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Research Article

Relative Abundance of Ammonia Oxidizers, Denitrifiers, and Anammox Bacteria in Sediments of Hyper-Nutrified Estuarine Tidal Flats and in Relation to Environmental Conditions

The relative abundances of nitrifying, denitrifying and anammox prokaryotes in sediments of three hyper-nutrified estuarine tidal flats of Laizhou Bay, Bohai Sea, China were investigated. Quantitative PCR estimates indicated that in most cases archaeal (AOA) *amoA* genes were more abundant than bacterial (AOB) *amoA* genes, and ratio of AOA/AOB was correlated with pH, Cd, and Cu. Of the denitrifiers, *nirK*-type outnumbered *nirS*-type, with *nosZ*-type being the lowest. Variation of the ratio between *nirK* and *nirS* abundance depends on pH, nitrite, nitrate, and Cd. The combination of (*nirS* + *nirK*-*nosZ*), an indicator of genetic potentials for N_2O emission, was only related to the temperature. Anammox bacterial 16S rRNA gene abundances were correlated with salinity, pH, nitrite, and Cu. In contrast, the contribution of anammox to N_2 production, by using anammox bacterial 16S rRNA/*nosZ* ratio as a proxy, was correlated to temperature, ammonium and dissolved oxygen in the overlying water, ratio of organic carbon to nitrogen, and arsenic in sediments. Our study stresses that abundances of N-cycling functional groups respond differently to variations of environmental conditions, and multiple factors including heavy metals with relatively low concentrations may play a role in shaping nitrogen cycling processes in these estuarine tidal flats.

Keywords: Community size; Environment factor; Gene copy number ratio; Heavy metal; Nitrogen cycle

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1 Introduction

A tidal flat is a multifunctional ecosystem characterized by high primary production, intense nutrient remineralization, and pollutant transportation and transformation in sediments. In estuarine tidal flats, microbial processes play an important role in self-purification by transformation, degradation, and alleviation of these pollutant and nutrient overloads [1, 2]. Microorganism-mediated nitrogen cycling processes are of particular concern with respect to the ecosystem functions and services provided by estuaries [3] given that nitrogen limits primary oceanic production, and nitrogen overloading may be implicated in coastal eutrophication.

Nitrification, denitrification, and anaerobic ammonium oxidation (anammox) are tightly coupled nitrogen cycling processes in marine sediments, in which nearly 50% of marine nitrogen removal occurs [4]. Ammonia oxidation is the first and rate-limiting step of nitrification in which ammonia (NH_3) is oxidized to nitrite (NO_2^-) by ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB). The ammonia monooxygenase gene (*amoA*) has been used extensively as a molecular marker for studying both types of ammonia oxidizers in environmental samples [5–19]. Denitrification is an anaerobic reduction of nitrate, through nitrite and to nitrous oxide (N_2O) and eventually dinitrogen (N_2). Two functionally similar but structurally different genes, *nirK* and *nirS*, encoding the copper (NirK) and cytochrome *cd1*-containing nitrite reductase (NirS), have not been found in the same organism and show different distributions in environments [20]. Both *nirK* and *nirS* genes are responsible for the rate-limiting step (NO_2^- to NO), and the *nosZ* gene codes for nitrous oxide reductase which catalyzes the reduction of N_2O to N_2, the final step of denitrification [21, 22]. Like denitrification, anammox represents a net loss of nitrogen from the system by

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Abbreviations: **AMB**, anammox bacteria; ***amoA***, ammonia monooxygenase gene; **AOA**, ammonia-oxidizing archaea; **AOB**, ammonia-oxidizing bacteria; **LZB**, Laizhou Bay; **NH_4-N**, ammonium nitrogen; ***nirK***, gene encoding the copper-containing nitrite reductase; ***nirS***, gene encoding the cytochrome *cd1*-containing nitrite reductase; ***nosZ***, gene encoding nitrous oxide reductase; **psu**, practical salinity units; **qPCR**, quantitative PCR; **rRNA**, ribosomal RNA; %R_{amx}, percentage of N_2 production due to anammox

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the production of N_2 via the reduction of NO_2^- coupled to the oxidation of ammonium (NH_4^+). Specific primers targeting 16S rRNA genes have been used for detecting anammox bacteria in environments [21].

It has been shown that community size or abundance of a functional community involved in nitrogen cycling is frequently correlated with the process rate, which reflects the ecosystem functions provided by the community [23–25]. For example, Hallin et al. [23] found that abundance, rather than composition, correlated with denitrification rates and nitrification rates by ammonia-oxidizing archaea in agricultural soils. Bacterial *amoA* gene abundance and ammonium content best explained potential nitrification rates in Alaskan soils [25], whereas *nosZ*, *nirS*, and *nirK* gene abundance and nitrate best explained potential denitrification rates in estuarine sediments [26]. Denitrifying gene abundances could be used as proxies for predicting N_2O emissions in soil and wetland environments [24, 27]. A positive correlation between anammox cell number and activities was reported in both, water columns and sediments, the abundance of anammox bacteria therefore can be considered as a proxy for anammox activities in environments [6, 28–30].

Earlier studies of marine sediments from a variety of estuarine locations have shown different patterns of AOA/AOB dominance [7–10]. For denitrifying genes, *nirS* was more abundant than *nirK* in the San Francisco Bay estuary and subtropical Fitzroy estuary [10, 31]. The contribution of anammox to N_2 production was estimated to be relatively low in coastal environments, and dissolved oxygen, nutrient load and salinity have been suggested as controls of the contribution of anammox to nitrogen removal [30, 32]. However, only a few studies have examined the abundances of ammonia oxidizers, different types of denitrifiers, and anammox bacteria in a single survey; and the genetic potential for N_2O emissions and contribution of anammox to N_2 production and their spatiotemporal pattern have seldom been explored for estuarine tidal flats.

Recent studies have showed that heavy metal accumulation may be an important factor in regulation of nitrogen transformations, because some critical enzymes involved in the microbial nitrogen cycle are metalloenzymes [33]. For example, the AMO coded by *amoA* gene is a Cu/Fe containing enzyme [34], and the NIR coded by *nirK* gene has been shown to contain Cu [35]. The effect of heavy metals on nitrogen cycling populations in sediments has not been studied sufficiently [31]. Moreover, most of the studies that have been performed have targeted only the top layer of sediments, and few have examined variability in N-cycling microbes' distributions in deeper layers. Surprisingly, AOB 16S rRNA genes had been detected at depths of up to 40 cm in sediments, and rates of nitrification down to 8 cm depth were also detectable, whereas the depth distribution of AOA in estuarine sediments is largely unknown [36, 37]. Recently, it was also demonstrated that the greatest potential for anammox was localized to the upper 2 cm and could be detected down to a depth of 4.5 cm in the sediments [38].

In the past decades, the Laizhou Bay (LZB), Bohai Sea, northern China has been hyper-nutrified due to huge in-terrestrial input (most commonly, dissolved inorganic nitrogen, DIN) from about 20 rivers of the coastal zone [39, 40]. In the meantime, in order to supply more tideland for economic developments (e.g., aquaculture, industry, harbor construction) in the coastal-line region of southern bank of LZB, tidal flat reclamation projects have been extensively planned and will be implemented in the near future. Thus, the environmental and ecological impacts of these reclamation projects are of great concern. In this study, we aimed to provide a baseline examination of ecosystem functions provided by nitrogen cycling microbes in sediments of LZB estuarine tidal flats, by using the gene abundance as indicators. For this purpose, abundances of N-cycling gene markers (*amoA*, *nirS*, *nirK*, *nosZ*, and anammox 16S rRNA) for ammonia oxidization, denitrification, and anammox processes were determined by quantitative PCR (qPCR). Copy number ratios of related genes and their relationship with environmental factors were calculated in order to explore the environmental impact on dominance of functional phylotypes, genetic potentials for N_2O emission, and contribution of anammox to N_2 production in coastal sediments. In addition, our study provides additional data for the links between microbial nitrogen cycle and trace metal levels.

2 Materials and methods

2.1 Study sites and sampling

Estuarine tidal flats of Bailang River (BL), Di River (Di), and Jiaolai River (JL), which flow into the Laizhou Bay (LZB), Bohai Sea, were investigated (Fig. 1). These three rivers have different pollution history. The Bailang River is 127 km long and drains a catchment of about 1237 km^2; it used to receive a lot of sewage and industrial wastewater as it flowed through Weifang City. However, the execution of a remediation project has significantly improved water quality of this river since 2006. The estuarine area covering three sampling sites (BL1-3) has been enclosed for mariculture since May 2011 and this led to less tidal exchange and higher salinity due to evaporation especially in summer. The Jiaolai River is about 130 km long, with a catchment area of 5478 km^2, receiving extensive agricultural and industrial discharges. The Di River is small sewage river (23 km long, catchment area 119 km^2), loaded with a large amount of wastewater from dyeing and subsurface brine industries, resulting in relatively high alkalinity and salinity.

In each tidal flat (location), three sites (JL1-3, BL1-3, and Di1-3) located in lower tidal area were sampled (Fig. 1). Sediment samples were collected in November 2010 (winter) and August 2011 (summer). Sediment cores (7.5 cm internal diameter) were taken with a custom-made corer. The top/upper layer (0–2 cm) and the lower layer (2–5 cm) of sediments were sliced and put into sterile plastic bags. Samples were stored in an ice box (4°C) and immediately frozen at −80°C after transferred to the laboratory.

2.2 Analysis of environmental factors

Dissolved oxygen (DO), pH, salinity, and the temperature of overlying water were measured on site with a Hydrolab MS5 water quality probe (HACH, USA). Lyophilized sediments were leaching liquor with KCl (2M), then nitrate (NO_3^--N), nitrite (NO_2^--N), and ammonium (NH_4^+-N) were measured with a nutrient AutoAnalyser (Seal, Germany). Total organic carbon and nitrogen contents of sediments were measured with a Vario Micro Cube Elemental Analyser (Elementar, Germany). A Marlvern Mastersizer 2000F granulometer (Malvern, England) was used for sediment grain size analysis. Sediments were treated with 1 M HCl [41], and concentrations of eight heavy metals (As, Co, Cd, Cr, Cu, Ni, Pb, and Zn) were determined with an ELAN DRC II plasma-MS (ICP-MS; PerkinElmer, Hong Kong).

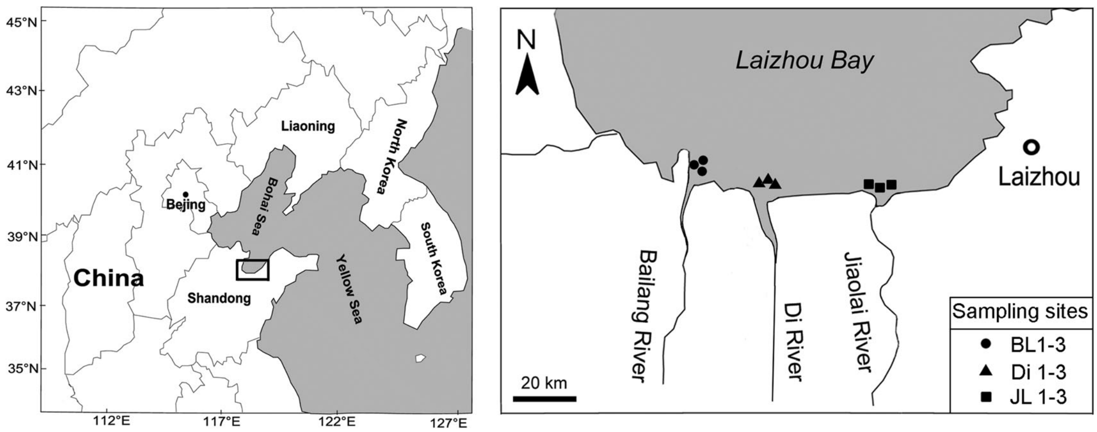

Figure 1. Location of the sampling sites in estuarine tidal flats of Laizhou Bay.

2.3 DNA extraction and quantitative PCR assay

Total genomic DNA of the sediment samples was extracted using the UltraClean Soil DNA Isolation kit (MO-BIO, USA) according to the manufacturer's instructions. To examine the spatiotemporal variation of community size of N-cycling groups in estuarine sediments, the abundances of several genes (anammox bacterial 16S rRNA, AOB-*amoA*, AOA-*amoA*, *nirS*, *nirK*, and *nosZ*) were quantified using qPCR. Primers used are listed in Tab. 1. Plasmids containing cloned gene PCR amplicons were extracted with the TIANpure Midi Plasmid Kit (Tiangen, Beijing, China) for use in standard curves: archaeal *amoA* clone AOALZB-1, bacterial *amoA* clone AOBLZB-8, anammox bacterial 16S rRNA clone AMX-LZB-11, *nirS* clone NIRSLZB-7, *nirK* clone NIRKLZB-3, and *nosZ* clone NOSZLZB-6. Concentrations of sediment and standard DNA were measured with a NanoDrop 2000C spectrophotometer (Thermo, Wilmington, DE, USA). A serial tenfold dilutions (10^{-2} to 10^{-7}) were used to obtain standard curves. All sample and standard reactions were performed in triplicate with an ABI 7500 Fast Real-time PCR System (Applied Biosystems, Foster City, CA, USA), and an average value was calculated. The quantification was based on the fluorescent dye SYBR Green I. Each reaction was performed in 25 μL containing 1 μL of DNA template, 0.5 μL of each primer (10 μM) and 12.5 μL of Maxima SYBR Green/ROX qPCR Master Mix 2× (Fermentas, USA). The PCR cycle started with 2 min at 50°C and 10 min at 95°C, followed by a total of 40 cycles of 30 s at 95°C, 40 s at 57°C for *nirS* genes (55°C for AMB 16S rRNA gene, 58°C for AOB-*amoA* and AOA-*amoA* genes, and 51°C for *nirK* gene), and 40 s at 72°C. Melt curves were obtained to check the specificity of amplification. The PCR amplification efficiencies were 85–113%, and correlation coefficients (R^2) for all assays were >0.99.

2.4 Statistical analysis

Student's *t*-test (two-tailed) was used to compare mean values of environmental variables as well as gene abundance between tidal flats, seasons, or layers. Spearman's correlation coefficient (ρ) between microbial abundances and environmental variables were calculated. All these analyses were performed using the program SPSS 13.0 for windows (SPSS, Chicago, USA).

3 Results

3.1 Environmental characteristics

Thirty-six sediment samples were taken from two layers, nine sites in two seasons. Environmental factors during the samplings are shown in Supporting Information Fig. S1. Water temperature ranged from 7.6 to 33.7°C, with significant differences between winter (mean 10.8°C) and summer (mean 30.4°C; *t*-test, $p < 0.01$). Although the water salinity varied greatly from 11.1 to 50.3 practical salinity units (psu), there was no statistically significant difference between the

Table 1. Primers for target genes used in this study

Target gene	Primer	Sequence (5′–3′)	Length of amplicon (bp)	Ref.
AOA-*amoA*	Arch-amoA-for	CTGAYTGGGCYTGGACATC	256	[11]
	Arch-amoA-rev	TTCTTCTTTGTTGCCCAGTA		
AOB-*amoA*	amoA1F	GGGGTTTCTACTGGTGGT	490	[12, 42]
	amoAr new	CCCCTCBGSAAAVCCTTCTTC		
nirS	Cd3aF	GTSAACGTSAAGGARACSGG	425	[43]
	R3cd	GASTTCGGRTGSGTCTTGA		
nirK	F1aCu	ATCATGGTSCTGCCGCG	472	[13]
	R3Cu	GCCTCGATCAGRTTGTGGTT		
nosZ	nosZ2F	CGCRACGGCAASAAGGTSMSSGT	267	[46]
	nosZ2R	CAKRTGCAKSGCRTGGCAGAA		
Anammox bacterial 16S rRNA	AMX808F	ARCYGTAAACGATGGGCACTAA	232	[30]
	AMX1040R	CAGCCATGCAACACCTGTRATA		

seasons or locations ($p > 0.05$; Supporting Information Fig. S1). A similar situation occurred for other environmental factors such as pH (5.97–8.61), dissolved oxygen (228.1–361.3 μM), and nitrate (2.71–15.54 mg/kg), nitrite (0.03–2.37 mg/kg), ammonium (5.67–11.79 mg/kg), and ratio of organic carbon to organic nitrogen (C/N; 6.73–11.89), except that average concentrations of nitrate (4.52 mg/kg) and nitrite (0.21 mg/kg) in JL were significantly lower than those (12.19 and 0.82 mg/kg) in Di tidal flat.

The median sediment grain size ranged from 14.1 to 86.6 μm, with larger size in BL and Di, whereas there was no significant difference between seasons or layers. According to the three quality grades of marine sediments defined by the National Standard of China (NSC) GB18668-2002 [14], concentrations of heavy metals As (0.84–3.91 mg/kg), Co (1.75–5.78 mg/kg), Cd (0.03–0.08 mg/kg), Cr (1.49–6.09 mg/kg), Cu (1.32–11.06 mg/kg), Ni (2.35–8.58 mg/kg), Pb (3.43–14.27 mg/kg), and Zn (5.05–14.05 mg/kg) in the sampling sites were lower than the first grade quality, representing a status of none to slight contamination. A previous study on surface sediments of LZB also showed similar levels of metal concentrations, which were lower than other coastal area in China (e.g., Jiaozhou Bay, Hongkong coast) and oversea (e.g., Thermaikos Gulf, Greece) [40]. Nevertheless, among the three tidal flats, JL generally had the highest levels of heavy metals. Significant differences in concentrations of Cd, Cu, Ni, Pb, and Zn were only found between BL and Di ($p < 0.05$, Supporting Information Fig. S1). No significant differences were detected for Cr or Co among all these three tidal flats ($p > 0.05$). The average concentration of As was 2.84 mg/kg at BL, representing the highest among the three areas. Metal concentrations seemed higher in the summer than in the winter, but only As concentrations were significantly different between the two seasons ($p < 0.05$, Supporting Information Fig. S1). In addition, there was no significant effect of sediment layer on heavy metal concentrations (Supporting Information Fig. S1).

Among the eight heavy metals determined, six metals (Co, Cr, Cu, Ni, Pb, and Zn) were collinear ($\rho > 0.64$, $p < 0.05$). In contrast, As and Cd did not follow the pattern of other metals, as significant correlations were only observed between As and temperature ($\rho = 0.74$, $p < 0.01$), NH_4-N ($\rho = 0.61$, $p < 0.05$) and C/N ($\rho = 0.66$, $p < 0.05$), and between Cd and nitrite ($\rho = -0.73$, $p < 0.01$) and pH ($\rho = -0.76$, $p < 0.01$).

3.2 Abundances and ratios of N-cycling gene markers

Comparison of gene abundances is shown in Fig. 2 (for raw data of copy numbers see Supporting Information Tab. S1). The copy numbers of anammox bacterial 16S rRNA and N-cycling functional genes were determined for the 36 sediment samples. AOA-*amoA* genes varied between 1.0×10^3 and 3.3×10^5 copies g^{-1} sediment (Fig. 2A–C; Supporting Information Tab. S1), which was between 0.5 and 26.6 times the level of AOB-*amoA* found in the samples (Tab. 2). Compared with AOB-*amoA*, AOA-*amoA* appeared to be more abundant across all the samples ($p < 0.05$, $n = 36$; Fig. 2A–C). Among the denitrifying genes, *nirK* appeared to be the most abundant, with copy numbers ranging from 2.7×10^3 to 1.6×10^7 g^{-1} sediment (Fig. 2D–F). The mean abundance of *nirS* was about half of *nirK* but 1.0- to 217.7-fold as much as that of *nosZ* genes (Tab. 2). The ratio between nitrite and nitrous oxide reductases (*nirK* + *nirS*)/*nosZ* varied greatly from 1.3 to 473.0. The abundance of AMB 16S rRNA gene ranged from 2.2×10^3 to 1.3×10^3 copies g^{-1} sediment (Fig. 2A–C), with a mean value approaching to that of *nosZ* genes, whereas their ratios varied greatly from 0.1 to 11.6 (Tab. 2).

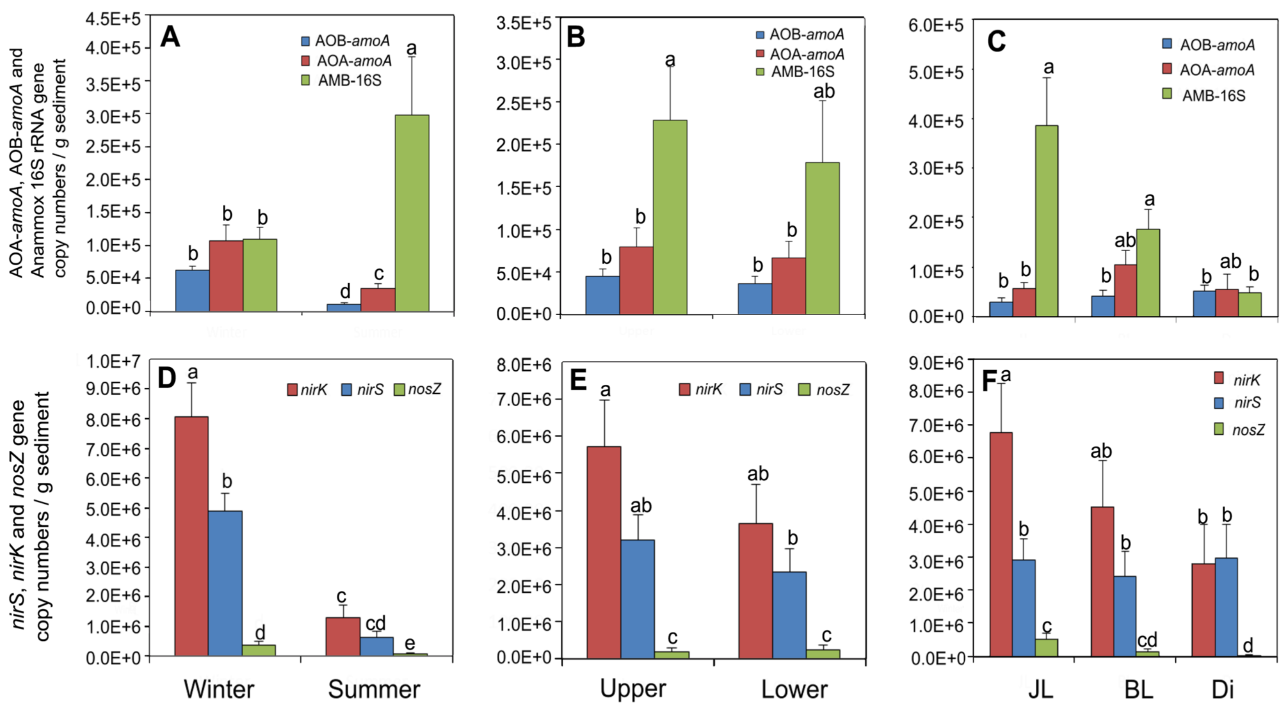

Figure 2. Copy numbers of anammox bacterial 16S rRNA and AOB-*amoA*, AOA-*amoA* (A–C), *nirS*, *nirK* and *nosZ* genes (D–F) determined for sediment samples (g^{-1} wet wt) from Laizhou Bay estuaries. Standard errors of the mean are indicated. Different letters above the bars indicate significant differences ($p < 0.05$) between sampling time or sampling layer or sampling site. Samples from all sites were subjected to seasonal or layer comparisons.

Table 2. Ratios or relative abundance between target genes involved in N-cycling of Laizhou Bay estuarine tidal flats

	JL				BL				Di			
	Winter		Summer		Winter		Summer		Winter		Summer	
	U	L	U	L	U	L	U	L	U	L	U	L
AOA/AOB	1.0	3.3	0.9	6.6	3.7	1.0	26.6	6.4	0.5	1.7	0.8	0.7
nirK/*nirS*	2.6	2.2	1.7	4.4	1.8	2.0	2.3	0.2	1.2	0.7	0.2	0.2
nirK/*nosZ*	13.4	12.3	21.2	16.2	121.7	16.1	47.2	16.7	255.3	66.5	0.2	0.3
nirS/*nosZ*	5.2	5.6	12.5	3.7	67.6	8.2	20.9	68.0	217.7	90.6	1.0	1.4
(*nirK* + *nirS*)/*nosZ*	18.6	17.9	33.7	19.9	189.3	24.3	68.1	84.8	473.0	157.1	1.3	1.6
AMB/*nosZ*	0.1	0.1	4.3	4.1	1.7	0.4	7.3	11.6	2.9	1.2	0.1	2.2

AMB, anammox 16S rRNA gene; L, lower layer; U, upper layer.

3.3 Correlation analysis

In order to explore the relationship between gene abundances and environmental variables, Spearman's correlations were performed (Tab. 3). Because the six heavy metals (Co, Cr, Cu, Ni, Pb, and Zn) were collinear, only Cu was considered as a representative here. For all gene abundances examined, most were negatively correlated with temperature ($\rho = -0.75$ to -0.91, $p < 0.01$), except for AOA *amoA* and AMB 16S rRNA genes which showed no significant correlations ($p > 0.05$). AOA also showed no correlation with other environmental variables, whereas AOB *amoA* was negatively correlated with the metal As ($\rho = -0.62$, $p < 0.05$). Among the denitrifying genes and their combination examined, only *nosZ* was significantly correlated with Cd ($\rho = 0.63$, $p < 0.05$) and NO_2-N ($\rho = -0.74$, $p < 0.01$). Notably, significant correlations between abundances of AMB 16S rRNA genes and Cu ($\rho = 0.82$, $p < 0.01$), nitrite, pH and salinity ($\rho = -0.64$ to -0.79, $p < 0.05$) were observed (Tab. 3).

The correlations between ratios of gene copy numbers and environmental variables may give a clue to understanding the regulating mechanisms for genes of identical functions. The AOA/AOB *amoA* copy number ratios were significantly correlated with pH ($\rho = -0.52$, $p < 0.05$) and the metals Cd ($\rho = 0.62$, $p < 0.05$) and Cu ($\rho = 0.58$, $p < 0.05$). The *nirK*/*nirS* ratios showed positive correlation with Cd ($\rho = 0.87$, $p < 0.01$), and negative with NO_2-N ($\rho = -0.85$, $p < 0.01$), NO_3-N ($\rho = -0.58$, $p < 0.05$) as well as pH ($\rho = -0.71$, $p < 0.01$). However, correlations between other denitrifying gene ratios (i.e., *nirS*/*nosZ*, *nirK*/*nosZ*, and (*nirS* + *nirK*)/*nosZ*) and variables examined were not supported. AMB/*nosZ*, a potential indicator for the relative contribution of anammox and denitrification processes to N_2 production, was correlated with C/N ($\rho = 0.78$, $p < 0.01$), temperature ($\rho = 0.70$, $p < 0.05$), dissolved oxygen ($\rho = -0.68$, $p < 0.05$) and NH_4-N ($\rho = 0.59$, $p < 0.05$). In addition, a positive correlation was identified between AMB/*nosZ* and As ($\rho = 0.61$, $p < 0.05$; Tab. 3).

Correlations between different genes in samples cross locations and seasons were analyzed (Tab. 4). When all 36 sediment samples from both the upper and lower layers were considered, there was no correlation between AOA and AOB *amoA* genes; whereas *nirS* and *nirK* abundances were significantly correlated ($\rho = 0.93$, $p < 0.01$). Interestingly, all the three denitrifying genes were positively correlated with AOB *amoA* gene ($\rho = 0.42$–0.63, $p < 0.05$), whereas only *nirS* and AMB 16S rRNA genes were positively correlated with AOA *amoA* ($\rho = 0.38$, $p < 0.05$). No correlation was observed between AMB and AOB. A similar pattern of these correlations was identified when only the upper layer samples were analyzed. Nevertheless, significant correlations between *nirK* and AOA ($\rho = 0.59$, $p < 0.05$) were only obtained from the dataset for the upper layers; correlations between

Table 3. Spearman's correlation coefficients (ρ) between environmental factors and gene abundance and ratio of target genes across seasons, locations and layers[a)]

	As	Cd	Cu	C/N	DO	Grain	NH_4-N	NO_2-N	NO_3-N	pH	Salinity	*T*
Abundance												
AOA	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
AOB	−0.62	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	**−0.75**
nirS	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	**−0.75**
nirK	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	**−0.91**
nosZ	ns	0.63	ns	ns	ns	ns	ns	**−0.74**	ns	ns	ns	**−0.76**
nirS + *nirK*-*nosZ*	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	**−0.85**
AMB	ns	ns	**0.82**	ns	ns	ns	ns	**−0.79**	ns	−0.64	**−0.78**	ns
Ratio												
AOA/AOB	ns	0.62	0.58	ns	ns	ns	ns	ns	ns	−0.52	ns	ns
nirK/*nirS*	ns	**0.87**	ns	ns	ns	ns	ns	**−0.85**	−0.58	**−0.71**	ns	ns
nirK/*nosZ*	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
nirS/*nosZ*	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
(*nirS* + *nirK*)/*nosZ*	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
AMB/*nosZ*	0.61	ns	ns	**0.78**	−0.68	ns	0.59	ns	ns	ns	ns	0.70

AMB, anammox 16S rRNA gene; DO, dissolved oxygen; Grain, sediment grain size; ns, not significant; *T*, temperature.
[a)] Only the significant correlations ($p < 0.05$) are shown, and the highly significant correlations ($p < 0.01$) are highlighted in bold.

Table 4. Spearman's correlation between N-cycling gene abundances across season, site and layer (lower triangle) or upper layer only (upper triangle)[a)]

	AOB	AOA	nirS	nirK	nosZ	AMB
AOB	–	ns	**0.80**	0.63	ns	ns
AOA	ns	–	0.51	0.59	ns	ns
nirS	**0.63**	0.38	–	**0.92**	0.50	ns
nirK	**0.54**	ns	**0.93**	–	**0.67**	ns
nosZ	0.42	ns	**0.67**	**0.78**	–	0.49
AMB	ns	0.34	ns	ns	0.35	–

AMB, anammox bacterial 16S rRNA gene; ns, not significant.
a) Only the significant correlations ($p < 0.05$) are shown, and the highly significant correlations ($p < 0.01$) are highlighted in bold.

AOA and *nosZ* or AMB were observed from the dataset for both layer samples, but not the upper layer samples (Tab. 4).

4 Discussion

4.1 Ammonia oxidizers

In this study, both ammonia oxidizers had significantly higher abundance in winter than in summer (Fig. 2). This seasonal change may be explained by strongly negative correlation between AOB and temperature (Tab. 3). However, because no significant correlations were found between AOA and the environmental factors measured in this study, factors contributing to seasonal changes of AOA in LZB estuaries remain unclear. This contrasts with previous studies showing that both AOA and AOB abundance were negatively correlated with the salinity gradient ranging from 0.6 to 31 psu in the San Francisco Bay estuary [8], and that the highest AOA and AOB abundance were recorded at 20 psu when a similar salinity range was examined in the Plum Island Sound estuary [15]. This discrepancy may be due to the high salinity (36.3–50.3 psu) for most of our samples. Nevertheless, the high salinity explains that both AOB and AOA *amoA* gene copy numbers determined in this study were about two to three order of magnitude lower than these reported for other estuaries [16–18, 44].

AOA outnumbered AOB in a majority of our samples, which is consistent with previous studies which showed that AOA usually dominated in more saline environments [19, 44–46]. However, AOB dominated in samples from Di and JL with salinity around 50 psu, suggesting that the salinity is not the only determining variable in the LZB estuaries, other variables such as nitrite, pH, and C/N ratio may also impact the abundances of ammonia oxidizers [8, 17, 47, 48]. Our further analysis indicated a negative correlation between AOA/AOB ratio and pH (Tab. 3), which is consistent with previous notion that AOA outnumbers AOB in soils with lower pH [49]. Furthermore, we found a negative correlation between As (0.84–3.91 mg/kg) and AOB abundance, and that the AOA/AOB ratio was also significantly positively correlated to both Cd (0.03–0.08 mg/kg) and Cu (1.32–11.06 mg/kg) in the LZB estuarine sediments (Tab. 3), contrasting to the study in the San Francisco Bay estuary, where Ni and Pd showed strong correlations with abundance of AOA [8]. Nevertheless, our results suggest different responses of AOA and AOB to heavy metal contaminated sediments, with AOB seemingly being more sensitive to As contamination. A similar pattern has been observed in highly contaminated soils (As, 10 000 mg/kg; Pb, 200 mg/kg; Cu, 1600–2400 mg/kg) [50, 51], where AOB was negatively affected whereas AOA seemed more tolerant.

4.2 Denitrifiers

Of the three types of denitrifiers examined, the *nirK*-type was more abundant than *nirS*-type and *nosZ*, especially in BL and JL, suggesting a dominant role of *nirK*-type denitrifiers in denitrification in the LZB estuaries. The lowest *nirK*-type abundances were recorded in summertime samples from Di where both the water salinity (50.3 psu) and pH (8.61) were the highest. The dominance of *nirK*-type and lowest abundance of the *nosZ*-type denitrifiers in LZB estuarine tidal flats are consistent with a study on sediment of a constructed wetland [24], but contrast with several reports that *nirS*-type dominated in estuarine sediments [10, 31, 52]. This is consistent with the idea that *nirS*- and *nirK*-type denitrifiers have different habitat preferences, as suggested by several studies [23, 53]. In sediments of the hypernutrified Colne estuary, the decrease of *nirS* gene abundance in the more marine sites might be related to nitrate and ammonia gradient [54], whereas *nirS* was highest in the more marine sites and *nirK* abundance was highest in riverine sites [31]. In this study, however, the higher ratio of *nirK*/*nirS* was shown to be related to lower pH and NO_x-N. The effects of these environmental variables on dominance of the two denitrifier types are thus confounding at present.

Trace metal availability may affect denitrification through the metalloenzymes and toxicity to benthic denitrifiers [33], and more generally, negative relationships between metals and the activity or the abundance of denitrifiers are found. For example, *nirK* was negatively correlated with Ag and methyl-mercury in the San Francisco Bay estuary [31]; both the abundance of *nosZ* gene and the denitrification rates in sediment decreased by the amendment of Cu (up to 79 mg/kg of wet sediment) in the Douro River estuary [52, 55]; lowest Cu concentrations (0–0.277 mg/kg of dry sediment) could yield a drastic decrease in the abundance of *nirK*, *nirS*, and *nosZ* genes in salt marsh sediments [56]. Nevertheless, a positive correlation between *nirS* and Pb was also observed [31]. In this study, we found a significantly positive correlation between Cd (0.03–0.08 mg/kg) and *nosZ* and the ratio of *nirK*/*nirS* in these three tidal flats (Tab. 3). Such previous data along with ours thus suggest that heavy metals, even presenting at relatively low levels, may be important factors influencing specific denitrifying populations or their relative abundance in estuarine sediments.

The high abundance of *nirS* and *nirK* genes compared with *nosZ* gene is an indicator of the genetic capacity of the system to potentially accumulate the N_2O intermediary [24]. Therefore, the value of *nirS* + *nirK*-*nosZ* or the ratio of (*nirS* + *nirK*)/*nosZ* could be used as predictors of the potential for N_2O emission. The seasonal effect on *nirS* + *nirK*-*nosZ* was significant (*t*-test, $p < 0.01$; Fig. 2D), predicting a higher N_2O emission potential in winter than in summer in the LZB estuarine tidal flats. This seasonal pattern of N_2O emission has long been observed [57], although the N_2O flux rate was not determined in this study. Nevertheless, no significant correlations were found between metals and *nirS* + *nirK*-*nosZ* or (*nirS* + *nirK*)/*nosZ* in our study, contrasting to a previous investigation showing that N_2O production was stimulated with the progressive increase of metals (Cu, Zn, Cr, and Cd) to estuarine sediments [52]. Relatively lower concentrations of heavy metals in the LZB estuarine tidal flats might explain this discrepancy.

4.3 Anammox bacteria

We found that AMB abundances were negatively correlated with salinity, pH and nitrite, and positively with Cu in the LZB estuarine sediments. This result is basically consistent with several previous studies which have showed that salinity [30], and nitrite concentration are significantly correlated with abundance of anammox bacteria [58]. Both high salinity and nitrite toxicity can inhibit anammox bacteria, which is understandable as the higher salinity challenges microorganisms with higher osmotic pressure, and nitrite suppresses the anammox process at certain threshold concentrations [59]. The inhibition on physiology of AMB may eventually lead to population decreases due to grazing pressure in the benthic microbial food webs. As for the positive correlation between Cu and AMB abundance found in this study, we are unable to give a proper explanation, since few studies have been carried out on effect of heavy metals on AMB [59]. Nevertheless, it is noteworthy that a previous study on closed marine systems has demonstrated that the addition of trace metals (Fe, Mn, Cu, Zn, and Mo) enhances the denitrification processes [60], which could supply more NO_2^- as substrate for anammox.

To our knowledge, this study for the first time investigated the abundance ratio between a marker gene of AMB (i.e., specific 16S rRNA gene) and a nitrous oxide reductase gene (i.e., *nosZ*) in sediment samples, as a potential indicator of the relative contribution of anammox to N_2 production (%R_{amx}). The rationale is that both the AMB and the *nosZ*-type denitrifiers are responsible for N_2 production, the abundance of these marker genes may be proxy of the capability of their functions. Although the relative contributions of these two processes were not investigated using ^{15}N stable isotope labeling in this study, the variations of AMB/*nosZ* in relation to environmental factors might give a clue to further studies. Our correlation analysis showed that the ratio of AMB/*nosZ* was significantly and positively correlated with C/N ratio, temperature, concentrations of As and NH_4-N in sediments, and negatively with dissolved oxygen in the overlying water (Tab. 3). In agreement, the %R_{amx} was positively correlated with both the organic carbon content of the sediment and the concentration of NO_3^- in the overlying water from intertidal flats in southeast England [61]. In the Chesapeake Bay, the %R_{amx} ranged from 0 to 22%, with the highest rate in the freshwater portion of the main stem of upper Chesapeake Bay, where water column NO_3^- concentrations are consistently high [62]. Nevertheless, it has also been shown that higher bottom water oxygen concentrations inhibited dissimilatory nitrate reduction to ammonium (DNRA) and denitrification but stimulated both anammox activity and the %R_{amx} [30]. Therefore, the usefulness of the abundance ratio of AMB 16S rRNA/*nosZ* as an indicator of AMB's contribution to N_2 production is partly supported by these previous observations. Further studies are needed to reveal the relationships between this or alternative indicators (e.g., the ratio of anammox functional gene to *nosZ* copy numbers) and %R_{amx}, which could benefit to large-scale investigations and our understanding on the spatiotemporal variations on the contribution of anammox to N_2 production in coastal ecosystems.

In summary, we have examined the spatiotemporal pattern of the abundance of N-cycling microbes in sediments of three hypernutrified LZB tidal flats where the heavy metal contamination were at relatively low levels. Overall, function-similar groups have showed niche specialization in the LZB estuarine sediments. AOA are more abundant than AOB in most cases, and their relative abundance is correlated significantly with the concentration of heavy metals (e.g., Cd and Cu) and pH. For denitrifiers, *nirK*-type usually outnumbers *nirS*-type, and *nosZ*-type is the lowest. The variations in abundances of *nirS* and *nirK* show a similar pattern by correlating with temperature, whereas their copy number ratio is correlated with pH, nitrite, nitrate, and cadmium. The variation of the ratio of *nirK* and *nirS* abundance depends on nitrogen load and cadmium. Abundance of *nosZ*-type is correlated with not only temperature, but also nitrite and Cd in sediments. Anammox bacterial abundances are strongly correlated with salinity, pH, nitrite and Cu. Furthermore, we have explored for the first time the associations between environmental factors and combinations or ratios of copy numbers of nitrogen cycling genes. For example, the abundance ratio of anammox bacterial 16S rRNA gene to denitrifying *nosZ* gene, which could be a potential indicator for the contribution of anammox to N_2 production, is correlated to temperature, dissolved oxygen in overlying water, and ratio carbon to nitrogen and concentrations of ammonium and As in sediments. For the two indicators of genetic potentials for N_2O emission (*nirS* + *nirK*-*nosZ*) and (*nirS* + *nirK*)/*nosZ*, the former is only correlated to the temperature, whereas the latter shows no correlations with any variables determined. Taken together, our study stresses that abundances of N-cycling functional groups respond differently to variations of environmental conditions, and multiple factors including heavy metals with relatively low concentrations may play a role in shaping nitrogen cycling processes in these estuarine tidal flats.

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