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Abundance and composition of ammonia-oxidizing bacteria and archaea in different types of soil in the Yangtze River estuary*

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Abstract: Tidal flats are soil resources of great significance. Nitrification plays a central role in the nitrogen cycle and is often a critical first step in nitrogen removal from estuarine and coastal environments. We determined the abundance as well as composition of ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA) in different soils during land reclamation process. The abundance of AOA was higher than that of AOB in farm land and wild land while AOA was not detected in tidal flats using real-time polymerase chain reaction (PCR). The different abundances of AOB and AOA were negatively correlated with the salinity. The diversities of AOB and AOA were also investigated using clone libraries by amplification of *amoA* gene. Among AOB, nearly all sequences belonged to the *Nitrosomonas* lineage in the initial land reclamation process, i.e., tidal flats, while both *Nitrosomonas* and *Nitrosospira* lineages were detected in later and transition phases of land reclamation process, farm land and wild land. The ratio of the numbers of sequences of *Nitrosomonas* and *Nitrosospira* lineages was positively correlated with the salinity and the net nitrification rate. As for AOA, there was no obvious correlation with the changes in the physicochemical properties of the soil. This study suggests that AOB may be more import than AOA with respect to influencing the different land reclamation process stages.

Key words: Wetland, Nitrification, Ammonia-oxidizing microorganisms, Abundance, Composition
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1 Introduction

Nitrification is the microbial oxidation of ammonia to nitrate via nitrite, which links the mineralization of organic matter-derived nitrogen to its ultimate removal as gaseous products by denitrification or anaerobic ammonia oxidization in estuarine and continental shelf sediments (Bernhard *et al*., 2007). Ammonia oxidation, the first and rate-limiting step in the nitrification process, is an important component of the nitrogen cycle. Aerobic lithotrophic nitrifiers comprise bacterial and archaeal ammonia oxidizers (Rotthauwe *et al.*, 1997; Francis *et al.*, 2005; Zhang *et al*., 2010).

Autotrophic ammonia-oxidizing bacteria (AOB) are affiliated with the β-Proteobacteria including genera *Nitrosomonas* and *Nitrosospira* (Head *et al*., 1993; Purkhold *et al*., 2000; 2003) and the γ-Proteobacteria including genus *Nitrosococcus* (Ward and O′Mullan, 2002). Environmental studies have focused primarily on the β-Proteobacteria

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because of their relative abundance in many environments especially in soils and sediments (Jia and Conrad, 2009; Wells *et al*., 2009). Recently, archaea that oxidize ammonia have been discovered (Venter *et al*., 2004; Könneke *et al*., 2005; Treusch *et al*., 2005) and several studies revealed that ammonia-oxidizing archaea (AOA), which belong to a novel phylum Thaumarchaeota (Brochier-Armanet *et al*., 2008; Spang *et al*., 2010), are the predominant ammonia oxidizers in various soil environments (Leininger *et al*., 2006; Zhang *et al*., 2010). Given its functional significance and conserved phylogeny, the ammonia monooxygenase (*amoA*) gene (Sharp *et al*., 1984), which catalyzes the first step of ammonia oxidation, has been used as a molecular marker to study both the diversities and abundances of AOB and AOA in marine and terrestrial environments, such as water columns (Santoro *et al*., 2008), sediments (Moin *et al*., 2009), activated sludge (Wells *et al*., 2009), and soils (Di *et al*., 2010). These new findings have stimulated reassessment of the ecological importance of these groups (AOB and AOA) in agricultural ecosystems.

China faces severe problems in terms of inadequate resources and environment contamination as the result of population growth and reduction in the amount of arable land. Wetlands, especially in coastal estuaries and muddy tidal flats, have a large potential for use as land resources. Reclamation of tidal flats into arable land is of great significance because it helps to alleviate the problem of limited soil resources.

The estuary of the Yangtze River is the largest in China (Li *et al*., 2010). The Dongtan Wetland is an intertidal estuarine wetland that is located at the eastern end of Chongming Island in the Yangtze River estuary. Chongming Island is the third largest island in China and the largest alluvial island in the world (Gan *et al*., 2009). The precursor of the present Chongming Island was a small inter-tidal shoal, and it has doubled in size over the last 50 years to about 120×10^3 ha, and is still growing by more than 200 ha per year through the increase of alluvial and land reclamation in tidal flats (Li *et al*., 2010). Land reclamation in tidal flats is a common practice globally. The agricultural soil present on Chongming Island increased by 729 ha per year via land reclamation in the past 20 years (Gao and Zhao, 2006). Sea dikes can be used to drain the salt marshes of tidal wetlands to create wild land. Tidal flats, wild land, and farm land are three typical stages of the soil utilization process. A recent study pointed out that the primary controller of the distribution of soil bacteria was soil habitat characteristics (Fierer and Jackson, 2006). Soil types were considered to be the primary factor influencing bacterial composition and abundance (Girvan *et al*., 2003). A number of studies have shown that the community structures of AOA and AOB change in response to fertilizer management (Enwall *et al*., 2005; He *et al*., 2007). The composition and abundance of ammonia oxidizers could be used as one important biological indicator in monitoring soil quality for agriculture (Bastida *et al*., 2008). Therefore, the analysis of correlations between the ammonia oxidizers and soil characteristics may provide useful information in guiding efficient use of emerging land resources formed by the Yangtze River.

In this study, we examined the ecology of ammonia-oxidizing microbial communities in soils in the Dongtan Wetland of Chongming Island by examining the diversity and abundance of the *amoA* gene for both AOB and AOA in three types of soils (tidal flats, wild land, and farm land). The abundances of AOB and AOA were quantified with real-time polymerase chain reaction (PCR) techniques, and the community compositions of the AOB and AOA were analyzed using *amoA* gene clone libraries. The effects of the soil physicochemical properties on the AOB and AOA communities were determined using statistical analysis.

2 Materials and methods

2.1 Experimental sites and sampling

Three different sites were selected as study sites in the Dongtan Wetland of Chongming Island, Shanghai, China. The coordinates of each site were as follows: tidal flat (T) (31°30′ N, 121°58′ E), wild land (W) (31°30′ N, 121°57′ E), and farm land (F) (31°31′ N, 121°51′ E). The coordinates were determined using the Global Positioning System (Explorist 110, Magellan). These sites represent a typical process in soil development in the Yangtze River estuary. The tidal flat was characterized by high primary production rates, and a consequent intense remineralization in the sediment without any interruption from activities of

human beings. The wild land was the land at the other side of the dike and had been left unused after the sea dike construction for six years after a period of reclamation. The farm land has been a part of a long-term reclamation program since 1980 with a wheat-rape rotation system. Each site was sampled in March 2007 at a depth of 0–10 cm. Six soil cores (approximately 5 cm diameter) were taken from each site and the distance between two cores was 2 m. Each sample was placed in a sterile plastic bag and transported to the laboratory. All samples were stored at 4 °C for analysis of soil characteristics, and subsamples were stored at −20 °C for DNA extraction.

2.2 Physicochemical analysis

Six soil cores were taken from each site and the physicochemical analyses of the six cores were measured separately. Soil pH was determined using a HANNA HI 9025 pH meter (Hanna, Italy). Water content and salinity were determined using a WET sensor (Delta-T, UK). Soil bulk density was determined by oven-drying a fixed volume of each soil core at 105 °C to constant weight. Total carbon (TC) and total nitrogen (TN) were analyzed using a FLASH EA 1112 series, CHNSO analyzer (Thermo, Italy). Ammonium and nitrate levels were determined by indophenol blue colorimetry (Solorzano, 1969) and ultraviolet spectrophotometry (Norman and Stucki, 1981), respectively. The soil net nitrification rate was determined as previously described (Fernandez *et al*., 2000; Kelly *et al*., 2011). To measure the net nitrification rate, 50 g of fresh soil was added to each bottle and bottles were covered loosely. Water was added every 2 or 3 d to maintain constant water content and aerobic conditions. The ammonia and nitrate concentrations were determined again after soil samples were incubated at 25 °C for 20 d. The net nitrification rate was equal to the average difference between the nitrate concentrations before and after incubation.

2.3 DNA extraction and preparation

Four gram of a composite soil sample for six cores of each site was used to DNA extraction according to the procedure described by Zhou *et al*. (1996). Deoxyribonucleic acid was purified using the Wizard DNA Clean-Up system (Promega, USA) according to the manufacturer's protocol. The concentration of extracted DNA was determined using a NanoDrop 1000 UV-Vis spectrophotometer (Thermo Fisher, USA).

2.4 Construction of *amoA* gene clone libraries of AOB and AOA

The *amoA* gene was amplified with the primer pair *amoA*-1F and *amoA*-2R (Rotthauwe *et al.*, 1997). The archaeal *amoA* gene was amplified with the primer pair Arch-amoAF and Arch-amoAR (Francis *et al*., 2005). In all PCR amplifications, reactions were performed with Taq DNA MasterMix (Tiangen, China) with a total volume of 50 μl and 10 ng DNA added as the template. The PCR product was gel-purified with the QIAquick Gel Extraction Kit (Qiagen, USA) according to the manufacturer's instructions and then ligated into the pMD-18 vector (TaKaRa, Japan). The resulting ligation products were used to transform *Escherichia coli* TG1 competent cells following the instructions of the manufacturer. Clones were randomly selected for sequencing using the primer M13F (5′-CTG GTA TCG GAT CGG CTG-3′).

2.5 Richness, phylogenetic, and statistical analyses

Sequences were compared with those in GenBank (National Center of Biotechnology Information, NCBI). Nucleotide sequences were assembled and edited using BioEdit (Hall, 1999), and nucleotide and amino acid sequence alignments were generated using ClustalX v1.83 (Jeanmougin *et al*., 1998). Operational taxonomic units (OTUs) were defined as amino acid sequence groups in which sequences differed by ≤5%. The richness was determined using the Chao1 estimator by Mothur v.1.21.1 (Schloss *et al*., 2009). The estimated coverage of the constructed *amoA* gene sequences was calculated as $C=[1-(n_1/N)]\times100\%$, where n_1 is the number of singletons sequences, and N is the total number of sequences (Good, 1953). Neighbor-joining phylogenetic trees (based on Jukes-Cantor distances) were constructed based on alignments of amino acid sequences using MEGA4.0 (Kumar *et al*., 2004). Distance-based bootstrap analyses were conducted using MEGA4.0 and were used to estimate the reliability of phylogenetic reconstructions by running 1000 replicates.

The Pearson's correlation coefficients describing the relationship between AOB or AOA abundance

and soil physicochemical properties were calculated using SigmaPlot Version 11. The Pearson's correlation coefficients for the relationships between the proportion changes of the sequences of the major lineages of AOB (*Nitrosospira* lineage and *Nitrosomonas* lineage) or AOA (soil/sediment lineage and water column/sediment lineage) and soil physicochemical properties were also calculated. Analysis of similarities (ANOSIM) was performed to evaluate the similarities between each of the two clone libraries using Mothur v.1.21.1 (Schloss *et al*., 2009). The correspondence analysis (CA) among the proportion changes of the sequences of the major lineages of AOB and AOA versus soil physicochemical properties was performed using MVSP Version 3.1.

2.6 Real-time PCR assay

The clones containing the bacterial and archaeal *amoA* gene sequences confirmed by sequencing were cultured using Lysogeny Broth (LB) liquid medium with ampicillin. Plasmid DNA was extracted with an Axygen plasmid purification kit (Axygen, USA) and quantified using a NanoDrop 1000 UV-Vis spectrophotometer. The copy numbers of the *amoA* genes were calculated directly from the concentration of the extracted plasmid DNA, which was subjected to a real-time PCR assay in triplicate to generate an external standard curve.

Real-time PCR was performed on an Mx3000P real-time PCR system (Stratagene, USA). Primers and TaqMan probes are listed in Table 1. A total of 10 ng of each DNA extract was used as the template in each reaction mixture. Bacterial *amoA* genes were quantified using the primer pair *amoA*-1F/*amoA*-2R (Rotthauwe *et al*., 1997) with SYBR® Premix Ex Taq™ (TaKaRa, Japan). The primer pair Arch amo196F/Arch amo277R and the probe TM amo247F (Treusch *et al*., 2005) were used for quantification of the archaeal *amoA* gene with real-time PCR master mix (Toyobo, Japan). The efficiency and coefficient (r^2) of the real-time PCR, for the amplification of *amoA* gene from AOA and AOB, were 102% and 108%, 0.997 and 0.989, respectively. Real-time PCR was conducted using the protocol for each target group as shown in Table 1. For the bacterial *amoA* gene assay, a melting curve analysis was performed to confirm PCR product specificity after the temperature increased from 55 °C to 95 °C following the three temperature steps.

2.7 Sequence accession numbers

The sequences obtained in this study have been deposited in the GenBank database under the accession Nos. HQ888765–HQ888800 (bacterial *amoA* gene sequences and deduced amino acid sequences of *amoA* gene) and HQ888801–HQ888822 (archaeal *amoA* gene sequences and deduced amino acid sequences of *amoA* gene).

3 Results

3.1 Soil physicochemical properties

Significant differences (P<0.05) in the soil physicochemical properties of the three environments were observed (Table 2). Tidal flats had the highest pH (8.90±0.04) and salinity ((1.35±0.26)%). The lowest pH (7.51±0.25) and salinity ((0.09±0.01)%) were recorded in farm land. Farm land that received organic fertilizer treatment had the highest TC

Table 1 Primers and probe for the *amoA* gene used for PCR and quantitative PCR (qPCR) analysis

Target	Primer or probe	Name	Sequence (5′ to 3′)	Amplicon length (bp)	Reference
Ammonia-oxidizing bacteria (AOB)	Primer	*amoA*-1F	GGGGTTTCTACTGGTGGT	491	Rotthauwe *et al*., 1997
	Primer	*amoA*-2R	CCCCTCKGSAAAGCCTTCTTC		
Ammonia-oxidizing archaea (AOA)	Primer	Arch amo196F	GGWGTKCCRGGRACWGCMAC	123	Treusch *et al*., 2005
	Primer	Arch amo277R	CRATGAAGTCRTAHGGRTADCC		
	Probe	TM amo247F	CAAACCAWGCWCCYTTKGCDACCCA		
	Primer	Arch-amoAF	STAATGGTCTGGCTTAGACG	635	Francis *et al*., 2005
	Primer	Arch-amoAR	GCGGCCATCCATCTGTATGT		

Table 2 Physiochemical properties and net nitrification rates of the tidal flats of Chongming Island in different development stages

Parameter	Tidal flats	Wild land	Farm land
pH	8.90±0.04 a	8.39±0.19 b	7.51±0.25 c
Density (g/cm^3)	1.49±0.09 a	1.76±0.05 b	1.47±0.14 a
Salinity (%)	1.35±0.26 a	0.15±0.06 b	0.09±0.01 b
Water content (%)	46.25±4.29 a	41.66±1.41 b	30.18±1.98 c
TC (g/kg)	13.22±0.73 a	15.02±1.53 b	20.02±0.39 c
TN (g/kg)	0.43±0.06 a	0.75±0.16 b	1.35±0.09 c
C/N ratio	31.09±4.80 a	20.34±2.37 b	14.83±0.69 c
NH_4^+-N (mg/kg)	9.47±1.11 a	7.91±2.16 a	4.82±0.73 b
NO_3^--N (mg/kg)	10.38±0.47 a	9.76±0.68 a	20.18±2.64 b
Net nitrification rate (mg/(kg·d))	0.18±0.16 a	0.77±0.18 b	0.67±0.21 b

Values are expressed as mean±SD (n=6). Values within the same line followed by the same letter do not differ significantly (P>0.05)

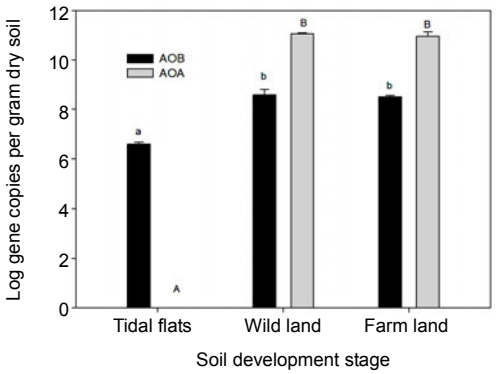

Fig. 1 Average gene copy numbers of bacterial and archaeal *amoA* genes in three soil development stages
The *amoA* gene of AOA in tidal flats site was not detected. The columns with same letter are not significantly different from each other (P>0.05, n=3). The lower case letters showed significant differences in abundances of AOB and capital letters showed those in AOA

and TN levels. The NH_4^+-N level in tidal flats was higher than that in farm land, while the converse was true for NO_3^--N.

The net nitrification rates were distinctly different between the primary soil and agricultural soil. The lowest nitrification rate was recorded in tidal flats.

3.2 Quantification of ammonia-oxidizing microorganisms

The abundances of AOB and AOA were determined using a real-time PCR assay targeting the *amoA* gene. The smallest AOB community size was detected in the tidal flats, followed by the farm land and wild land; the content of AOB in the wild land and farm land was about 100 times higher than that of the tidal flats (Fig. 1). No significant differences were observed between the abundances in farm land and wild land (P=0.56). Similar to AOB, the AOA population sizes were high in the farm land and wild land. However, AOA were not detected in the tidal flats by real-time PCR. There was no significant difference in the population size between farm land and wild land (both mean $\log_{10}$ ratio AOA:AOB, 1.29) (P=0.45).

3.3 Bacterial and archaeal *amoA*-based community structures

Both AOB and AOA partial *amoA* genes were sequenced in the three sites. A total of 292 AOB *amoA* gene sequences and 139 AOA *amoA* gene sequences were obtained from six clone libraries and were translated into amino acid sequences for analysis. The clone numbers for AOB in farm land, wild land, and tidal flats were 94, 102, and 97, respectively, while those for AOA in these three samples were 65, 39, and 35, respectively. These deduced amino acid sequences were grouped into OTUs based on 0.05 amino acid substitutions per amino acid position (Figs. 2 and 3).

The Chao1 total estimators of AOB and AOA were calculated for bacterial and archaeal *amoA* genes using a 5% amino acid sequence divergence with randomly selected 35 sequences. The Chao1 values in each AOB sample from farm land, wild land, and tidal flats were 12, 11, and 8, respectively. Values of AOA for the three samples were 5, 7, and 9, respectively. There was a striking difference in bacterial and archaeal *amoA* gene diversities as measured by richness at the three soil development stages. The richness in the three soil development stage sites showed opposite trends for the bacterial and archaeal *amoA* genes. The greatest richness of the bacterial and archaeal *amoA* genes appeared in farm land and tidal flats, respectively. The estimated coverages of AOB for farm land, wild land, and tidal flats were 94%, 96%, and 98%, respectively. The values of AOA for the three samples were 98%, 95%, and 91%, respectively.

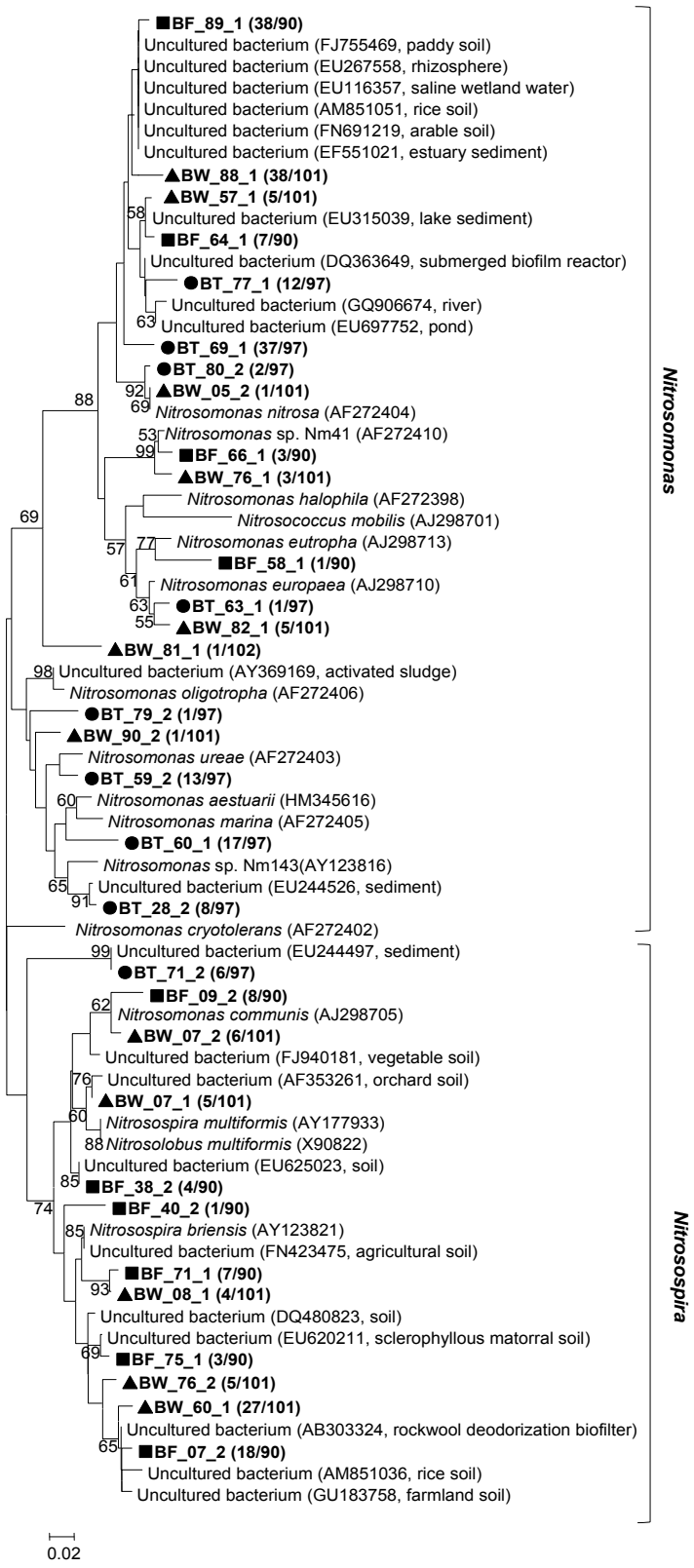

Fig. 2 Phylogenetic tree showing the evolutionary relationships among the deduced amino acid sequences of bacterial *amoA* genes in different soil development stages

Tidal flat: ●; Wild land: ▲; Farm land: ■. The first number in brackets is the number of the sequences which could be assigned to this OTU and the second number is the total sequence number of this clone library

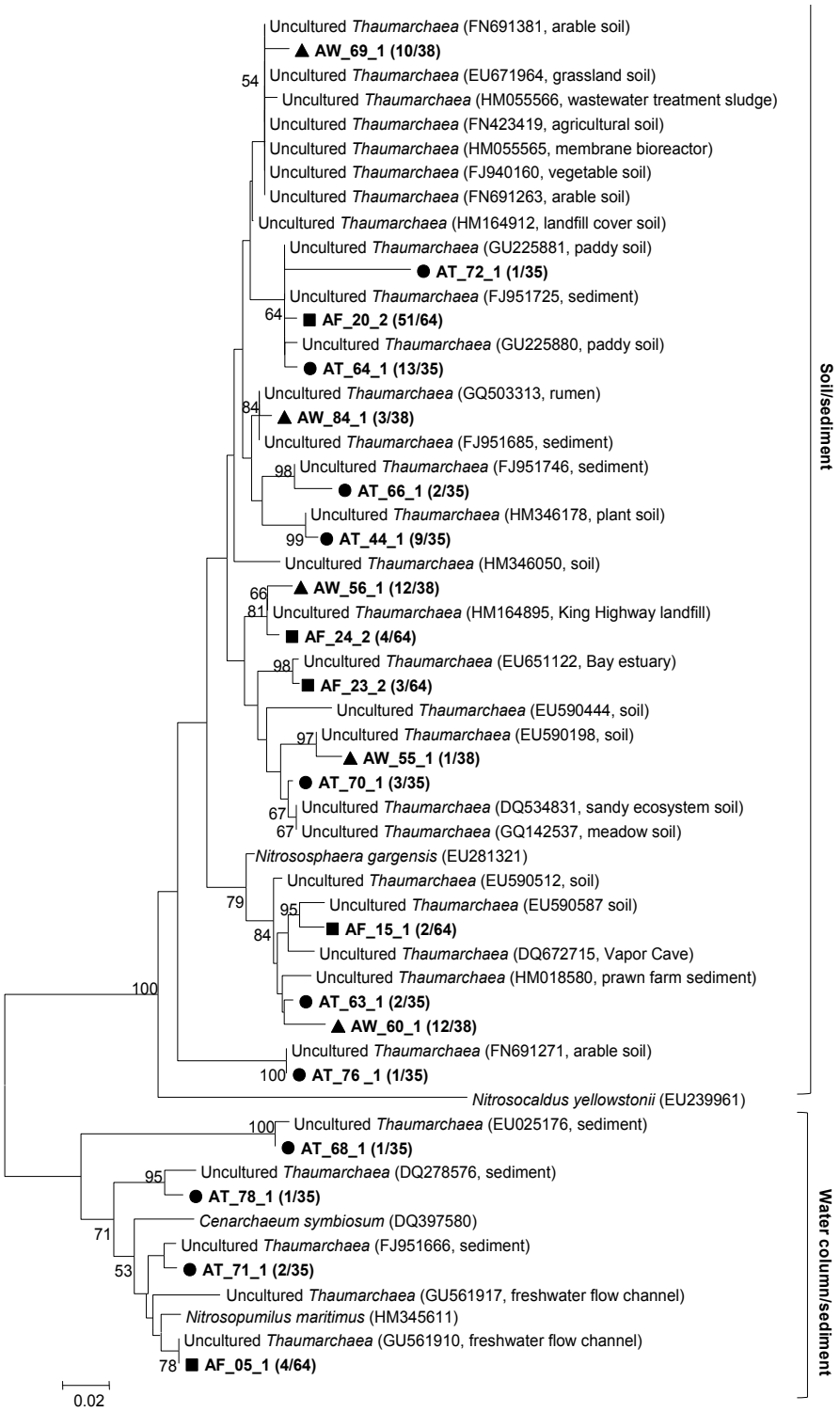

Fig. 3 Phylogenetic tree showing the evolutionary relationships among the deduced amino acid sequences of archaeal *amoA* genes in different soil development stages

Tidal flat: ●; Wild land: ▲; Farm land: ■. The first number in brackets is the number of the sequences which could be assigned to this OTU and the second number is the total sequence number of this clone library

Both the *Nitrosomonas* and *Nitrosospira* lineages of AOB were detected in this study (Fig. 2). Nearly all sequences from the tidal flats belonged to the *Nitrosomonas* lineage. In contrast, in farm land and wild land, more than half of the sequences belonged to the *Nitrosospira* lineage, and about 40% of sequences could be affiliated to the *Nitrosomonas* lineage. In AOB, the most abundant OTUs (clones BT_69_1, BW_88_1, and BF_89_1) were similar to the sequences of uncultured bacterium clones detected in different sources that were 95% similar to *Nitrosomonas nitrosa* (AF272404). Nearly one-third of the AOB deduced amino acid sequences of *amoA* gene in three soil development stages belonged to this OTU. The sequences belonging to the *Nitrosospira* lineage were almost all from farm land and wild land.

All sequences from AOA grouped within the *Thaumarchaea* (Fig. 3). The AOA community diversity was lower than that of AOB in this study. In tidal flats, 11% of sequences belonged to the water column/sediment cluster, related to *Nitrosopumilus maritimus* (HM345611); in wild land and farm land, respectively, 3% and 6% of all sequences belonged to this cluster, whereas the remainder fell within the soil/sediment cluster (Francis *et al.*, 2005). The most abundant OTUs were similar to sequences of uncultured *Thaumarchaea*, most of which have been detected in agricultural soils. About 80% of the sequences from farm land could be affiliated with the OTU AF_20_2.

3.4 Correlations between ammonia-oxidizing microbes and soil physicochemical properties

The relationships between AOB and AOA abundances and soil physicochemical properties were investigated using Pearson's correlation coefficients (Table 3). The logs of both the AOB and AOA *amoA* gene copy numbers showed significant negative correlations with salinity (R<0.05). The abundances did not show significant correlations with other soil physicochemical properties in this analysis.

The relationship between the major lineages of AOB and AOA and both soil chemical properties and potential net nitrification rates are shown in Table 3. The *Nitrosospira* lineage showed a significant correlation with the net nitrification rate, while the *Nitrosomonas* lineage showed a significant correlation with salinity. The AOA lineages did not show any significant correlation with the soil physicochemical properties.

Table 4 shows the R value between each of the two clone libraries of ANASIM analysis. The three sites exhibited different communities for both AOB and AOA. For AOB, the significant difference was observed mainly between tidal flats and farm and wild lands. There was no significant difference of AOB communities between farm land and wild land. For AOA, the results showed significant difference among communities in three sites.

The correlations between soil physicochemical properties and the major AOB and AOA groups were analyzed using CA (Fig. 4). The results of CA showed

Table 3 Pearson's correlation coefficients describing the relationship between soil and ammonia oxidizers' characteristics

Parameter	Correlation coefficient				
	AOB abundance[a]	AOA abundance[a]	*Nitrosospira* lineage[b]	*Nitrosomonas* lineage[b]	Soil/sediment and water column/sediment lineage[c]
Salinity	−0.997*	−0.999*	−0.991	0.998*	−0.911
Net nitrification rate	0.992	0.989	0.998*	−0.990	0.976
pH	−0.758	−0.775	−0.722	0.767	−0.492
Density	0.476	0.453	0.523	−0.464	0.746
Water content	−0.696	−0.715	−0.656	0.706	−0.412
TC	0.680	0.698	0.639	−0.690	0.391
TN	0.744	0.761	0.707	−0.753	0.473
C/N ratio	−0.931	−0.940	−0.910	0.936	−0.752
NH_4^+-N	−0.734	−0.752	−0.697	0.744	−0.461
NO_3^--N	0.422	0.446	0.373	−0.435	0.0902

[a] AOB and AOA abundances were measured by real-time PCR of the *amoA* gene; [b] The *Nitrosospira* and *Nitrosomonas* lineages were calculated by the sequence proportion; [c] The sequence proportion of soil/sediment and water column/sediment lineage showed similar correlation so the results were combined. * Statistically significant correlations with a P value <0.05

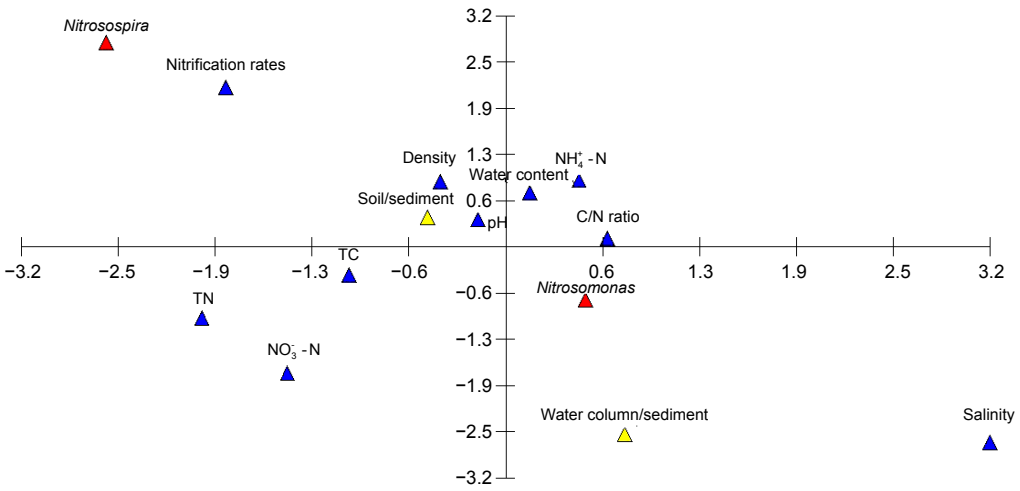

Fig. 4 Correspondence analysis based on the soil physiochemical properties and net nitrification rates and the proportion of major lineages of AOB (*Nitrosospira* lineage and *Nitrosomonas* lineage) and AOA (soil/sediment lineage and water column/sediment lineage)

The major lineages of AOB and AOA were showed by the red and yellow triangles, respectively. The soil physiochemical properties and net nitrification rates were showed by the blue triangle

Table 4 ANOSIM results of every two clone libraries for AOB and AOA

Clone library	*R* value	*P* value
AOB in farm land and tidal flats	0.058	<0.001
AOB in farm land and wild land	−0.001	0.445
AOB in tidal flats and wild land	0.047	0.002
AOA in farm land and tidal flats	0.190	0.001
AOA in farm land and wild land	0.214	<0.001
AOA in tidal flats and wild land	0.051	0.009

similar relationships as those determined by the Pearson's correlation coefficients. The AOB and AOA lineages appeared in the second and forth quadrants in addition to the net nitrification rate, density, pH, and salinity, indicating that these soil physicochemical properties must be important to the AOB and AOA lineages.

4 Discussion

4.1 Soil physicochemical properties

The soil physicochemical properties varied among the three soil development stages and were responsible for the variations in nitrification. In general, soil water content, pH, and NH_4^+-N content control nitrification (Paul and Clark, 1989; Rhoades and Coleman, 1999). Water in the tidal flats was a mixture of freshwater and seawater, having a high salinity. Effects of changes in physicochemical conditions along an estuarine gradient on nitrification rates have been well documented, indicating decreased nitrification as the salinity increases (Rysgaard *et al*., 1999; Bernhard and Bollmann, 2010), which was consistent with the change of nitrification rates in this study. Farm land was under a long-term reclamation, and thus the TN in the farm land was the highest. Salinity plays an important role in controlling NH_4^+-N adsorption capacity with increased NH_4^+-N efflux as salinity increases (Boynton and Kemp, 1985). The general pattern of NH_4^+-N is higher in the high salinity areas and NO_3^--N is enriched in the low salinity areas, which would be contributed by the difference of nitrification activity, and has been frequently observed in other estuaries (Windom and Niencheski, 2003; Charette and Buesseler, 2004; Santoro *et al*., 2008) and is consistent with our results.

4.2 Abundance and richness of ammonia oxidizers

In previous studies (Bernhard *et al*., 2005; 2007; Santoro *et al*., 2008), AOB and AOA community compositions and abundances appear to vary with soil conditions, which also showed significant difference among AOA and AOB communities in the different soil development stages in this study. There was a dramatic difference in the abundances of AOB and AOA in the tidal flats. The abundances of both AOB

and AOA showed a significant negative correlation with salinity, which is consistent with the results of previous studies covering estuaries salinity gradients (Bernhard *et al*., 2007; Santoro *et al*., 2008). Salinity is a particularly important parameter for ammonia oxidation, in part because of its influence on NH_4^+ adsorption (Boatman and Murray, 1982). It is thus reasonable that salinity has a strong influence on microbial community composition and size. It also has been proposed as potentially significant environmental divers of AOB and AOA abundance or community structure in various environments, especially in marine or estuary ecosystems (Sahan and Muyzer, 2008; Santoro *et al.*, 2008). In both the farm land and wild land, AOA outnumbered AOB. This finding is consistent with the results of other reports (Leininger *et al*., 2006; Zhang *et al*., 2010). One intriguing result was that AOA were not detected in the tidal flats using real-time PCR even though they were detected using normal PCR. This primer set used in real-time PCR was widely used in previous studies (Leininger *et al*., 2006; Boyle-Yarwood *et al*., 2008; Mertens *et al*., 2009) Furthermore, when comparing the primer set sequences to the sequences achieved from clone libraries of AOA, they matched perfectly. It means that the real-time PCR conditions for AOA analysis are appropriate for amplification. Some reports specifically described the low sensitivity of real-time PCR compared with normal PCR (Hafez *et al*., 2005; Bastien *et al.*, 2008). Hence, real-time PCR is not always more sensitive than normal PCR. Therefore, the result of a decrease in the abundance of AOA in the tidal flats was still credible. As the decrease of AOB and AOA abundance in tidal flats, net nitrification rate in tidal flats was also observed lower than that in farm and wild lands, indicating that the decrease of ammonia oxidizer's activity in tidal flats. Although previous studies have suggested that in soil environments AOA are often more diverse and abundant than AOB (Santoro *et al*., 2008; Zhang *et al*., 2010), our results revealed a lower richness of AOA than that of AOB in the three soil development stages.

4.3 Community diversities of AOA and AOB

Previous studies have suggested the predominance of *Nitrosospira* over *Nitrosomonas* in terrestrial ecosystems (Kowalchuk *et al*., 2000; Avrahami *et al*., 2002; Avrahami and Conrad, 2003). Both lineages of AOB were detected in this study. The sequences of the bacterial *amoA* genes in farm land and wild land were consistent with the reported results. Interestingly, no *Nitrosospira*-like sequence was found in the saline tidal flats site. Of the physicochemical parameters, salinity and the net nitrification rate of the farm land and wild land showed significant differences from those of tidal flats (Table 2), indicating that the salinity and the net nitrification rate can be the important factors determining the AOB assemblages in different soil development stages. The results of CA (Fig. 4) showed that the proportion of *Nitrosomonas* lineages was positively correlated with salinity and negatively correlated with net nitrification rate, density, and pH.

The enormous differences in the AOB communities among the first soil stage of the tidal flats, the last stage of farm land, and the middle development stage of wild land revealed a difference with respect to the soil development stage. Several studies concerning the factors affecting the distribution of AOB have suggested that salinity is one of the important factors (Bernhard *et al*., 2005; 2007), which was supported by the results of this study. Members of the *Nitrosospira* lineage were only detected in the farm land and wild land. However, the *Nitrosospira* lineage is often dominant in salty environments such as estuarine or marine systems (Ward *et al*., 2007; Bernhard and Bollmann, 2010). An estuarine environment is the interface of land, freshwater and marine environments (Dang *et al*., 2010). Tidal flats in this study are coastal wetlands which are different from the study site of the Yangtze River estuary (Dang *et al*., 2010), so the result of no *Nitrosospira*-like sequences in tidal flats was not surprising. On the other hand, in studies which were focused on agricultural soil AOB communities (Chu *et al*., 2007; Chen *et al*., 2008), the *Nitrosospira* lineage was also found to be dominant in agricultural soils. In our results, more than half of the sequences from farm land and wild land belonged to the *Nitrosomonas* lineage, and the tidal flats were also dominated by *Nitrosomonas*. Those results indicated that the tidal flats are significantly different from the developed soil stages. *Nitrosomonas* has been detected in high-ammonia environments, such as sediment from surface-flow wetland mesocosms (Allen *et al*., 2010), manure-treated wetlands (Ibekwe *et al*., 2003), and

waste water treatment plants (Park *et al*., 2006), which is coincident with the higher ammonia content of the tidal flats than that of farm land and wild land. The differences in the AOB community structure can be used as a biological indicator of different soil development stages.

Phylogenetic analysis reveals a high AOB diversity in three soil development stages. The most abundant bacterial *amoA* sequence types (clones BT_69_1, BW_88_1, and BF_89_1) of the three sites were from the *Nitrosomonas* lineage. This type of sequence was found to predominate in different sources such as agriculture soil (Wang *et al*., 2009), wetland water sample (Dorador *et al*., 2008), and estuary sediment (Sahan and Muyzer, 2008). In the three different soil development stages, about one-third of AOB *amoA* gene sequences belonged to this type, indicating that this type of *Nitrosomonas* is distributed equally in the different environments. In the *Nitrosomonas* lineage, the second most abundant bacterial *amoA* gene sequence type (clone BT_60_1) was affiliated with the *Nitrosomonas marina* cluster but with more than 6% dissimilarity with respect to the isolated *Nitrosomonas marina*. All sequences belonging to this type were from the tidal flats. This type was seldom detected in soil and is typically detected in marine-related environments, such as coastal sediments (Dang *et al*., 2010) and estuaries (Purkhold *et al*., 2003). The water in tidal flats is mixed freshwater and seawater. Some soil characteristics, such as salinity and water content, of the tidal flats are more similar to those of marine environments than those of farm land and wild land. Although most marine environments show relatively high proportions of *Nitrosospira* lineage of AOB, the most abundant sequence type in the *Nitrosospira* lineage detected in this study has most often been detected in agriculture soil in previous studies (Chu *et al*., 2007; Wang *et al*., 2009), which is consistent with the soil development stages of farm land and wild land.

The AOA community diversity was lower than that for AOB in this study. Most of the sequences from the three soil development stages could be affiliated with the soil/sediment cluster, and were closely related to sequences identified in studies of soil and sediment (Leininger *et al*., 2006; Shen *et al*., 2008; Zhang *et al*., 2009), suggesting a similar environment globally. The AOA diversities did not show significant differences among the three soil development stages. However, it had no obvious correlation with the differences in the soil physicochemical properties. As for the results of CA (Fig. 4), the percentage of soil/sediment lineage was positively correlated with net nitrification rate, density, and pH and negatively correlated with salinity.

5 Conclusions

The results of this study showed that salinity, pH, and net nitrification rate in different soil development stages had a greater influence on the abundances and community compositions of AOB and AOA than other soil physicochemical properties in different soil development stages of tidal flats. However, AOB and AOA displayed differences in the response to the different soil development stages. Among the AOB, the ratio of the number of sequences belonging to *Nitrosomonas* and *Nitrosospira* showed significant correlations with salinity and net nitrification rates, which showed significant differences between the primary soil and agricultural soil. In contrast, the AOA community composition showed no significant correlation with soil development stage. The differences in the AOB community structure can be used as a biological indicator of different soil development stages, which could be used to monitor the resource utilization of tidal flats.

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