



Contrasting spatiotemporal patterns and environmental drivers of diversity and community structure of ammonia oxidizers, denitrifiers, and anammox bacteria in sediments of estuarine tidal flats

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1 **Contrasting spatiotemporal patterns and environmental drivers of diversity and community structure of**
2 **ammonia oxidizers, denitrifiers, and anammox bacteria in sediments of estuarine tidal flats**

3

4 **Anjing Yang Xiaoli Zhang Hélène Agogue Christine Dupuy Jun Gong**

5

6 A. Yang X. Zhang* J. Gong*

7 Laboratory of Environmental Microbiology, Key Laboratory of Coastal Environmental Processes, Yantai Institute of
8 Coastal Zone Research, Chinese Academy of Sciences, Yantai, China

9 *email: xlzhang@yic.ac.cn; jgong@yic.ac.cn

10

11 A. Yang

12 University of Chinese Academy of Sciences, Beijing, China

13

14 H. Agogue C. Dupuy

15 Littoral, Environnement et Sociétés (LIENSS) UMR 7266 CNRS – University of La Rochelle, La Rochelle, France

16

17 **Abstract**

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19 The spatial and temporal patterns of diversity, community structure, and their drivers are fundamental issues in
20 microbial ecology. This study aimed to investigate the relative importance of spatial and seasonal controls on the
21 distribution of nitrogen cycling microbes in sediments of estuarine tidal flats, and to test the hypothesis that metals
22 impact the distribution of nitrogen-cycling microbes in the coastal system. Two layers of sediment samples were
23 collected from three estuarine tidal flats of Laizhou Bay in 2010 winter and 2011 summer. The alpha diversities
24 (Shannon and Simpson indices) and community structure of ammonia oxidizing bacteria (AOB) and archaea (AOA),
25 denitrifier and anammox bacteria (AMB) were revealed using denaturing gradient gel electrophoresis and clone library
26 analysis of *amoA*, *nosZ* and 16S rRNA gene markers. We found that both AOB and AMB exhibited distinct seasonal
27 patterns in either alpha diversity or community turnover; AOA had different alpha diversities in two layers, but neither
28 spatial nor seasonal patterns were found for their community turnover. However, no distinct spatiotemporal pattern was
29 observed for either diversity or community composition of *nosZ*-type denitrifiers. For correlations between alpha
30 diversities and environmental factors, significant correlations were found between AOB and ammonium, temperature
31 and As, between denitrifiers and nitrite, salinity and Pb, and between AMB and Pb, C/N, ammonium, pH and DO.
32 Salinity and sediment grain size were the most important factors shaping AOB and AOA communities, respectively;
33 whereas AMB community structure was mostly determined by temperature, dissolved oxygen, pH and heavy metals As
34 and Cd. These results stress that ammonia oxidizers, denitrifiers and anammox bacteria have generally different
35 distributional patterns across time and space, and heavy metals might have contributed to their differentiated
36 distributions in coastal sediments.

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38 **Keywords** Biogeography Community turnover Environmental factors Heavy metals Nitrogen cycle

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41 **Introduction**

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43 Microbe-driven nitrification, denitrification, and the anaerobic ammonium oxidation (anammox) play a pivotal role in
44 nitrogen cycling in estuarine and coastal systems (Spencer and MacLeod 2002; Howarth and Marino 2006; Lam et al.
45 2007). These processes can relieve nitrogen load delivered to coastal waters, reducing the risk of eutrophication
46 (Howarth 2008).

47 The spatial and temporal patterns of the diversity and distribution of N-cycling functional groups underlie the
48 changes of N-cycling processes. Based on the *amoA* gene (encoding ammonia monooxygenase), Ammonia oxidation
49 was previously thought to be restricted to ammonia-oxidizing bacteria (AOB), which are mostly presented by the
50 β -proteobacterial *Nitrosomonas* and *Nitrospira*, and a few in the γ -proteobacterial *Nitrosococcus* (Purkhold et al. 2000;
51 Horz et al. 2004). Dang et al. (2010b) reported that nearby wastewater treatment plants and polluted rivers could have
52 significant impact on AOB composition and distribution of Jiaozhou Bay estuary. Afterwards, ammonia-oxidizing
53 archaea (AOA) belonging to the Crenarchaeota Group 1.1a and Group 1.1b (now known as a separate clade,
54 Thaumarchaeota, Brochier-Armanet et al. 2008) were revealed to be critical for the global nitrogen cycle, which also
55 had functional *amoA* gene (Francis et al. 2007; Zhang et al. 2012). Francis et al. (2005) observed that AOA were
56 pervasive in marine water columns and sediments, and diverse and distinct AOA communities were associated with
57 each of these habitats. The *nosZ* gene codes for nitrous oxide reductase which catalyzes the reduction of N_2O to N_2 , the
58 final step of denitrification. Scala and Kerkhof (1999) investigated the diversity of *nosZ* gene in sediments obtained
59 from the Atlantic Ocean and Pacific Ocean continental shelves and found that denitrifier communities might be
60 restricted geographically. Like denitrification, anammox contributed significantly to the production of N_2 . The
61 anammox bacterial specific 16S rRNA (AMB 16S rRNA) gene in Mai Po estuary sediment performed strong seasonal
62 dynamics due to the anthropogenic and terrestrial inputs (Li et al. 2011).

63 Salinity has been reported as the most important driver for ammonia oxidizer communities. The shift from
64 low-salinity *Nitrosomonas* communities to high-salinity *Nitrospira* communities has been observed in many estuarine
65 systems (Francis et al. 2003; Bernhard et al. 2005; Jin et al. 2011). AOA *amoA* sequences often formed distinct groups
66 according to salinity (Moister and Francis 2008; Abell et al. 2010). Additionally, AOA were proposed to be important
67 actors in low-nutrient, low-pH, and sulfide-containing environments (Erguder et al. 2009). There were spatial and
68 temporal variations of denitrifying communities at the Fitzroy River and San Francisco Bay estuaries, and salinity,
69 organic carbon, nitrogen, chlorophyll- α and some metals were found to be factors influencing the community structure
70 (Abell et al. 2010; Moister and Francis 2010). The anammox bacterial distributions presented strong spatial and

seasonal variations along the Cape Fear River estuarine gradient, which were also highly correlated with salinity variation (Dale et al. 2009). Nevertheless, our knowledge about spatiotemporal patterns and controls of N-cycling microbial communities in tidal flats with high anthropogenic disturbance is limited. The dynamics of these nitrogen-removal groups are likely to be complex and tightly coupled when they compete for a common ecological niche. However, studies examining and comparing the seasonal and spatial patterns of ammonia oxidizers, denitrifiers, and anammox bacteria in a single survey are still rare.

Estuarine sediments are also sinks of metals from different origins. Trace amounts of some metals, which are necessary cofactors in enzymes or co-enzymes and electron transport chains, can be stimulatory to microbial activity (Granger and Ward 2003, Yang et al. 2013). There are evidences that metals may be an important factor in regulating nitrogen transformations in sediment habitats. For example, in the Douro River estuary (north-west Portugal), the transcription diversity of *nosZ* gene showed a drastic decrease with the increase of Cu concentration (Magalhães et al. 2011). The metal toxicity was modulated by sediment properties (metal concentrations, grain size, organic carbon to nitrogen ratio, etc.), and denitrification revealed high sensitivity to heavy metals Cu, Cr, Pb, Zn and Cd in sandy sites but not in muddy sites (Magalhães et al. 2007). However, the effect of heavy metals on nitrogen cycling populations in sediments has not been studied sufficiently, and little is known about the linkage between metals and the nitrogen microbial community structure in estuarine systems.

Estuarine tidal flats of the Laizhou Bay (LZB), a semi-enclosed bay of the Bohai Sea, northern China, have been hypernutrified due to high level of dissolved inorganic nitrogen (DIN), organic pollutants and heavy metals discharged from coastal industries (e.g. aquaculture, subsurface brine industries and dyeing industries) and agriculture (Hu et al. 2010; Zhang et al. 2014). These tidal flats thus present ideal environments for studying the multiple environmental stresses on nitrogen-removal microorganisms. We have carried out an ecological study on benthic microbial nitrogen cycling in this area, and demonstrated that abundances of N-cycling functional groups respond differently to variations of environmental conditions, and metals Cu and Cd affect AOA/AOB dominance (Zhang et al. 2014). As another contribution, this study focused on the following objectives: (1) to investigate the relative importance of spatial (locations, layers) and seasonal controls on the distribution of ammonia oxidizers, denitrifiers, and anammox bacteria, and (2) to test the hypothesis that metals impact the diversity and community composition of nitrogen microbial community in the estuarine system.

Materials and Methods

101 Sampling and environmental parameters analysis

102

103 The physical conditions of three hypernutrified estuarine tidal flats in mouths of Jiaolai River (JL), Bailang River (BL)
104 and Di River (Di) of the Laizhou Bay has been described previously in detail (Zhang et al. 2014). These three rivers
105 have different pollution history and discharge sources (e.g. dying industries, mariculture and brine industries). The
106 sampling and analysis of the environmental parameters including dissolved oxygen, pH, salinity, and temperature of
107 overlying water, and nitrate, nitrite, ammonium, total organic carbon and nitrogen contents, sediment grain size, and
108 trace metal As, Co, Cd, Cr, Cu, Ni, Pb, Zn levels of sediments, were also performed as described in the previous
109 publication (Zhang et al. 2014).

110 In brief, surface sediment samples were collected in 2010 winter (November) and 2011 summer (August), and
111 three sediment replicates (JL1-3, BL1-3 and Di1-3) were randomly sampled in each estuarine tidal flat. The sediment
112 cores were sectioned into the upper layer (0-2 cm) and the lower layer (2-5 cm). The sediment samples were referred to
113 as the location, layer and season collected (e.g. JL-U-W and JL-L-W).

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115 DNA extraction and PCR amplification

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117 The environmental genomic DNA was extracted from 0.5 g of sediment using the Ultra Clean Soil DNA Isolation kit
118 (Mo-Bio, USA). DNA concentration was quantified using a NanoDrop 2000C Spectrophotometer (Thermo Scientific,
119 USA). The DNA was diluted 10 times before the PCR amplification.

120 PCR primers used in this study were shown in Table 1. The PCR amplification procedure was performed with a
121 Tprofessional Thermocycler (Biometra, Germany) using the PCR kit DreamTaqTM Green PCR Master Mix (Fermentas,
122 USA). The reactions were set up in volumes of 25 µl containing 1 µl template DNA, 400 nM of each primer, and 12 µl
123 of PCR Master Mix. PCR programs were as follows: 95°C for 3 min, 30–35 cycles of 95°C for 30 s, 57°C
124 (amoA1F/amoA-rnew) or 56°C (Arch-amoAF/Arch-amoAR) or 53°C (nosZF/nosZ1622R) or 58°C
125 (Pla46f-GC/Amx368r) for 40 s, followed by 72°C for 40 s, and finally 72°C for 10 min.

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127 Denaturing gradient gel electrophoresis, cloning and sequencing

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129 PCR products of AMB 16S rRNA gene were analyzed by denaturing gradient gel electrophoresis (DGGE) with a
130 DCode mutation detection system (Bio-Rad, USA). Forty µl PCR products (100-200 ng/µl) were loaded onto a 6%

polyacrylamide gel with a denaturant gradient between 20% and 80% (100% denaturant containing 7 M urea and 40% formamide). Electrophoreses were run at a constant voltage of 200 V and 60°C for 5h. Subsequently, the gels were stained for 30 min in 1× GeneFinder (Bio-V, China), and then visualized in an imaging system (Syngene, USA). The main bands were excised and incubated overnight at 4°C in 30μl sterilized deionized water as templates for reamplification. The PCR products were checked for single bands on DGGE, purified using a Purification Kit (Tiangen Biotech, China), and sequenced by a commercial company (Sangon, Shanghai, China). DGGE images were analyzed using Quantity One 2.1 (Bio-Rad, USA) to generate a densitometric profile. The peak areas of the fingerprint patterns were used to indicate the intensities. Bands with a relative intensity of less than 0.5% of the sum of all band intensities were discarded.

As for AOB *amoA*, AOA *amoA* and *nosZ* genes, triplicate of PCR products were pooled, ligated into the pTZ57R/T vector (Fermentas), and transferred into competent *Escherichia coli* TOP10 cells (Tiangen). Therefore, 12 clone libraries of each gene were constructed for 3 sampling locations, 2 layers and 2 seasons. Positive recombinants were selected using X-Gal-IPTG LB indicator plates amended with ampicillin (100 mg/ml). The insertion was determined by PCR amplification with the universal primer set M13F and M13R. Amplicons of correct size were digested separately with endonucleases (Fermentas) *HhaI*, *RsaI* for *amoA* gene (Jin et al. 2010), and *MspI*, *RsaI* for *nosZ* gene (Rich et al. 2003). Restriction fragments were resolved by electrophoresis on 2.5% agarose gels. Each restriction fragment length polymorphism (RFLP) pattern was defined as an operational taxonomic unit (OTU), and representative clones were randomly selected for sequencing (Sangon).

Alpha diversities (local diversities of a given community), such as Shannon (*H*) and Simpson (*D*) indices, were calculated based on number and intensities of DGGE bands, or number and relative abundance of OTUs in libraries. These indices were calculated with an online diversity calculator (<http://www.changbioscience.com>). The coverage (*C*) of clone libraries was calculated as $C=[1-(n1/N)]\times 100$, where *n1* is the number of unique (frequency=1) RFLP pattern detected in a library and *N* is the total number of clones in the same library (Mullins et al. 1995).

Phylogenetic analysis and sequence deposition

Possible chimerical DNA sequences were checked with programs CHIMERA_CHECK (Gontcharova et al. 2010) and Bellerophon (Huber et al. 2004). Nucleotide sequences were aligned with GenBank sequences using ClustalW (Thompson et al. 1994). Phylogenetic trees were constructed with MEGA 5.0 (Tamura et al. 2011) using the neighbor-joining method, and bootstrap resampling analysis for 1,000 replicates was performed to estimate the

161 confidence of the tree topologies.

162 The nucleotide sequences obtained in this study have been deposited in the GenBank database under accession
163 numbers JX465173 to JX465197 (AMB 16S rRNA), JX465198 to JX465201 (AOA *amoA*), JX465202 to JX465230
164 (AOB *amoA*), and JX465231 to JX465276 (*nosZ*).

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166 Statistical analyses

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168 Mean values of alpha diversities were compared with the pairwise *t-test* or one-way ANOVA analysis following by a
169 least significance difference (LSD) test at the 0.05 confidence level. Spearman's correlation coefficient (ρ) was
170 calculated to explore the relationship between alpha diversities and environmental variables. These analyses were
171 performed using the statistic software SPSS 13.0 for windows (SPSS, Chicago, USA).

172 Community clustering of nitrogen functional groups was analyzed with the principal coordinate analysis (PCoA)
173 using the UniFrac program (Lozupone and Knight 2005), according to the instructions at the UniFrac website
174 (<http://bmf2.colorado.edu/unifrac/index.psp>). Differences in community composition clustered by sampling location,
175 layer and season, were pairwise or globally tested based on weighted UniFrac metric. Relationships between microbiota
176 and environmental factors were analyzed using the software CANOCO (version 4.5, Microcomputer Power, Ithaca,
177 USA) (Ter-Braak and Smilauer 2002). A detrended correspondence analysis (DCA) was conducted in order to decide
178 whether a canonical correspondence analysis (CCA) or redundancy analysis (RDA) should be used in ordination
179 (Ysebaert and Herman 2002). The statistical significance of the variable added was tested using a Monte Carlo
180 permutation test (999 permutations).

181

182 Results

183

184 DGGE and clone library analyses

185

186 A total of 321 bands were detected in DGGE gels of AMB 16S rRNA (Fig. S1). The bands from triplicate samples were
187 combined for subsequent analyses, and then the number of bands per sample varied between 7 and 16 (Table S1). Of 12
188 *amoA* genes of AOB and AOA, and *nosZ* gene clone libraries, 375, 394 and 380 insert-positive clones were identified,
189 resulting in 29, 4 and 46 unique OTUs, respectively. The numbers of OTUs ranged from 2 to 9 (for AOB), from 2 to 4
190 (for AOA), and from 4 to 10 (for *nosZ*) (Table S1). The coverage (*C*) values of bacterial and archaeal *amoA* and *nosZ*

191 gene libraries were more than 80%, indicating that most ammonia oxidizer and denitrifier had been detected. Archaeal
192 *amoA* gene exhibited relatively lower diversities (H 0.10 ~ 0.60; D 0.08 ~ 0.28), while AMB 16S rRNA genes appeared
193 highly diverse (H 1.86 ~ 2.68; D 0.83 ~ 0.93) (Table S1).

194

195 Spatial and seasonal patterns of alpha diversities

196

197 ANOVA and *t-test* analyses were performed to compare the alpha diversity indices of ammonia oxidizers, denitrifiers
198 and anammox bacteria from different sampling locations (JL, BL and Di), layers (upper and lower) and seasons (winter
199 and summer) (Table 2). The results showed that the alpha diversities of AMB varied greatly among sampling locations,
200 layers, or seasons, with significantly lower indices in Di tidal flat (vs. JL and BL) ($P=0.004$ for H ; $P=0.001$ for D), in
201 upper layer (vs. lower) ($P=0.003$ for H ; $P=0.018$ for D) and in winter (vs. summer) ($P=0.015$ for H ; $P=0.022$ for D).
202 Alpha diversities of AOB *amoA* gene were significantly higher in winter than in summer ($P=0.025$ for H ; $P=0.048$ for
203 D). Nevertheless, AOA *amoA* gene was more diverse in the lower than in the upper layer in terms of D index ($P=0.043$),
204 but not in H ($P=0.068$). No differences were found for alpha diversities of *nosZ* gene.

205

206 Correlations between alpha diversities and environmental factors

207

208 In order to explore the relationship between gene diversities and environmental variables, Spearman's correlations were
209 performed (Table 3). Among the eight metals determined, six (Co, Cr, Cu, Ni, Pb and Zn) were collinear ($\rho > 0.64$,
210 $P < 0.05$), and thus only Pb, As and Cd was retained for subsequent correlation analyses. H and D of bacterial *amoA* were
211 negatively correlated with the concentration of $\text{NH}_4^+\text{-N}$ ($\rho = -0.76$ and -0.72 , $P < 0.01$, respectively), temperature ($\rho = -0.69$,
212 $P < 0.05$; $\rho = -0.72$, $P < 0.01$, respectively) and metal As ($\rho = -0.61$, $P < 0.05$), whereas H and D of archaeal *amoA* exhibited
213 no correlation with all environmental variables examined. *NosZ* gene diversities had positive correlations with
214 concentration of $\text{NO}_2^-\text{-N}$ ($\rho = 0.60$, $P < 0.05$ for H) and salinity ($\rho = 0.69$, $P < 0.05$ for H), and negative correlations with Pb
215 ($\rho = -0.64$ and -0.68 , $P < 0.05$, for H and D , respectively). H and D of AMB 16S rRNA genes were positively correlated
216 with Pb ($\rho = 0.74$ and 0.76 , $P < 0.01$), ratio of organic carbon to nitrogen (C/N, $\rho = 0.73$ and 0.72 , $P < 0.01$, respectively) and
217 $\text{NH}_4^+\text{-N}$ ($\rho = 0.70$ and 0.71 , $P < 0.05$, respectively), but negatively with pH of overlying water ($\rho = -0.72$ and -0.75 , $P < 0.01$,
218 respectively) and DO ($\rho = -0.59$, $P < 0.05$).

219

220 Phylogenetic analyses

221

222 The phylogenetic tree based on partial AOB *amoA* gene sequences revealed that no OTUs were common across all
223 samples (Fig. S2). BLASTing against GenBank revealed that A large proportion (246 out of 375) of the clones closely
224 matched with these found from hypernutrified sedimentary environments including San Francisco Bay (Mosier and
225 Francis 2008), Pearl River (Jin et al. 2011), Jiulong River, and Jiaozhou Bay (Dang et al. 2010b), indicating anammox
226 bacterial assemblages in these coastal environments are phylogenetically similar regardless of geographic locations. All
227 OTUs of AOB *amoA* were affiliated to β -Proteobacteria and distributed into 8 clusters. Two clusters containing 120
228 clones appeared to be *Nitrospira*-related, with the remaining clusters *Nitrosomonas*-related. In the
229 *Nitrospira*-related cluster, some OTUs were first detected in this study, probably representing novel *Nitrospira*
230 lineages. A *Nitrosomonas*-related OTU (AOB8) was closely related to sequences from Jiaozhou Bay A5 station, which
231 was characterized by high efflux of heavy metals from wastewater treatment plant (Dang et al. 2010b).

232 Phylogenetic analysis of AOA *amoA* gene showed that a majority (364 out of 394) of sequences were placed in the
233 “marine sediment/water” Crenarchaeota clade along with *Nitrosopumilus maritimus* (HM345611), a representative of
234 Crenarchaeota group 1.1a (Fig. S3). Phylogenetically, these sequences were closely related to those from sediments of
235 Jiaozhou Bay estuary (Dang et al. 2008), Bahia del Tobari estuary (Beman and Francis 2006) as well as wastewater
236 treatment plants (WWTP). Only 30 clones (7.6%) fell exclusively into the “hot spring/soil” Crenarchaeota clade, which
237 includes the Crenarchaeota group 1.1b clone Candidatus *Nitrososphaera gargensis* GA15P03. Sequences between two
238 groups exhibited only 79~80% similarities at the nucleotide level. The most dominant OTU AOA1 (88.8%) occurred in
239 all clone libraries. Interestingly, OTU AOA4 was never recovered from the Di tidal flat.

240 All *nosZ* OTUs were grouped into 3 major clusters in the phylogenetic tree (Fig. S4). Cluster 1 was mainly
241 comprised of clones closely related to α - and β -Proteobacteria, cluster 2 mostly to γ -Proteobacteria, and cluster 3 to
242 α -Proteobacteria only. There were no common genotypes across all samples and no OTUs appeared particularly
243 dominant. More than half of *nosZ* sequences were affiliated with the α -proteobacterial genera, such as *Ruegeria*, *Nisaea*,
244 *Polymorphum*, *Rhodobacter*, *Azospirillum*, *Mesorhizobium* and *Bradyrhizobium*. These genera were previously found in
245 marine sediment habitats, polluted soils, and activated sludge (Huang et al. 2001; Urios et al. 2008; Huo et al. 2011; Nie
246 et al. 2012). The second most abundant sequences were related to γ -Proteobacteria, including genera *Halomonas*,
247 *Marinobacter*, *Pseudomonas* and *Alcaligenes*, which were used in discoloration (Mabinya et al. 2011), heavy metals
248 biosorption (Hussein et al. 2004) or reduction of chemical oxygen demand (COD) (Rajeshkumar and Jayachandran
249 2004) from high salt wastewater.

250 A total of 25 DGGE gel bands of AMB in different positions were excised and sequenced. All sequences grouped

251 into 4 clusters in the phylogenetic tree based on the 16S rRNA genes (Fig. S5). A large proportion (15 out of 25) of our
252 sequences was grouped into the cluster 1, a distinct clade with <87% sequence identity against GenBank, suggesting
253 novel genetic diversity of AMB species in the Laizhou Bay estuaries. In cluster 2, four sequences affiliated to
254 *Candidatus Scalindua* were only recovered from winter samples. None of our sequences was placed in Cluster 3, which
255 included other well-known AMB (e.g., Kuenenia, Brocadia, Jettenia asiatica, and Anammoxoglobus propionicus).
256 Cluster 4, a putative anammox or Planctomycetes cluster, contained the sequences only occurring in summer.

258 Spatial and seasonal patterns of community structure

260 According to weighted UniFrac significance tests, the community structure of AOB ($P=0.03$) and AMB ($P<0.01$) was
261 seasonally different. However, the community differences in AOA or *nosZ*-type denitrifiers were insignificant across
262 locations, layers or seasons ($P > 0.10$) (Table 4). These results were consistent with the PCoA plots (Fig. 1), in which
263 AOB and AMB were evidently separated into winter and summer groups, with the first principal coordinates (P1)
264 explaining 73.02% and 77.93% of the total community variability respectively, whereas no distinct patterns could be
265 recognized for either AOA or denitrifiers.

267 Environmental factors influencing community turnover

269 To further understand how environmental factors impacted target gene distributions, the weighted CCA analyses
270 were made. DCA results showed that CCA model better approximated AOB *amoA* and *nosZ* species relationship to the
271 explanatory variables (maximum gradient length greater than 4), but RDA model should be selected for AMB 16S
272 rRNA and AOA *amoA* (maximum gradient length less than 3). The optimal models were produced with manual
273 deselection of collinear variables and manual forward selection via Monte Carlo permutation significance tests. Of all
274 the environmental factors analyzed, salinity was significantly correlated with community changes of AOB ($P=0.009$;
275 Fig. 2A). Sediment grain size was the most important factor influencing the community structure of AOA ($P=0.048$)
276 (Fig. 2B). The metal As had the strongest correlation with *nosZ*-type denitrifier community changes, though no
277 variables were significant ($P=0.16$) (Fig. 2C). For AMB, several environmental factors, such as temperature ($P=0.001$),
278 dissolved oxygen ($P=0.007$), pH ($P=0.016$) and metals As ($P=0.029$) and Cd ($P=0.03$), presented significant
279 correlations (Fig. 2D); summertime samples of the Di tidal flat with high pH values grouped separately from these of JL
280 and BL (Fig. 2D).

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Discussion

Sediments provide a range of niches for N-cycling microbes which could co-occur or spatiotemporally isolate. Our study investigated the alpha diversities and community structure of four functional groups, which are responsible for interlinked N-cycling processes in sediment samples. We explored their spatial and seasonal patterns and associations with environmental conditions in estuarine tidal flats. It should be noted that the methodologies (i.e., DGGE and clone library analysis) we employed in this study may have some limitations. These include the non-intensive sampling of communities, which leads to the incapability to detect uncommon or rare phylotypes in the communities and thus underestimation of richness (Bent and Forney 2008). Nevertheless, Shannon and Simpson indices can be estimated more accurately because rare phylotypes generally have a smaller relative numerical impact, and the use of fingerprinting methods for many of samples, followed by cluster analysis or clone library analysis, are still useful in revealing distributional patterns and composition of microbial communities (Bent and Forney 2008). In this study, we found that ammonia oxidizers, denitrifiers, and anammox bacteria had contrasting distributional patterns across locations, layers or seasons, which provides an integrated view of the community assembly and underlying mechanisms of N-cycling functional groups in the these typical coastal habitats.

Ammonia oxidizers

Differences in ammonia oxidizers distribution in estuaries are undoubtedly determined by the complex interplay of biological and environmental variables. In this study, both ANOVA and UniFrac significance tests indicated that AOB diversity and community turnover in sediment exhibited distinct seasonal patterns (Fig. 1, Table 2). Furthermore, our correlation analysis indicated that the lower diversity of AOB in summer could be due to higher concentrations of ammonium and As in the sediment and the higher temperature (Table 3). The higher temperature will increase the activity of heterotrophic microbes in sediment, leading to the increase of remineralization which supplies more ammonium for ammonia oxidizers, of which increase of abundance should be expected. In contrast, we observed a negative correlation between AOB abundance and temperature (Zhang et al. 2014). Despite that AOB diversity was demonstrated to be higher in low-salinity sites than in high-salinity sites (Sahan and Muyzer 2008), no significant correlations between salinity and alpha diversities were found in our study. These indicate that temperature should represent a highly limited factor for AOB diversity and community size, for which differentiated ecology of AOB clades

at different temperatures could be accounted. For instance, Sahan and Muyzer (2008) showed that *Nitrosomonas* spp. often was dominant in high-temperature (May, July and September) seasons, while *Nitrospira* was abundant in low-temperature seasons in estuarine sediments. Tourna et al. (2008) demonstrated that in slurry incubation, *Nitrospira* cluster 1 completely disappeared at high temperature (30°C). Our CCA showed that salinity was the most influential physicochemical factor structuring AOB community composition (Fig. 2), which is consistent with previous studies (Francis et al. 2003; Bernhard et al. 2005; Mosier and Francis 2008; Jin et al. 2011). AOB at the high-salinity sites were most similar to *Nitrospira* clade, but at the mid- and low-salinity sites were distributed among *Nitrospira* and *Nitrosomonas* sequences (Bernhard et al. 2005). Taken together, these data suggest that alpha diversity and community composition of AOB in surface sediment are primarily shaped by temperature and salinity, respectively, which, in turn, account for the seasonal patterns of AOB distribution in these environments.

Our data suggest that AOA have rather stable diversity and composition in estuarine sediments, regardless of reason and location (Table 2, 4). The only significant factors we observed for Simpson index and community structure lie in the vertical distribution (upper and lower layers) and grain size of sediment (Table 2, Fig. 2). Nevertheless, although this study demonstrates clearly the distributional patterns of AOA in both diversity and composition in estuarine sediments, the underlying environmental mechanisms remain inconclusive, as the different layers and grain sizes of sediment are closely related to other environmental parameters (Andrieux-Loyer and Aminot 2001), such as dissolved oxygen and pH, which were not measured for sediment in this study. In fact, sediment pH was found to be important for AOA distribution in sediments and soils (Erguder et al. 2009; Li et al. 2011).

The negative correlation between AOB diversity and metal As found in this study is coincident with our recent report that the AOB abundance was negatively correlated with As in the studied samples (Zhang et al. 2014), suggesting that AOB are sensitive to As, as have been demonstrated in a laboratory study that As (III and V) decreased soil potential nitrification activity and was toxic to AOB (Gong et al. 2002). The metal As is toxic to bacteria by inhibiting basic cellular functions, which are often linked with energy metabolism, microbial biomass C and respiration (Ghosh et al. 2004). Nevertheless, no correlation between AOA and metals was found in this study, suggesting that AOA could be more tolerant to As than AOB. In contrast, metal As contributed the most to AOA distinction in polluted mangrove sediments (Cao et al. 2011), possibly because As presented with much higher concentrations in the mangrove sediment.

Denitrifiers

Similar to AOA, *nosZ*-type denitrifiers exhibited neither spatial nor seasonal patterns of diversity and composition in

341 this study. This is contrast to the notion that salinity is a major driver of denitrifying communities in aquatic
342 environments on the global scale (Jones and Hallin 2010). The insignificant effect of salinity on community turnover of
343 *nosZ*-type denitrifiers could be due to the fact that most of our samples were much more saline (36.3–50.3 psu) than
344 generally expected for estuarine samples. The positive correlation between salinity and *nosZ* gene diversity in this study
345 remain unexplainable (Table 3), as many bacteria cannot survive in high-salinity environments, thus the high salinity
346 should limit the bacterial diversity.

347 Pb was negatively correlated with alpha diversities of *nosZ* gene (Table 3), suggesting a potentially toxic effect of
348 Pb on *nosZ*-type denitrifiers. The links between heavy metals and denitrifiers have been demonstrated in previous
349 studies. For example, increase of Pb concentration in soils led to greater sensitivity of N₂O reductase (Bollag and
350 Barabasz 1979). Pb also affected the diversity of soil *nirK*-type denitrifiers even at the low concentration (1 ppm)
351 (Sobolev and Begonia 2008). In San Francisco Bay estuary, Pb was also closely correlated with *nirS*-type denitrifier
352 abundance (Moister and Francis 2010). Nevertheless, these potential effects on denitrifiers should be only attributed to
353 Pb, the associations with other metals (Cu, Co, Cr, Ni and Zn), which are collinear with Pb, are also potential
354 contributors (Cao et al. 2008).

355

356 Anammox bacteria

357

358 Comparing with other estuarine environments, such as Cape Fear River estuary (Dale et al. 2009), Mai Po Nature
359 Reserve estuary (Li et al. 2011) and Yodo River estuary (Amano et al. 2007), the diversity of AMB in Laizhou Bay
360 estuaries was relatively lower, as only the “*Scalindua*”-like phylotypes were recovered. This result is consistent with
361 well-known adaptation of “*Scalindua*” to saline environments, and “*Kuenenia*” and “*Brocadia*” are low-salinity taxa
362 (Dale et al. 2009; Junier et al. 2010). Similar to a previous study of Jiaozhou Bay estuaries (Dang et al. 2010a), this
363 study of LZB estuaries also revealed some phylotypes which presented as putatively new clusters of AMB within the
364 phylum Planctomycetes (Fig. S5). Despite of the lower alpha diversities, the abundance of AMB was high in the
365 investigated sites (Zhang et al. 2014), suggesting an important role of AMB in N₂ production in the sediments of LZB
366 tidal flats.

367 In the high-salinity estuaries of LZB, we identified temperature and pH were significantly correlated with
368 community changes of AMB (Fig. 2). High temperature environments provide special niches for some AMB, such as
369 the presence of “*Brocadia*” and “*Kuenenia*” phylotypes in hot spring and deep-sea hydrothermal vents (Byrne et al.
370 2009; Jaeschke et al. 2009), and a novel “*Scalindua* sinooilfield” clade in high-temperature oil reservoirs (Li et al. 2010).

pH can affect the equilibrium of $\text{NH}_3/\text{NH}_4^+$ in environments. Higher pH is conducive to the formation of free NH_3 , of which higher concentrations could inhibit anammox (Aktan et al. 2012). This explains the negative correlations of pH with diversity (Table 3), and composition of AMB (Fig. 2), and a separate AMB assemblage in the Di estuary where a large amount of alkaline water was discharged. In the meantime, our data showed that higher C/N ratio could promote the diversity of AMB. However, Hu et al. (2012) observed higher diversity of AMB in samples with lower organic contents. The strong competition or cooperation for nitrite may occur between AMB and denitrifiers in low organic environments, leading to higher diversity of AMB (Dalsgaard and Thamdrup 2002; Rysgaard et al. 2004). In addition, heavy metals (e.g. Pb, As and Cd) also strongly influenced AMB diversity and composition in LZB sediment samples (Fig. 2, Table 3). Similarly, a significant correlation between Pb and *hzo* gene (a functional gene of AMB) diversity was revealed (Li et al. 2011).

381

382 **Conclusions**

383

384 In summary, we investigated the spatial and seasonal patterns of alpha diversities and community turnover of
385 co-occurring AOA, AOB, *nosZ*-type denitrifiers and AMB in sediment samples of LZB estuarine tidal flats. Generally
386 speaking, these four N-cycling functional groups exhibited contrasting distribution patterns: seasonality was evident for
387 diversities and community composition of AOB and AMB, but not for AOA and *nosZ*-type denitrifiers; diversities of
388 these N-cycling groups seldom varied with location of sampling sites or sediment layer, except for AMB which
389 exhibited significant difference among locations and between layers. Further analyses identified different sets of
390 physicochemical parameters that could explain the distributional patterns of these functional groups. In particular, we
391 found that the distributions of bacterial N-cycling players (e.g., AOB, *nosZ*-type denitrifier and AMB) were potentially
392 related to several heavy metals in sediments, which, however, was never found for AOA in this study. Overall, our
393 results highlight the differentiated distributional patterns of co-occurring N-cycling functional microbes in coastal
394 sediments.

395

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400

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568

569 **Tables**

570 **Table 1** Characteristics of the PCR primers used in the study

Application	Target gene	Primer	Sequence (5'-3')	Length of amplicon (bp)	Reference	
Cloning	AOB <i>amoA</i>	amoA1F	GGGGTTTCTACTGGTGGT	490	Agogu�� et al. 2008	
		amoArnew	CCCCTCBGSAAAVCCTTCTTC			
	AOA <i>amoA</i>	Arch-amoAF	STAATGGTCTGGCTTAGACG	635	Agogu�� et al. 2008	
		Arch-amoAR	GCGGCCATCCATCTGTATGT			
DGGE	AMB16S rRNA	<i>nosZ</i>	nosZ-F	CGYTGTTCMTCGACAGCCAG	267	Zhou et al. 2011
			nosZ1622R	CGSACCTTSTTGCCSTYGCG		
			Pla46f	GGATTAGGCATGCAAGTC	453	Sanchez-Melsio et al. 2009
			AMX368r-GC*	CCTTTCGGGCATTGCGAA		
571	GC*=CCGCCGCGCGGCGGGCGGGGCGGGGCACGGGGGG					
572						

573 **Table 2** Comparison of Shannon (*H*) and Simpson (*D*) diversities of target genes across sampling locations or layers or
574 seasons using the one-way ANOVA or *t*-test analyses*

	AOB <i>amoA</i>		AOA <i>amoA</i>		<i>nosZ</i>		AMB 16S rRNA	
	<i>H</i>	<i>D</i>	<i>H</i>	<i>D</i>	<i>H</i>	<i>D</i>	<i>H</i>	<i>D</i>
Location								
JL	1.14±0.17	0.63±0.06	0.38±0.06	0.18±0.04	1.31±0.26	0.63±0.10	2.43±0.13 ^a	0.9±0.01 ^a
BL	1.10±0.31	0.56±0.10	0.31±0.10	0.14±0.05	1.35±0.09	0.71±0.02	2.48±0.06 ^a	0.9±0.01 ^a
Di	1.21±0.23	0.61±0.09	0.20±0.05	0.09±0.02	1.65±0.18	0.76±0.05	1.96±0.04 ^b	0.84±0.00 ^b
<i>P</i> -value	0.951	0.844	0.270	0.264	0.417	0.379	0.004	0.001
Layer								
Upper	0.99±0.16	0.55±0.06	0.21±0.05	0.09±0.02	1.43±0.11	0.71±0.04	2.23±0.12	0.88±0.01
Lower	1.31±0.19	0.64±0.06	0.38±0.06	0.18±0.03	1.45±0.20	0.69±0.07	2.35±0.12	0.89±0.01
<i>P</i> -value	0.194	0.283	0.068	0.043	0.901	0.782	0.003	0.018
Season								
Winter	1.43±0.13	0.70±0.03	0.29±0.05	0.13±0.03	1.50±0.14	0.74±0.03	2.17±0.10	0.87±0.01
Summer	0.87±0.16	0.50±0.06	0.31±0.08	0.14±0.04	1.38±0.17	0.65±0.07	2.41±0.13	0.90±0.01
<i>P</i> -value	0.025	0.048	0.848	0.845	0.661	0.344	0.015	0.022

575 *Values are provided as mean and standard errors. Different superscript letters indicate significant pairwise difference
576 ($P<0.05$) in multiple comparison following ANOVA. All significant differences ($P<0.05$) are highlighted in bold.
577

578 **Table 3** Spearman's correlation coefficients (ρ) between environmental factors and Shannon (H) and Simpson indices
579 (D) of target genes across seasons, locations and layers*

	AOB <i>amoA</i>		AOA <i>amoA</i>		<i>nosZ</i>		AMB 16S rRNA	
	<i>H</i>	<i>D</i>	<i>H</i>	<i>D</i>	<i>H</i>	<i>D</i>	<i>H</i>	<i>D</i>
As	-0.61	-0.61	ns	ns	ns	ns	ns	ns
Cd	ns	ns	ns	ns	ns	ns	ns	ns
Pb	ns	ns	ns	ns	-0.64	-0.68	0.74	0.76
C/N	ns	ns	ns	ns	ns	ns	0.73	0.72
DO	ns	ns	ns	ns	ns	ns	-0.59	-0.59
Grain size	ns	ns	ns	ns	ns	ns	ns	ns
NH ₄ ⁺ -N	-0.76	-0.72	ns	ns	ns	ns	0.70	0.71
NO ₂ ⁻ -N	ns	ns	ns	ns	0.60	ns	ns	ns
NO ₃ ⁻ -N	ns	ns	ns	ns	ns	ns	ns	ns
pH	ns	ns	ns	ns	ns	ns	-0.72	-0.75
Salinity	ns	ns	ns	ns	0.69	ns	ns	ns
Temperature	-0.69	-0.71	ns	ns	ns	ns	ns	ns

580 *Only the significant correlations ($P<0.05$) are shown, and the highly significant correlations ($P<0.01$) are highlighted
581 in bold.

582 *C/N=ratio of organic carbon to nitrogen; DO = dissolved oxygen; ns= not significant.
583

584 **Table 4** Comparisons of community composition of target genes among different sampling stations, layers or seasons
585 using weighted UniFrac significance test*

Group pairs	<i>P</i> -value			
	AOB <i>amoA</i>	AOA <i>amoA</i>	<i>nosZ</i>	AMB 16S rRNA
Location				
JL vs. BL	0.96	1.00	0.99	1.00
BL vs. Di	1.00	1.00	1.00	1.00
JL vs. Di	1.00	1.00	1.00	0.45
Layer				
Upper vs. Lower	0.73	0.78	0.42	0.99
Season				
Winter vs. Summer	0.03	0.96	0.68	≤ 0.01

586 *The significant correlations ($P < 0.05$) are highlighted in bold.

587 **Figure Captions**

588

589 **Figure 1** Plots of principal coordinate analysis based on UniFrac weighted metric of AOB-*amoA* (A), AOA-*amoA* (B),
590 *nosZ* (C), and AMB-16S rRNA (D), showing spatial and/or seasonal changes in N-cycling microbial assemblages in
591 sediments of estuarine tidal flats

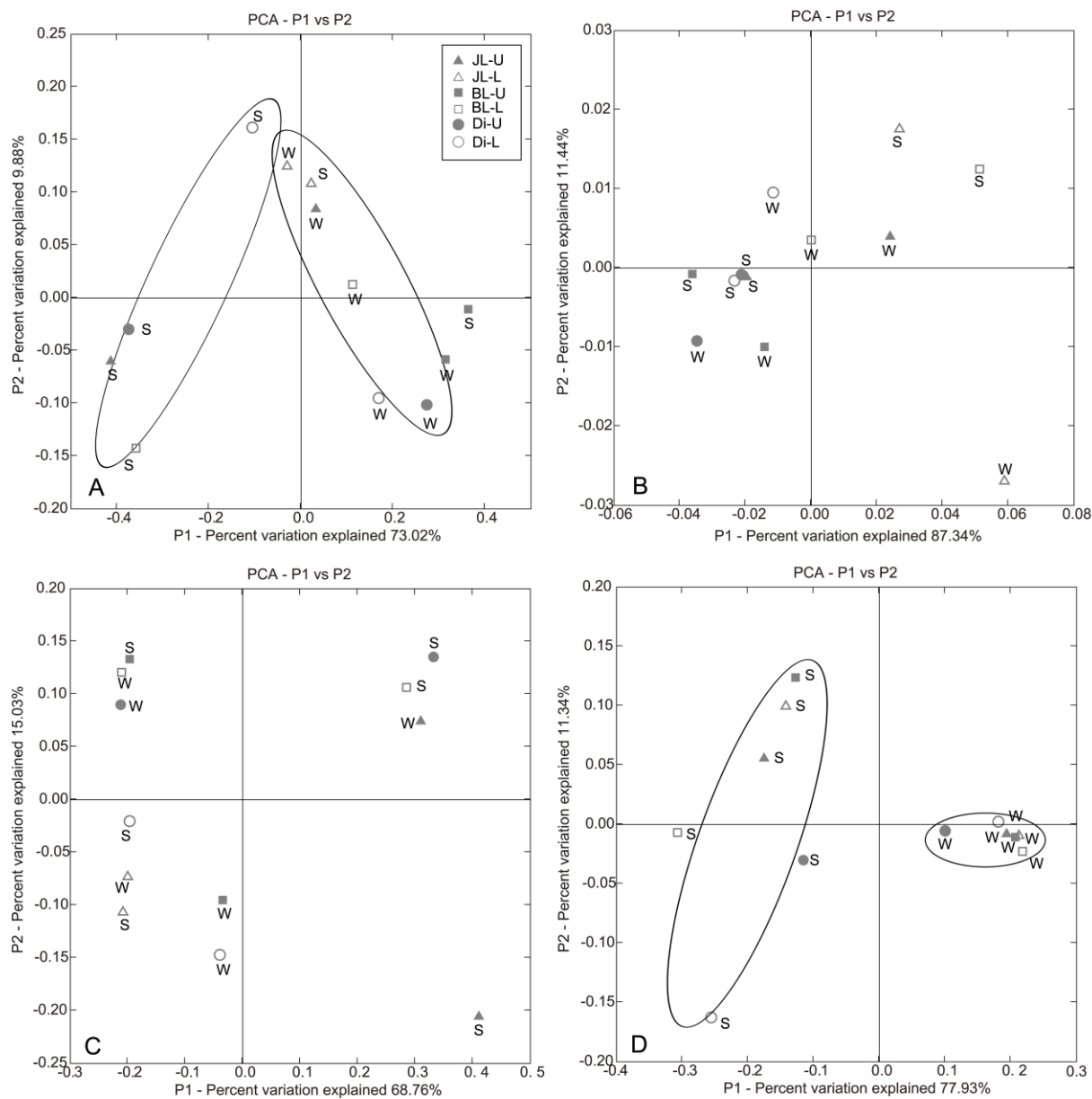
592

593 **Figure 2** Plots of canonical correspondence analysis and redundancy analysis showing the relationships between the
594 environmental factors and N-cycling microbial communities of AOB-*amoA* (A), AOA-*amoA* (B), *nosZ* (C), and
595 anammox bacterial 16S rRNA genes (D)

596

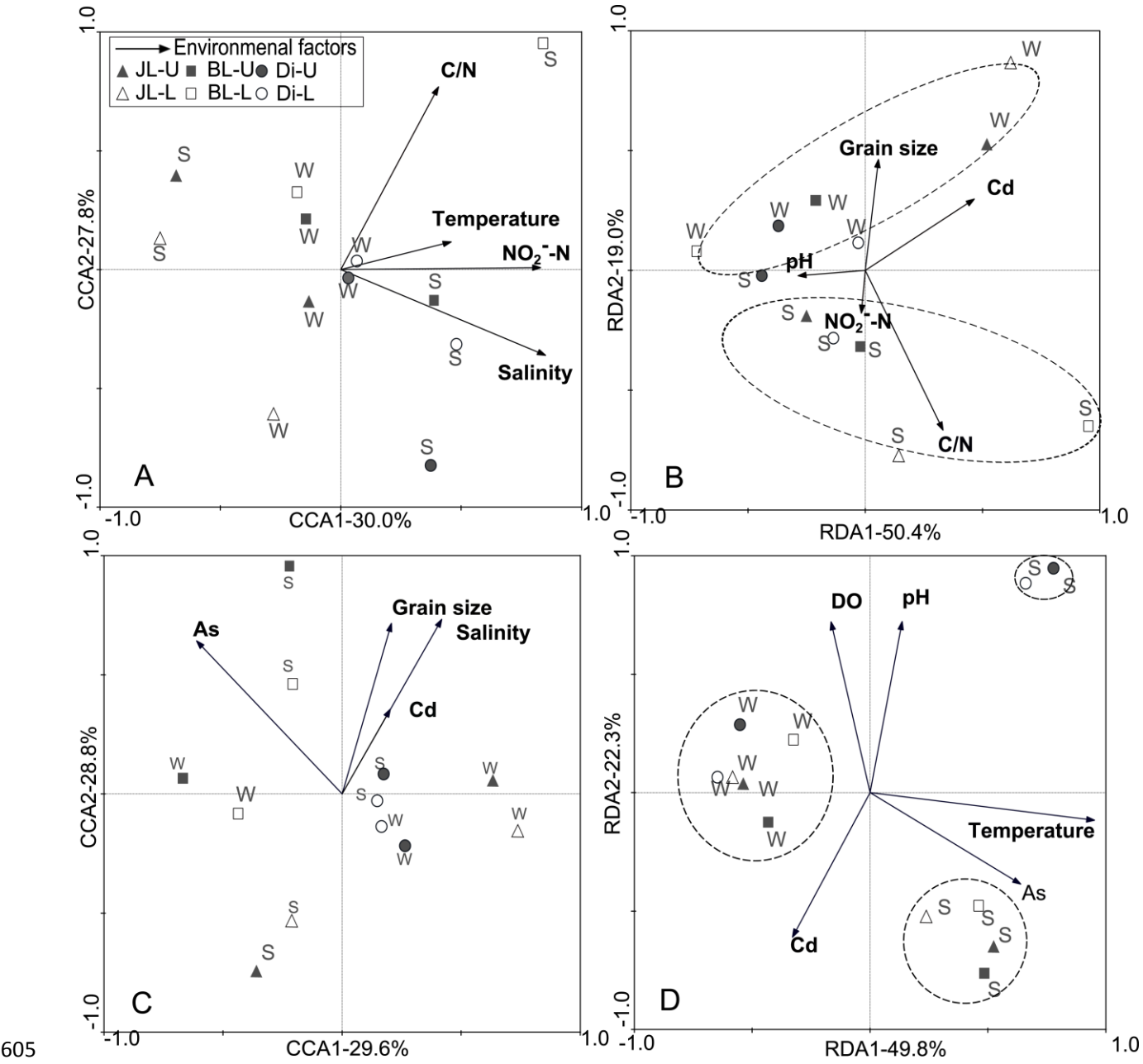
597

598 Figure 1



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603 Figure 2
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606 **Supporting Information**

607

608 **Table S1** Shannon (*H*) and Simpson (*D*) diversity and coverage (*C*%) estimates for AMB16S rRNA, AOB *amoA*, AOA *amoA*, and *nosZ* genes

Sample	DGGE			Clone libraries														
	AMB 16S rRNA			AOB <i>amoA</i>					AOA <i>amoA</i>					<i>nosZ</i>				
	Bands	<i>H</i>	<i>D</i>	No. of clones	OTUs	<i>C</i> %	<i>H</i>	<i>D</i>	No. of clones	OTUs	<i>C</i> %	<i>H</i>	<i>D</i>	No. of clones	OTUs	<i>C</i> %	<i>H</i>	<i>D</i>
JL-U-W	9	2.11	0.87	43	6	97.7	1.57	0.76	37	4	97.3	0.43	0.19	26	5	96.2	1.42	0.72
JL-L-W	11	2.32	0.90	30	5	96.7	1.25	0.67	37	3	97.3	0.42	0.22	27	8	96.3	1.99	0.86
BL-U-W	11	2.31	0.89	27	4	91.3	1.12	0.62	36	3	94.4	0.20	0.08	43	7	97.7	1.44	0.74
BL-L-W	13	2.46	0.91	32	9	87.5	1.98	0.82	32	3	96.9	0.27	0.11	25	4	96.0	1.07	0.65
Di-U-W	7	1.86	0.83	35	5	94.3	1.21	0.65	31	2	96.8	0.10	0.04	36	7	100.0	1.83	0.83
Di-L-W	8	1.96	0.85	33	9	85.8	1.48	0.65	30	3	96.7	0.32	0.15	44	6	97.7	1.27	0.68
JL-U-S	15	2.60	0.92	25	4	96.0	0.90	0.53	29	3	93.1	0.20	0.08	29	5	97.4	1.00	0.53
JL-L-S	16	2.68	0.93	50	3	100.0	0.83	0.54	33	3	100.0	0.48	0.24	38	5	97.4	0.85	0.40
BL-U-S	14	2.53	0.91	34	3	100.0	0.58	0.39	26	2	100.0	0.17	0.08	37	5	97.3	1.42	0.73
BL-L-S	15	2.60	0.92	44	3	100.0	0.73	0.41	40	4	97.5	0.60	0.28	44	7	95.5	1.48	0.70
Di-U-S	8	1.97	0.85	33	2	100.0	0.55	0.37	30	3	93.3	0.20	0.08	27	6	88.9	1.45	0.69
Di-L-S	9	2.06	0.85	36	6	97.2	1.60	0.77	33	3	93.9	0.20	0.08	30	10	86.7	2.05	0.85

609 **Figure S1** DGGE profile of anammox bacterial 16S rRNA gene from Laizhou Bay tidal flats in winter (A) and in
610 summer (B). Position of bands was highlighted with the numbered arrows

611

612 **Figure S2** A neighbor-joining tree showing the phylogenetic positions of ammonia-oxidizing bacterial *amoA* gene from
613 tidal flats of Laizhou Bay. Bootstrap values no less than 50% are shown at the nodes. The scale bar indicates 0.1
614 nucleotide substitution per site. The γ -proteobacterial *Nitrosococcus oceani* is used as the outgroup

615

616 **Figure S3** A neighbor-joining phylogenetic tree showing the affiliation of ammonia-oxidizing archaeal *amoA* genes
617 obtained from tidal flats of Laizhou Bay. Bootstrap values no less than 50% are shown at the nodes. The scale bar
618 indicates 0.05 nucleotide substitution per site. The ammonia-oxidizing bacteria (JX465202) from tidal flats of Laizhou
619 Bay is used as the outgroup

620

621 **Figure S4** Phylogenetic analysis of denitrifying *nosZ* gene obtained from Laizhou Bay estuarine tidal flats. Bootstrap
622 values no less than 50% were shown at the nodes. The scale bar indicates 0.1 nucleotide substitution per site. The
623 δ -proteobacterial *Anaeromyxobacter dehalogenans* is used as the outgroup

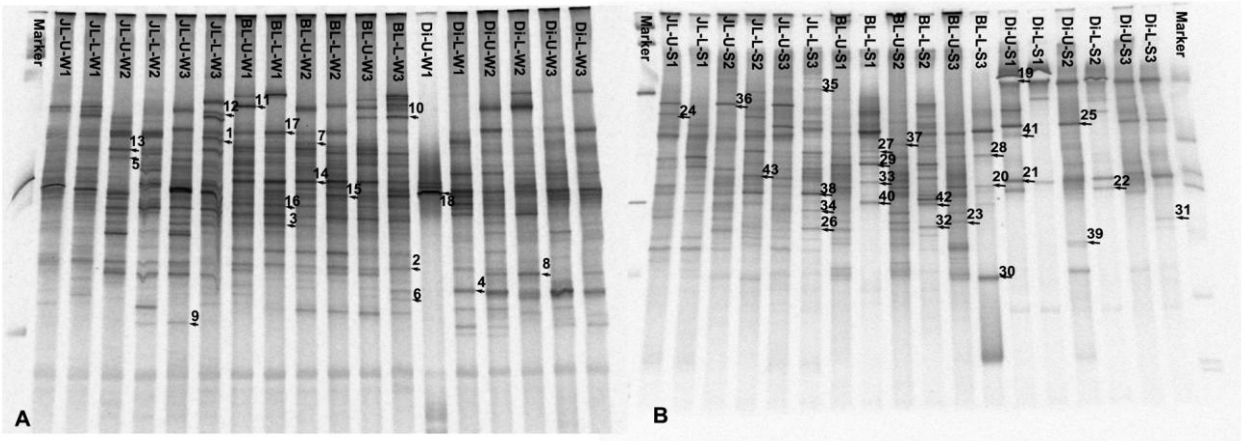
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625 **Figure S5** Phylogenetic relationships of anammox bacterial 16S rRNA genes as determined by neighbor-joining method.
626 Bootstrap values no less than 50% were shown at the nodes. The scale bar indicates 0.1 nucleotide substitution per site.
627 Two Planctomycetes species (*Isosphaera pallida* and *Pirellula* sp.) are used as the outgroup

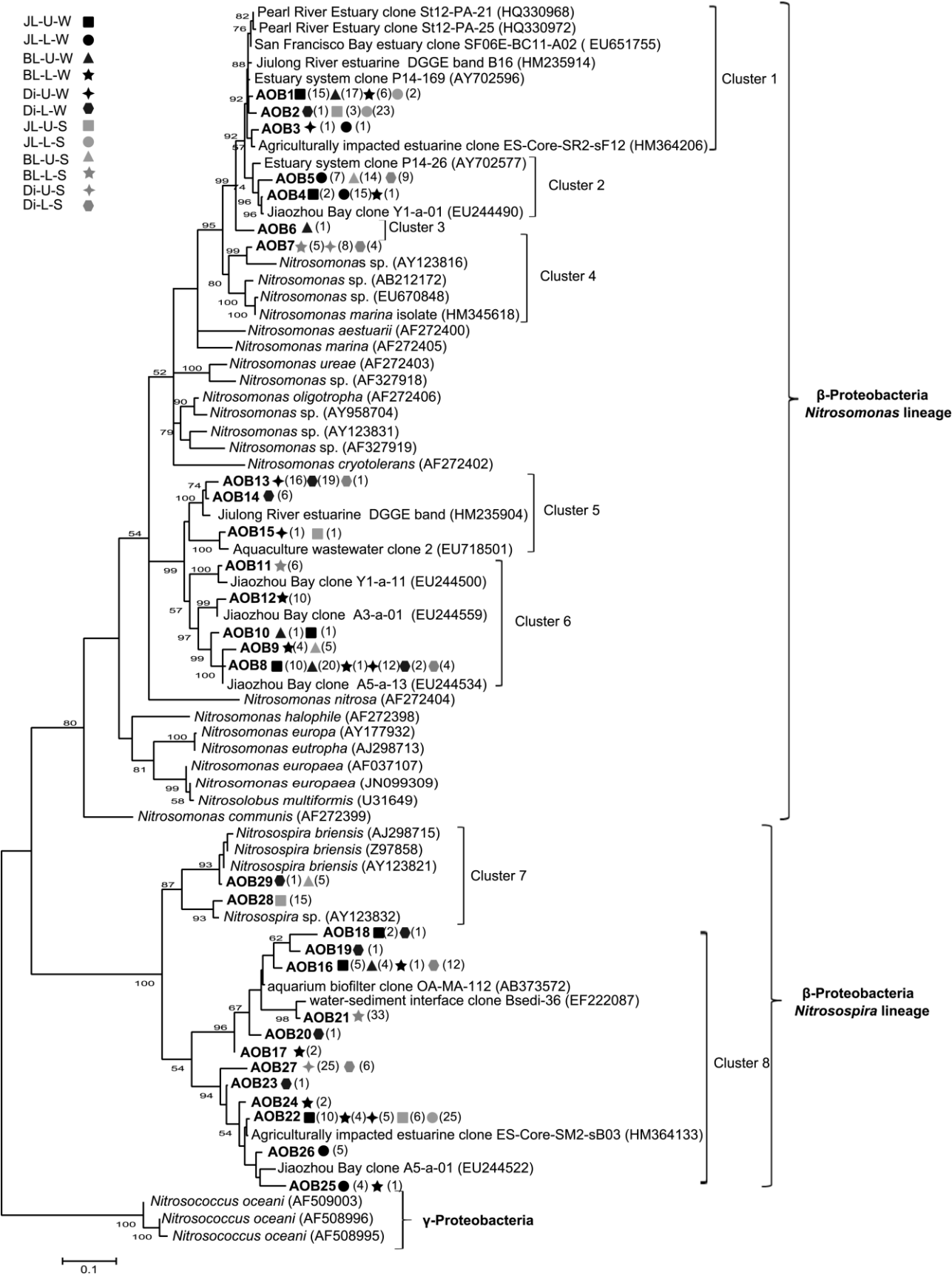
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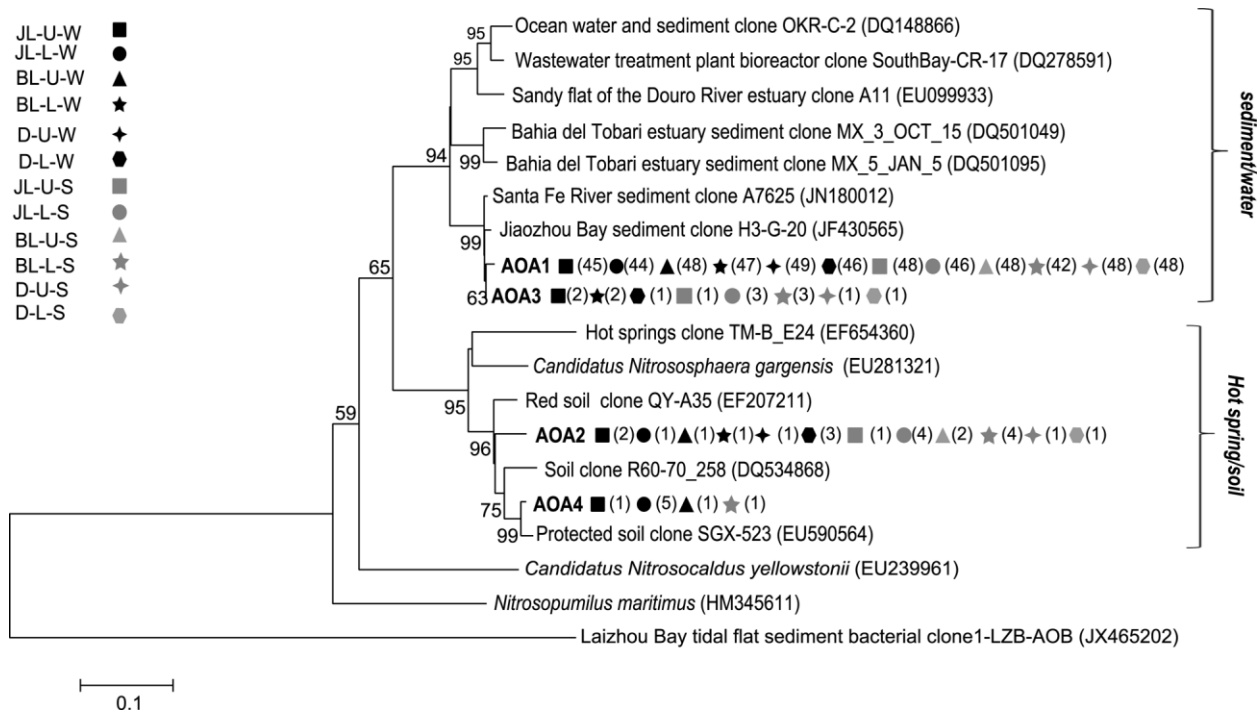
630 Figure
631 S1



632
633



637 Figure S3



638
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641
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