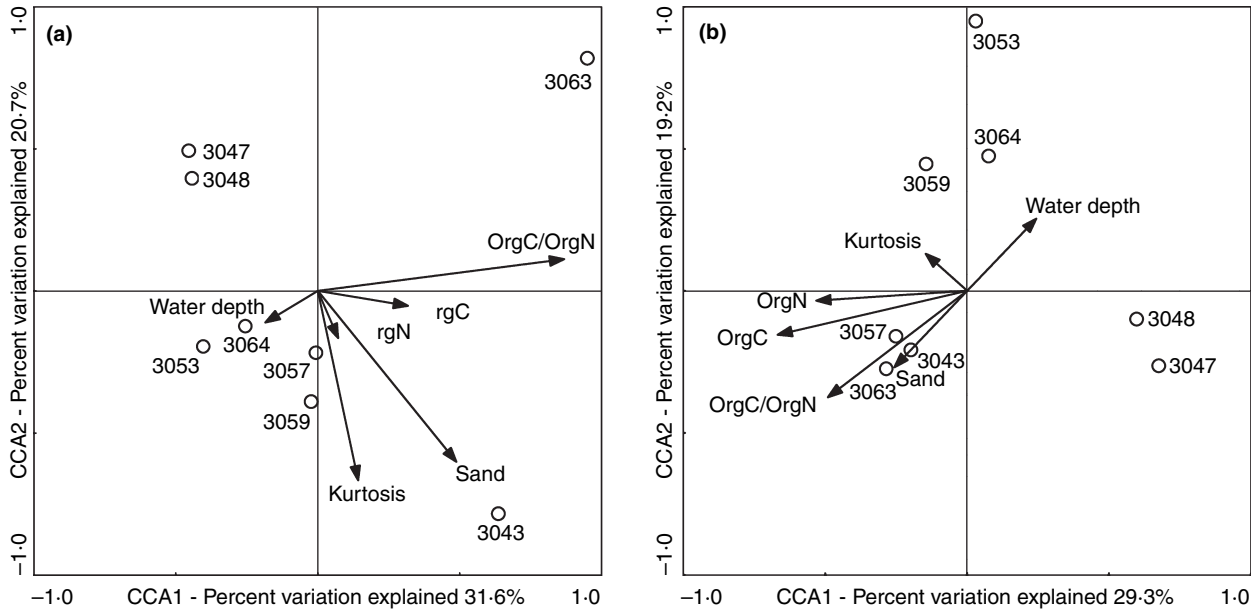

Figure 4 CCA ordination plots for the first two dimensions to show the relationship between the sedimentary AEA community with environmental parameters analysed using (a) the weighted OTU data (both sequences and their clone abundance were considered) and (b) the unweighted OTU data (omitting clone abundance for each AmoA sequence) in the cold deep-sea sediments of the Tropical West Pacific Continental Margin. Correlations between environmental variables and CCA axes are represented by the length and angle of arrows (environmental factor vectors). *Abbreviation*: Sand, sediment sand content. (○, Sampling station; ⟶, Environmental factors).

grouped together and their distribution was positively correlated with OrgC, OrgN, OrgC/OrgN and the sediment sand content and negatively correlated with water depth. In particular, the sediment OrgC appeared to be significant ($P = 0{\cdot}053$, 1000 Monte Carlo permutations) to the AEA–environment relationship. None of the other environmental factors contributed significantly ($P > 0{\cdot}200$) to the AEA–environment relationship. However, the combination of these environmental factors provided additionally 78·3% of the total CCA explanatory power.

Discussion

Deep sea sediments constitute the largest compartment of the global biosphere and as such are also the largest unexplored habitat on Earth (Whitman *et al.* 1998). Not surprisingly, studies of the distribution, abundance and functions of its resident microbial assemblages have begun only recently.

The colour of the sediments sampled in this study was mostly brown, yellow, olive green or olive grey thereby indicating that these sections were probably removed from the oxic or suboxic zones of the sediments (Diaz and Trefry 2006). Previous studies indicated that the AEA are particularly abundant in low oxygen environments, which is ecologically relevant (Park *et al.* 2006; Coolen *et al.* 2007; Lam *et al.* 2007; Santoro *et al.* 2008). Therefore, the presence of diverse archaeal *amoA* genes identified by our study may represent diverse AEA assemblages in deep-sea subsurface sediments with a comparable ecological relevance.

By comparison, the cold deep-sea sediment AEA diversity of the tropical West Pacific Continental Margin is higher than that of the other marine environments, such as the hypernutrified subtropical Bahía del Tóbari estuary (Beman and Francis 2006), the temperate Changjiang estuary and its adjacent East China Sea (Dang *et al.* 2008b). The Bohol Sea, Bashi Strait and Sibuyan Sea sediments had the highest *amoA* gene diversity (Table 2, Fig. S2) and also the highest proportion of the putative terrestrial or estuarine AmoA-related sequences (Fig. 1, Table 2). Previous studies indicated that the AEA communities in terrestrial environments are distinct from those in marine environments (Leininger *et al.* 2006), whereas other studies indicated that estuaries and coasts might harbour mixed populations of AEA from soil and sediments (Francis *et al.* 2005; Beman and Francis 2006; Dang *et al.* 2008b). The deposition of archaea from terrestrial input could potentially explain the existence of the putative terrestrial or estuarine AmoA-related sequences in some of the studied deep-sea sites. These

sites might harbour mixed AEA populations of both terrestrial and marine origins and thus, a higher microbial diversity.

Phylogenetic analysis of experimental and retrieved archaeal AmoA sequences resulted in two sequence clusters (Fig. 1) consistent with a previous report characterizing the AEA community in the Changjiang estuary and its adjacent East China Sea (Dang *et al.* 2008b). None of the AmoA sequences in Cluster II was deduced from sequenced DNA that originated from AEA resident in other deep-sea environments, such as the cold seep water column and sediments of the Northeastern Japan Sea (Nakagawa *et al.* 2007), which may receive little terrestrial influence. Besides this phylogenetic evidence, the spatial distribution of sediment AEA assemblages in response with environmental variables analysed via CCA statistics (Fig. 4) also indicates that certain AEA assemblages (stations 3043, 3057 and 3063) are positively correlated with the distribution of sediment OrgC/OrgN or OrgC and negatively correlated with water depth, which may serve as signatures of terrestrial input intensity (Kukal 1971). Hence, our data indicate that the marginal seas may serve as a conduit for the transport of microbes from the land or estuaries to the deep ocean. The putative terrestrial or estuarine AEA found in the cold deep sedimentary environments of the tropical West Pacific may serve as a bioindicator or biotracer for terrestrial impact on the deep-sea benthic microbial ecosystem.

The finding of terrestrial AmoA-like sequences in the deep-sea sediments supports our hypothesis that the frequent activities of volcanoes, earthquakes, tropical storms, typhoons, tsunamis, floods and landslides in the Philippine area may have intensified the transport of terrestrial microbes into the deep-sea sedimentary environment. The occurrence of the putative terrestrial AEA in the cold deep-sea subsurface sediments may thus relate to certain historical geological or climatic events. More importantly, our results suggest that certain terrestrial or estuarine AEA may have broader ecophysiological adaptation potential than previously appreciated.

The UniFrac analyses indicate a heterogeneous distribution of the sediment AEA community in the tropical West Pacific Continental Margin (Figs. 2, 3), which might be the result of the differential distribution of the putative terrestrial or estuarine AEA in the various deep-sea sampling sites (Table 2). Our UniFrac results also indicate the importance of both quantitative (based on both sequences and clones data) and qualitative (based solely on sequences data) community analyses. The unweighted PCoA analysis mainly illustrates the compositional difference of the AmoA assemblages among the sampling stations, while the weighted PCoA analysis can also illustrate the community structural difference of the AmoA assemblages. Although stations 3057 and 3059 had large proportions of the putative terrestrial or estuarine AmoA sequences (54·6% and 46·7%), the abundance of the corresponding clones was quite low (26·6% and 21·6%). The different PCoA classification results pertaining to sampling stations 3057 and 3059 (Fig. 3) may indicate a transitional status of their AmoA assemblages; from strongly land-impacted stations (3063 and 3043) to marine environment-dominated stations (3047, 3048 and 3053). Similarly, the comparison of the weighted and unweighted CCA analyses may also indicate the importance of employing both quantitative and qualitative analyses in decoding the AEA–environment relationship (Fig. 4).

A considerable proportion of our AmoA sequences were related to the GenBank sequences originally associated with community DNA retrieved from the deep-sea hydrothermal vent or terrestrial hot spring environments (Zhang *et al.* 2008). This finding indicates that deep-sea AEA either have a wide range of temperature adaptation or the AEA present in the modern deep-sea cold sediment environments of the West Pacific Continental Margin have a thermophilic evolutionary history (Hatzenpichler *et al.* 2008; de la Torre *et al.* 2008; Zhang *et al.* 2008). The existence of volcanic ash layers in some of the sediment cores (Institut Polaire Francais 2006) indicates that the latter inference is plausible, as geothermal events, such as volcanoes, happened historically in the studied area. Extant deep-sea cold sediment AEA assemblages may thus serve as a geomicrobiological record of the past geothermal events in the continental margin deep-sea sediments (Inagaki *et al.* 2001).

The continental margin is the most important interface between the terrestrial and marine environments, especially in the recycling of nutrients (Walsh 1991; Derry and Murray 2004). Relating to the environmental characteristics, several groups or subgroups of the AmoA sequences were found to be specific to the tropical West Pacific Continental Margin sedimentary environments (Fig. 1). These unique archaeal ammonia monooxygenase sequences may reflect specific adaptation (molecular evolution) of the sediment functional archaeal community including novel ammonia-oxidizing archaea. Environmental specificity of the AEA community has also been found in other deep-sea environments, such as the Sediment A-1 subgroup detected in the deep-sea cold seep pelagic brown sediments at the northeastern Japan Sea (Fig. 1) (Nakagawa *et al.* 2007). Related to environmental background and microbial evolutionary history, specific biogeographical distribution of certain microbial functional members in the deep-sea sedimentary environments may be a common phenomenon. Specifically, in the tropical West Pacific Continental Margin, the stations in the Philippine inland seas (including station 3043) may

represent AEA assemblages with various terrestrial influences, whereas the other stations connected directly to the open Philippine Sea may represent marine environment-dominant AEA assemblages.

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References

Altschul, S., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D.J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**, 3389–3402.

Arp, D.J. and Stein, L.Y. (2003) Metabolism of inorganic N compounds by ammonia-oxidizing bacteria. *Crit Rev Biochem Mol Biol* **38**, 471–495.

Beman, J.M. and Francis, C.A. (2006) Diversity of ammonia-oxidizing archaea and bacteria in the sediments of a hypernutrified subtropical estuary: Bahía del Tóbari, Mexico. *Appl Environ Microbiol* **72**, 7767–7777.

Beman, J.M., Roberts, K.J., Wegley, L., Rohwer, F. and Francis, C.A. (2007) Distribution and diversity of archaeal ammonia monooxygenase (*amoA*) genes associated with corals. *Appl Environ Microbiol* **73**, 5642–5647.

Beman, J.M., Popp, B.N. and Francis, C.A. (2008) Molecular and biogeochemical evidence for ammonia oxidation by marine *Crenarchaeota* in the Gulf of California. *ISME J* **2**, 429–441.

ter Braak, C.J.F. and Šmilauer, P. (2002) *CANOCO Reference Manual and CanoDraw for Windows User's Guide: Software for Canonical Community Ordination (ver. 4.5)*. Ithaca, NY: Microcomputer Power.

Caffrey, J.M., Bano, N., Kalanetra, K. and Hollibaugh, J.T. (2007) Ammonia oxidation and ammonia-oxidizing bacteria and archaea from estuaries with differing histories of hypoxia. *ISME J* **1**, 660–662.

Cavicchioli, R., Demaere, M.Z. and Thomas, T. (2007) Metagenomic studies reveal the critical and wide-ranging ecological importance of uncultivated Archaea: the role of ammonia oxidizers. *Bioessays* **29**, 11–14.

Coolen, M.J.L., Abbas, B., van Bleijswijk, J., Hopmans, E.C., Kuypers, M.M.M., Wakeham, S.G. and Sinninghe Damsté, J.S. (2007) Putative ammonia-oxidizing *Crenarchaeota* in suboxic waters of the Black Sea: a basin-wide ecological study using 16S ribosomal and functional genes and membrane lipids. *Environ Microbiol* **9**, 1001–1016.

Dang, H.Y. and Lovell, C.R. (2000) Bacterial primary colonization and early succession on surfaces in marine waters as determined by amplified rRNA gene restriction analysis and sequence analysis of 16S rRNA genes. *Appl Environ Microbiol* **66**, 467–475.

Dang, H.Y., Li, T.G., Chen, M.N. and Huang, G.Q. (2008a) Cross-ocean distribution of *Rhodobacterales* bacteria as primary surface colonizers in temperate coastal marine waters. *Appl Environ Microbiol* **74**, 52–60.

Dang, H.Y., Zhang, X.X., Sun, J., Li, T.G., Zhang, Z.N. and Yang, G.P. (2008b) Diversity and spatial distribution of sediment ammonia-oxidizing crenarchaeota in response to estuarine and environmental gradients in the Changjiang Estuary and East China Sea. *Microbiology* **154**, 2084–2095.

Derry, L.A. and Murray, R.W. (2004) Continental margins and the sulfur cycle. *Science* **303**, 1981–1982.

Diaz, R.J. and Trefry, J.H. (2006) Comparison of sediment profile image data with profiles of oxygen and Eh from sediment cores. *J Mar Syst* **62**, 164–172.

Falkowski, P.G. (1997) Evolution of the nitrogen cycle and its influence on the biological sequestration of CO_2 in the ocean. *Nature* **387**, 272–275.

Felsenstein, J. (1989) PHYLIP – phylogeny inference package (version 3.2). *Cladistics* **5**, 164–166.

Francis, C.A., Roberts, K.J., Beman, J.M., Santoro, A.E. and Oakley, B.B. (2005) Ubiquity and diversity of ammonia-oxidizing archaea in water columns and sediments of the ocean. *Proc Natl Acad Sci USA* **102**, 14683–14688.

Francis, C.A., Beman, J.M. and Kuypers, M.M.M. (2007) New processes and players in the nitrogen cycle: the microbial ecology of anaerobic and archaeal ammonia oxidation. *ISME J* **1**, 19–27.

Gruber, N. and Galloway, J.N. (2008) An Earth-system perspective of the global nitrogen cycle. *Nature* **451**, 293–296.

Hatzenpichler, R., Lebedeva, E.V., Spieck, E., Stoecker, K., Richter, A., Daims, H. and Wagner, M. (2008) A moderately thermophilic ammonia-oxidizing crenarchaeote from a hot spring. *Proc Natl Acad Sci USA* **105**, 2134–2139.

He, J.Z., Shen, J.P., Zhang, L.M., Zhu, Y.G., Zheng, Y.M., Xu, M.G. and Di, H. (2007) Quantitative analyses of the abundance and composition of ammonia-oxidizing bacteria and ammonia-oxidizing archaea of a Chinese upland red soil under long-term fertilization practices. *Environ Microbiol* **9**, 2364–2374.

Inagaki, F., Takai, K., Komatsu, T., Kanamatsu, T., Fujioka, K. and Horikoshi, K. (2001) Archaeology of Archaea:

geomicrobiological record of Pleistocene thermal events concealed in a deep-sea subseafloor environment. *Extremophiles* **5**, 385–392.

Institut Polaire Francais. (2006) *MD155 – Marco Polo 2 Images XIV Cruise Report*. Technopôle Brest-Iroise: Institut Polaire Francais.

Könneke, M., Bernhard, A.E., de la Torre, J.R., Walker, C.B., Waterbury, J.B. and Stahl, D.A. (2005) Isolation of an autotrophic ammonia-oxidizing marine archaeon. *Nature* **437**, 543–546.

Kowalchuk, G.A. and Stephen, J.R. (2001) Ammonia-oxidizing bacteria: a model for molecular microbial ecology. *Annu Rev Microbiol* **55**, 485–529.

Kukal, Z. (1971) *Geology of Recent Sediments*. p. 490. New York: Academic Press.

Lam, P., Jensen, M.M., Lavik, G., McGinnis, D.F., Muller, B., Schubert, C.J., Amann, R., Thamdrup, B. *et al.* (2007) Linking crenarchaeal and bacterial nitrification to anammox in the Black Sea. *Proc Natl Acad Sci U S A* **104**, 7104–7109.

Lansigan, F.P., de los Santos, W.L. and Coladilla, J.O. (2000) Agronomic impacts of climate variability on rice production in the Philippines. *Agric Ecosyst Environ* **82**, 129–137.

Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., Valentin, F., Wallace, I.M. *et al.* (2007) Clustal W and Clustal X version 2.0. *Bioinformatics* **23**, 2947–2948.

Lee, S.-Y., Huh, C.-A., Su, C.-C. and You, C.-F. (2004) Sedimentation in the Southern Okinawa Trough: enhanced particle scavenging and teleconnection between the Equatorial Pacific and western Pacific margins. *Deep Sea Res Part I Oceanogr Res Pap* **51**, 1769–1780.

Leininger, S., Urich, T., Schloter, M., Schwark, L., Qi, J., Nicol, G.W., Prosser, J.I., Schuster, S.C. *et al.* (2006) Archaea predominate among ammonia-oxidizing prokaryotes in soils. *Nature* **442**, 806–809.

Liu, Y.C., Santos, A., Wang, S.M., Shi, Y.L., Liu, H.L. and Yuen, D.A. (2007) Tsunami hazards along Chinese coast from potential earthquakes in South China Sea. *Phys Earth Planet Inter* **163**, 233–244.

Lozupone, C.A., Hamady, M., Kelley, S.T. and Knight, R. (2007) Quantitative and qualitative β diversity measures lead to different insights into factors that structure microbial communities. *Appl Environ Microbiol* **73**, 1576–1585.

Magalhães, C., Bano, N., Wiebe, W.J., Bordalo, A.A. and Hollibaugh, J.T. (2008) Dynamics of nitrous oxide reductase genes (*nosZ*) in intertidal rocky biofilms and sediments of the Douro River Estuary (Portugal), and their relation to N-biogeochemistry. *Microb Ecol* **55**, 259–269.

Mincer, Y.J., Church, M.J., Taylor, L.T., Preston, C., Karl, D.M. and DeLong, E.F. (2007) Quantitative distribution of presumptive archaeal and bacterial nitrifiers in Monterey Bay and the North Pacific Subtropical Gyre. *Environ Microbiol* **9**, 1162–1175.

Nakagawa, T., Mori, K., Kato, C., Takahashi, R. and Tokuyama, T. (2007) Distribution of cold-adapted ammonia-oxidizing microorganisms in the deep-ocean of the northeastern Japan Sea. *Microbes Environ* **22**, 365–372.

Nicol, G.W. and Schleper, C. (2006) Ammonia-oxidising *Crenarchaeota*: important players in the nitrogen cycle? *Trends Microbiol* **14**, 207–212.

Ozawa, A., Tagami, T., Listanco, E.L., Arpa, C.B. and Sudo, M. (2004) Initiation and propagation of subduction along the Philippine Trench: evidence from the temporal and spatial distribution of volcanoes. *J Asian Earth Sci* **23**, 105–111.

Park, H.D., Wells, G.F., Bae, H., Criddle, C.S. and Francis, C.A. (2006) Occurrence of ammonia-oxidizing archaea in wastewater treatment plant bioreactors. *Appl Environ Microbiol* **72**, 5643–5647.

Park, S.J., Park, B.J. and Rhee, S.K. (2008) Comparative analysis of archaeal 16S rRNA and *amoA* genes to estimate the abundance and diversity of ammonia-oxidizing archaea in marine sediments. *Extremophiles* **12**, 605–615.

Ramette, A. (2007) Multivariate analyses in microbial ecology. *FEMS Microbiol Ecol* **62**, 142–160.

Sahan, E. and Muyzer, G. (2008) Diversity and spatio-temporal distribution of ammonia-oxidizing Archaea and Bacteria in sediments of the Westerschelde estuary. *FEMS Microbiol Ecol* **64**, 175–186.

Santoro, A.E., Francis, C.A., de Sieyes, N.R. and Boehm, A.B. (2008) Shifts in the relative abundance of ammonia-oxidizing bacteria and archaea across physicochemical gradients in a subterranean estuary. *Environ Microbiol* **10**, 1068–1079.

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

Seitzinger, S.P. (1988) Denitrification in freshwater and coastal marine ecosystems: ecological and geochemical significance. *Limnol Oceanogr* **33**, 702–724.

Steger, D., Ettinger-Epstein, P., Whalan, S., Hentschel, U., de Nys, R., Wagner, M. and Taylor, M.W. (2008) Diversity and mode of transmission of ammonia-oxidizing archaea in marine sponges. *Environ Microbiol* **10**, 1087–1094.

de la Torre, J.R., Walker, C.B., Ingalls, A.E., Könneke, M. and Stahl, D.A. (2008) Cultivation of a thermophilic ammonia oxidizing archaeon synthesizing crenarchaeol. *Environ Microbiol* **10**, 810–818.

Treusch, A.H., Leininger, S., Kletzin, A., Schuster, S.C., Klenk, H.P. and Schleper, C. (2005) Novel genes for nitrite reductase and Amo-related proteins indicate a role of uncultivated mesophilic crenarchaeota in nitrogen cycling. *Environ Microbiol* **7**, 1985–1995.

Urakawa, H., Tajima, Y., Numata, Y. and Tsuneda, S. (2008) Low temperature decreases the phylogenetic diversity of ammonia-oxidizing archaea and bacteria in aquarium biofiltration systems. *Appl Environ Microbiol* **74**, 894–900.

Walsh, J.J. (1991) Importance of continental margins in the marine biogeochemical cycling of carbon and nitrogen. *Nature* **350**, 53–55.

Whitman, W.B., Coleman, D.C. and Wiebe, W.J. (1998) Prokaryotes: the unseen majority. *Proc Natl Acad Sci U S A* **95**, 6578–6583.

Wuchter, C., Abbas, B., Coolen, M.J., Herfort, L., van Bleijswijk, J., Timmers, P., Strous, M., Teira, E. *et al.* (2006) Archaeal nitrification in the ocean. *Proc Natl Acad Sci U S A* **103**, 12317–12322.

Zhang, C.L., Ye, Q., Huang, Z., Li, W., Chen, J., Song, Z., Zhao, W., Bagwell, C. *et al.* (2008) Global occurrence of archaeal *amoA* genes in terrestrial hot springs. *Appl Environ Microbiol* **74**, 6417–6426.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. The map of the sampling stations of the tropical West Pacific Continental Margin.

Figure S2. Rarefaction curve of the archaeal *amoA* gene clone libraries using the OTUs defined with 5% *amoA* sequence distance cutoff.

Figure S3. Pairwise weighted UniFrac significance test of the sedimentary AmoA assemblages calculated with the online UniFrac program.

Figure S4. Pairwise P significance test of the sedimentary AmoA assemblages calculated with the online UniFrac program.

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