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# 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

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16

17 **Abstract**

18

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.

37

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

39

40

## 41 **Introduction**

42

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  $\beta$ -proteobacterial *Nitrosomonas* and *Nitrospira*, and a few in the  $\gamma$ -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- $\alpha$  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

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

77 Estuarine sediments are also sinks of metals from different origins. Trace amounts of some metals, which are  
78 necessary cofactors in enzymes or co-enzymes and electron transport chains, can be stimulatory to microbial activity  
79 (Granger and Ward 2003, Yang et al. 2013). There are evidences that metals may be an important factor in regulating  
80 nitrogen transformations in sediment habitats. For example, in the Douro River estuary (north-west Portugal), the  
81 transcription diversity of *nosZ* gene showed a drastic decrease with the increase of Cu concentration (Magalhães et al.  
82 2011). The metal toxicity was modulated by sediment properties (metal concentrations, grain size, organic carbon to  
83 nitrogen ratio, etc.), and denitrification revealed high sensitivity to heavy metals Cu, Cr, Pb, Zn and Cd in sandy sites  
84 but not in muddy sites (Magalhães et al. 2007). However, the effect of heavy metals on nitrogen cycling populations in  
85 sediments has not been studied sufficiently, and little is known about the linkage between metals and the nitrogen  
86 microbial community structure in estuarine systems.

87 Estuarine tidal flats of the Laizhou Bay (LZB), a semi-enclosed bay of the Bohai Sea, northern China, have been  
88 hypernutrified due to high level of dissolved inorganic nitrogen (DIN), organic pollutants and heavy metals discharged  
89 from coastal industries (e.g. aquaculture, subsurface brine industries and dyeing industries) and agriculture (Hu et al.  
90 2010; Zhang et al. 2014). These tidal flats thus present ideal environments for studying the multiple environmental  
91 stresses on nitrogen-removal microorganisms. We have carried out an ecological study on benthic microbial nitrogen  
92 cycling in this area, and demonstrated that abundances of N-cycling functional groups respond differently to variations  
93 of environmental conditions, and metals Cu and Cd affect AOA/AOB dominance (Zhang et al. 2014). As another  
94 contribution, this study focused on the following objectives: (1) to investigate the relative importance of spatial  
95 (locations, layers) and seasonal controls on the distribution of ammonia oxidizers, denitrifiers, and anammox bacteria,  
96 and (2) to test the hypothesis that metals impact the diversity and community composition of nitrogen microbial  
97 community in the estuarine system.

98

## 99 **Materials and Methods**

100

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).

114

115 DNA extraction and PCR amplification

116

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 DreamTaq<sup>TM</sup> Green PCR Master Mix (Fermentas,  
122 USA). The reactions were set up in volumes of 25  $\mu$ l containing 1  $\mu$ l template DNA, 400 nM of each primer, and 12  $\mu$ l  
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.

126

127 Denaturing gradient gel electrophoresis, cloning and sequencing

128

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  $\mu$ l PCR products (100-200 ng/ $\mu$ l) were loaded onto a 6%

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

140 As for AOB *amoA*, AOA *amoA* and *nosZ* genes, triplicate of PCR products were pooled, ligated into the pTZ57R/T  
141 vector (Fermentas), and transferred into competent *Escherichia coli* TOP10 cells (Tiangen). Therefore, 12 clone  
142 libraries of each gene were constructed for 3 sampling locations, 2 layers and 2 seasons. Positive recombinants were  
143 selected using X-Gal-IPTG LB indicator plates amended with ampicillin (100 mg/ml). The insertion was determined by  
144 PCR amplification with the universal primer set M13F and M13R. Amplicons of correct size were digested separately  
145 with endonucleases (Fermentas) *HhaI*, *RsaI* for *amoA* gene (Jin et al. 2010), and *MspI*, *RsaI* for *nosZ* gene (Rich et al.  
146 2003). Restriction fragments were resolved by electrophoresis on 2.5% agarose gels. Each restriction fragment length  
147 polymorphism (RFLP) pattern was defined as an operational taxonomic unit (OTU), and representative clones were  
148 randomly selected for sequencing (Sangon).

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

154

155 Phylogenetic analysis and sequence deposition

156

157 Possible chimerical DNA sequences were checked with programs CHIMERA\_CHECK (Gontcharova et al. 2010) and  
158 Bellerophon (Huber et al. 2004). Nucleotide sequences were aligned with GenBank sequences using ClustalW  
159 (Thompson et al. 1994). Phylogenetic trees were constructed with MEGA 5.0 (Tamura et al. 2011) using the  
160 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*).

165

166 Statistical analyses

167

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 ( $\rho$ ) 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  $\beta$ -Proteobacteria and distributed into 8 clusters. Two clusters containing 120  
228 clones appeared to be *Nitrosospira*-related, with the remaining clusters *Nitrosomonas*-related. In the  
229 *Nitrosospira*-related cluster, some OTUs were first detected in this study, probably representing novel *Nitrosospira*  
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  $\alpha$ - and  $\beta$ -Proteobacteria, cluster 2 mostly to  $\gamma$ -Proteobacteria, and cluster 3 to  
242  $\alpha$ -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  $\alpha$ -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  $\gamma$ -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.

257

258 Spatial and seasonal patterns of community structure

259

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.

266

267 Environmental factors influencing community turnover

268

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).

281

282 **Discussion**

283

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

297

298 Ammonia oxidizers

299

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

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

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

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

337

338 Denitrifiers

339

340 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 N<sub>2</sub>O 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 N<sub>2</sub> 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* sinoilfield” clade in high-temperature oil reservoirs (Li et al. 2010).

371 pH can affect the equilibrium of  $\text{NH}_3/\text{NH}_4^+$  in environments. Higher pH is conducive to the formation of free  $\text{NH}_3$ , of  
372 which higher concentrations could inhibit anammox (Aktan et al. 2012). This explains the negative correlations of  
373 pH with diversity (Table 3), and composition of AMB (Fig. 2), and a separate AMB assemblage in the Di estuary  
374 where a large amount of alkaline water was discharged. In the meantime, our data showed that higher C/N ratio could  
375 promote the diversity of AMB. However, Hu et al. (2012) observed higher diversity of AMB in samples with lower  
376 organic contents. The strong competition or cooperation for nitrite may occur between AMB and denitrifiers in low  
377 organic environments, leading to higher diversity of AMB (Dalsgaard and Thamdrup 2002; Rysgaard et al. 2004). In  
378 addition, heavy metals (e.g. Pb, As and Cd) also strongly influenced AMB diversity and composition in LZB sediment  
379 samples (Fig. 2, Table 3). Similarly, a significant correlation between Pb and *hzo* gene (a functional gene of AMB)  
380 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		
	<i>nosZ</i>	nosZ-F	CGYTGTTCMTCGACAGCCAG	267	Zhou et al. 2011
		nosZ1622R	CGSACCTTSTTGCCSTYGCG		
DGGE	AMB16S rRNA	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>
<b>Location</b>								
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 <sup>a</sup>	0.9±0.01 <sup>a</sup>
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 <sup>a</sup>	0.9±0.01 <sup>a</sup>
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 <sup>b</sup>	0.84±0.00 <sup>b</sup>
<i>P</i> -value	0.951	0.844	0.270	0.264	0.417	0.379	<b>0.004</b>	<b>0.001</b>
<b>Layer</b>								
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	<b>0.043</b>	0.901	0.782	<b>0.003</b>	<b>0.018</b>
<b>Season</b>								
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	<b>0.025</b>	<b>0.048</b>	0.848	0.845	0.661	0.344	<b>0.015</b>	<b>0.022</b>

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 ( $\rho$ ) 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	
	$H$	$D$	$H$	$D$	$H$	$D$	$H$	$D$
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	<b>0.74</b>	<b>0.76</b>
C/N	ns	ns	ns	ns	ns	ns	<b>0.73</b>	<b>0.72</b>
DO	ns	ns	ns	ns	ns	ns	-0.59	-0.59
Grain size	ns	ns	ns	ns	ns	ns	ns	ns
NH <sub>4</sub> <sup>+</sup> -N	<b>-0.76</b>	<b>-0.72</b>	ns	ns	ns	ns	0.70	0.71
NO <sub>2</sub> <sup>-</sup> -N	ns	ns	ns	ns	0.60	ns	ns	ns
NO <sub>3</sub> <sup>-</sup> -N	ns	ns	ns	ns	ns	ns	ns	ns
pH	ns	ns	ns	ns	ns	ns	<b>-0.72</b>	<b>-0.75</b>
Salinity	ns	ns	ns	ns	0.69	ns	ns	ns
Temperature	-0.69	<b>-0.71</b>	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
<b>Location</b>				
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
<b>Layer</b>				
Upper vs. Lower	0.73	0.78	0.42	0.99
<b>Season</b>				
Winter vs. Summer	<b>0.03</b>	0.96	0.68	<b>≤ 0.01</b>

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

587 **Figure Captions**

588

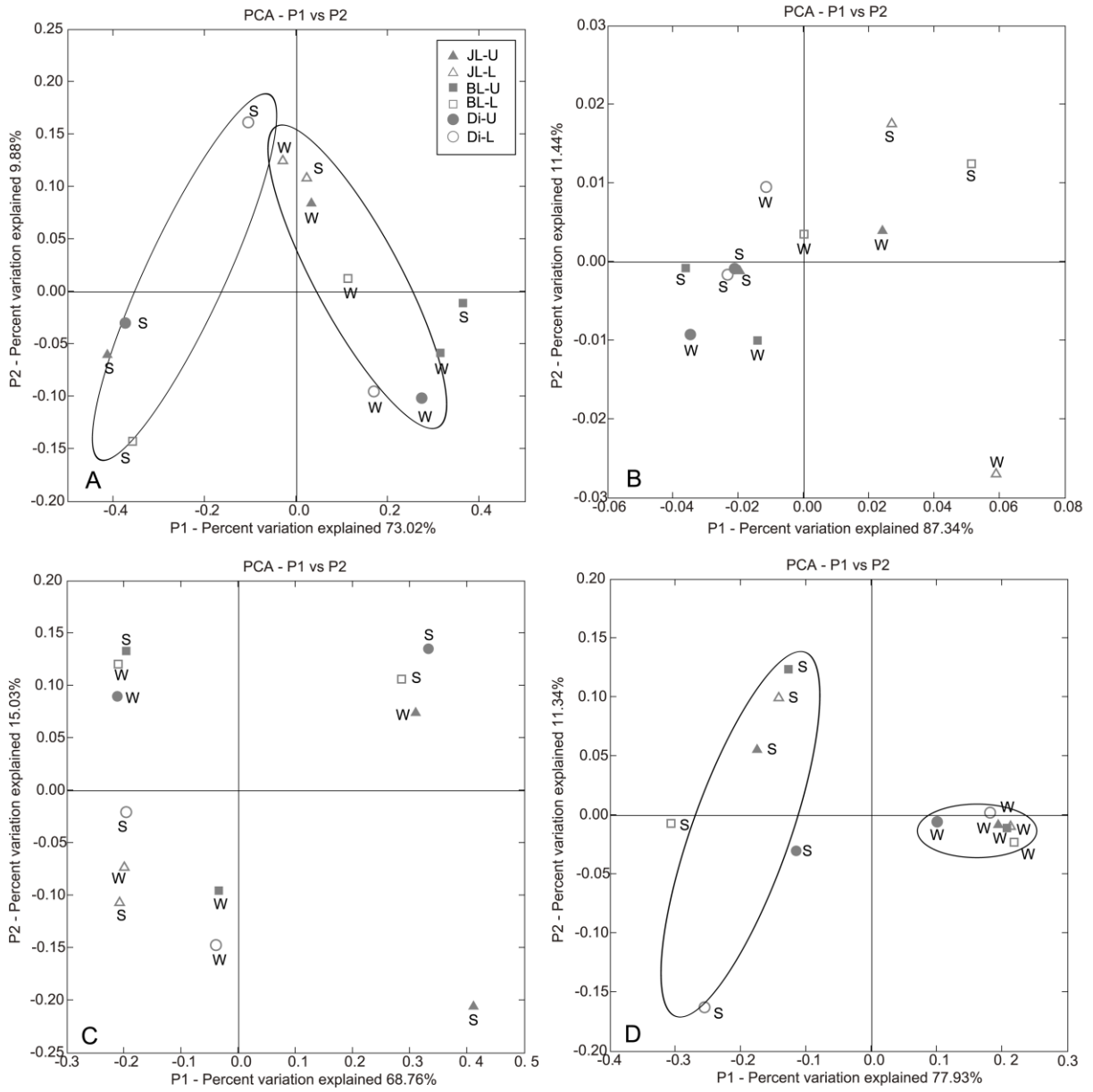
589 **Figure 1** Plots of principal coordinate analysis based on UniFrac weighted metric of AOB-*amoA* (A), AOA-*amoA* (B),  
590 *nosZ* (C), and AMB-16S rRNA (D), showing spatial and/or seasonal changes in N-cycling microbial assemblages in  
591 sediments of estuarine tidal flats

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



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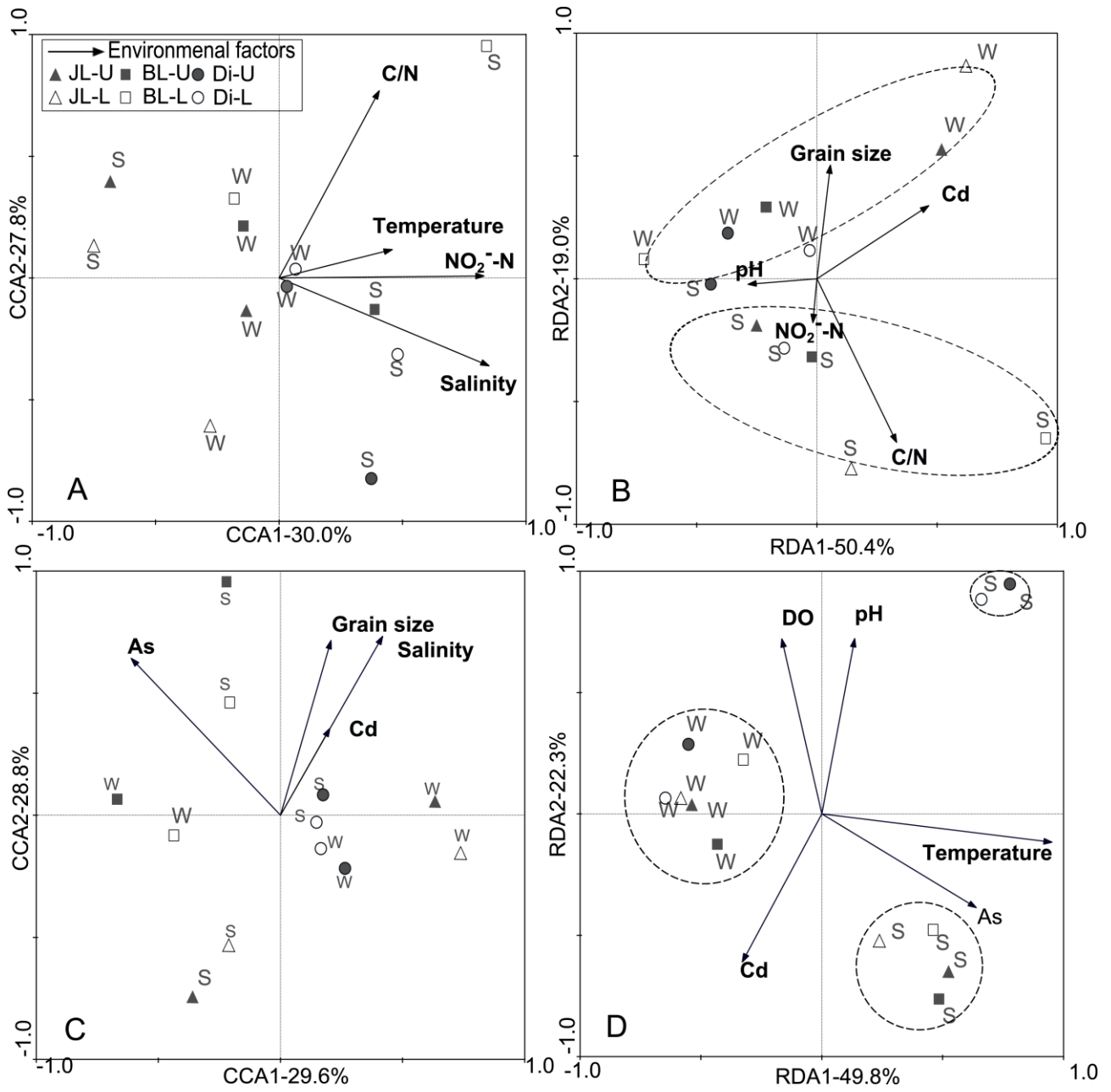
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602

603 Figure 2

604



605

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  $\gamma$ -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  $\delta$ -proteobacterial *Anaeromyxobacter dehalogenans* is used as the outgroup

624

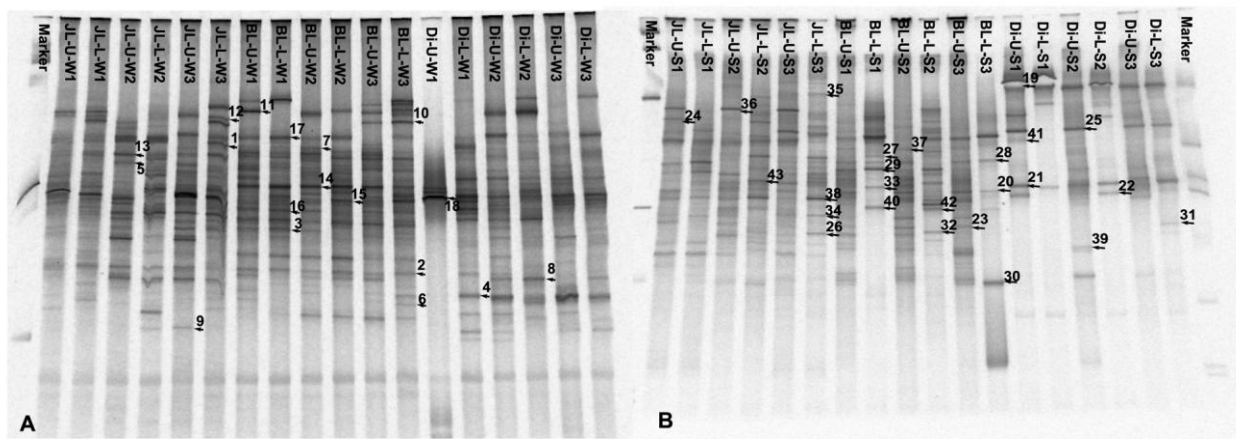
625 **Figure S5** Phylogenetic relationships of anammox bacterial 16S rRNA genes as determined by neighbor-joining method.  
626 Bootstrap values no less than 50% were shown at the nodes. The scale bar indicates 0.1 nucleotide substitution per site.  
627 Two Planctomycetes species (*Isosphaera pallida*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

628

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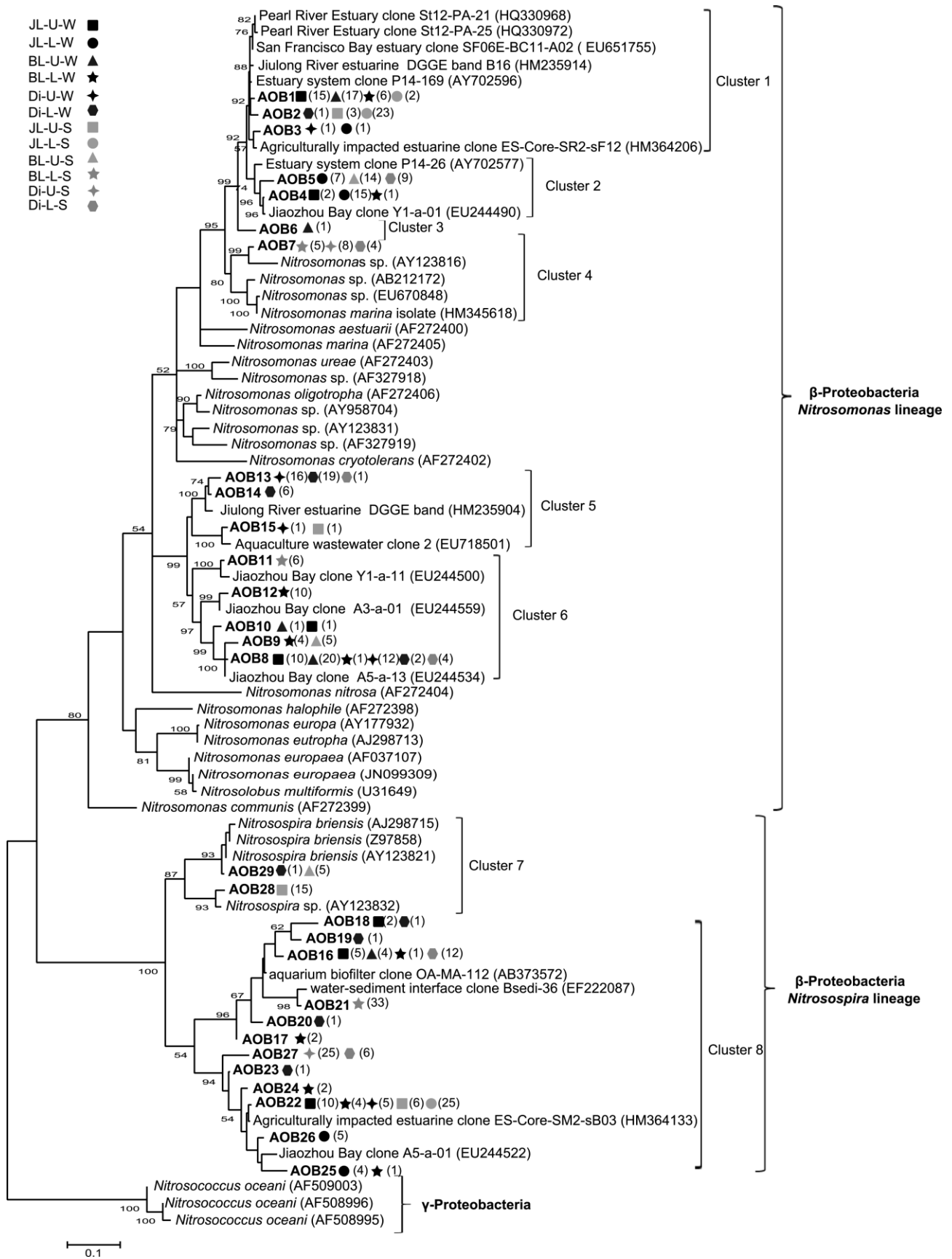
630 Figure

631 S1



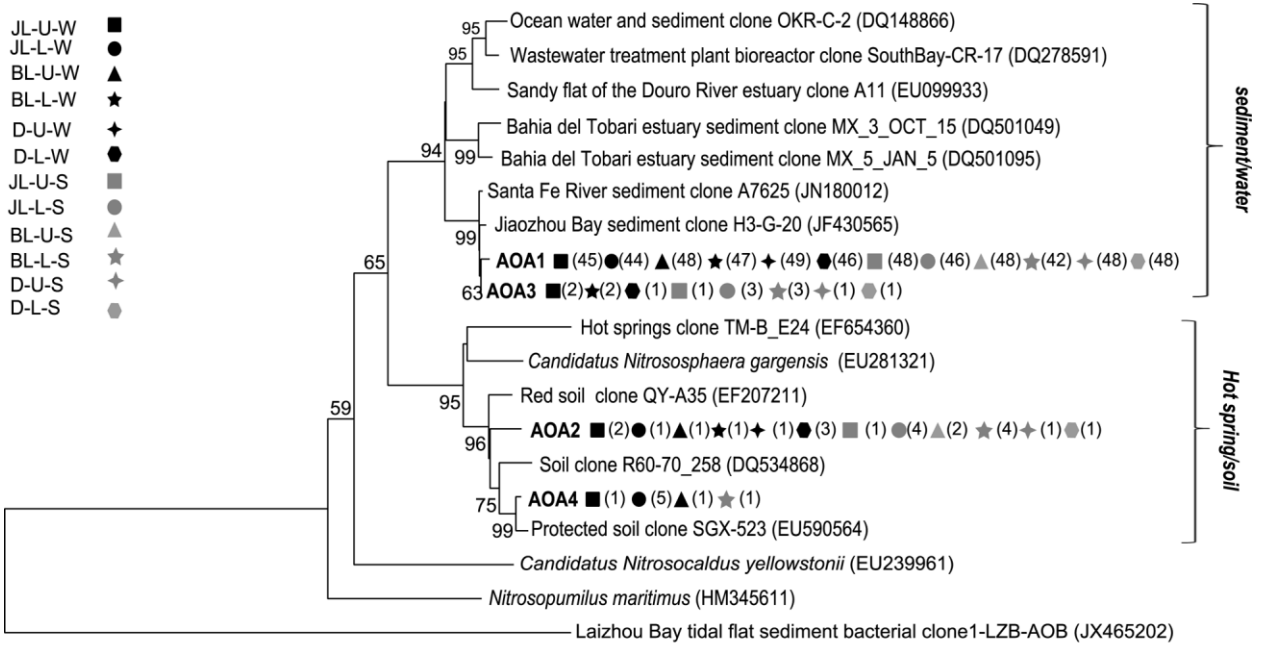
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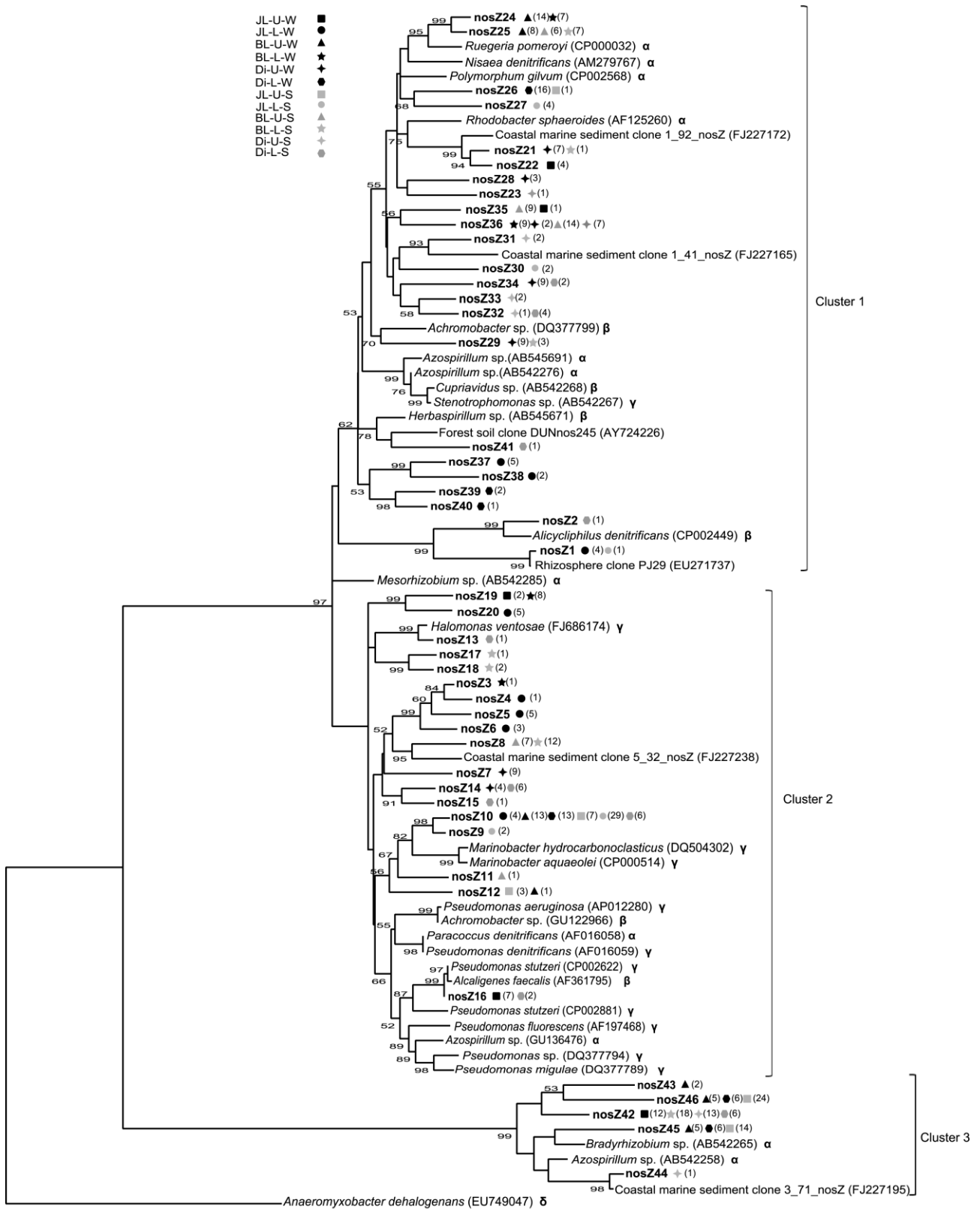


637 Figure S3



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