



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

Xiaoli Zhang, Hélène Agogué, Christine Dupuy, Jun Gong

► To cite this version:

Xiaoli Zhang, Hélène Agogué, Christine Dupuy, Jun Gong. Relative Abundance of Ammonia Oxidizers, Denitrifiers, and Anammox Bacteria in Sediments of Hypernutrified Estuarine Tidal Flats and in Relation to Environmental Conditions. *CLEAN - Soil, Air, Water*, 2014, 42 (6), pp.815-823. 10.1002/clen.201300013 . hal-01086924

HAL Id: hal-01086924

<https://hal.science/hal-01086924>

Submitted on 25 Nov 2014

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

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

Xiaoli Zhang¹, Hélène Agogue², Christine Dupuy², Jun Gong^{1*}

¹ Microbial Ecology Group, Key Laboratory of Coastal Biology and Bioresource Utilization, Yantai Institute of Coastal Zone Research, Chinese Academy of Sciences, Yantai, China,

² Littoral, Environnement et Sociétés (LIENSSE) UMR 7266 CNRS – University of La Rochelle, La Rochelle, France.

* Correspondence: Dr. Jun Gong, Yantai Institute of Coastal Zone Research, Chinese Academy of Sciences, Yantai, 264003, China. Email: jgong@yic.ac.cn

Running head: Abundance of Nitrogen Cycling Microbes in Tidal Flats

Abbreviations:

AMB, anammox bacteria; **amoA**, ammonia monooxygenase gene; **AOA**, ammonia-oxidizing archaea; **AOB**, ammonia-oxidizing bacteria; **C/N**, ratio of carbon to nitrogen; **DIN**, dissolved inorganic nitrogen; **DO**, dissolved oxygen; **LZB**, Laizhou Bay; **N₂**, dinitrogen gas; **NH₃**, ammonia; **NH₄⁺**, ammonium; **NH₄-N**, ammonium nitrogen; **nirK**, gene encoding the copper-containing nitrite reductase; **nirS**, gene encoding the cytochrome *cdl*-containing nitrite reductase; **NO₂⁻**, nitrite; **N₂O**, nitrous oxide; **NO₂-N**, nitrite-nitrogen; **NO₃-N**, nitrate-nitrogen; **nosZ**, gene encoding nitrous oxide reductase; **rRNA**, ribosomal RNA; **psu**, practical salinity units; **qPCR**, quantitative PCR; **As**, Arsenic; **Co**, cobalt; **Cd**, cadmium; **Cr**, chromium; **Cu**, copper; **Ni**, nickel; **Pb**, lead; **Zn**, zinc. **Fe**, iron; **%R_{amx}**, percentage of N₂ production due to anammox.

Abstract

The relative abundances of nitrifying, denitrifying and anammox prokaryotes in sediments of three hypernutrified 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 N₂O 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 N₂ 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: Environment factors; heavy metals; nitrogen cycle; community size; abundance; gene copy number ratio.

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, 15, 18-21, 33, 34, 37, 39, 40, 42-45]. 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 [6]. 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 [7, 8]. Like denitrification, anammox represents a net loss of nitrogen from the system by 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 [7].

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 [9-11]. For example, Hallin et al. (2009) found that abundance, rather than composition, correlated with denitrification rates and nitrification rates by ammonia-oxidizing archaea in agricultural soils [9]. Bacterial *amoA* gene abundance and ammonium content best explain potential nitrification rates in Alaskan soils [11], whereas *nosZ*, *nirS* and *nirK* gene abundance and nitrate best explain potential denitrification rates in estuarine sediments [12]. Denitrifying gene abundances can be used as proxies for predicting N_2O emissions in soil and wetland environments [10, 13]. 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 [14-17].

Earlier studies of marine sediments from a variety of estuarine locations have shown different patterns of AOA/AOB dominance [18-21]. For denitrifying genes, *nirS* was more abundant than *nirK* in the San Francisco Bay estuary and subtropical Fitzroy estuary [21, 22]. 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 [17, 23]. 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 N₂O emissions and contribution of anammox to N₂ 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 [24]. For example, the AMO coded by *amoA* gene is a Cu/Fe containing enzyme [25], and the NIR coded by *nirK* gene has been shown to contain Cu [26]. The effect of heavy metals on nitrogen cycling populations in sediments have not been studied sufficiently [22]. 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 [27, 28]. 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 [29].

In the past decades, the Laizhou Bay (LZB), Bohai Sea, northern China has been hypernutrified due to huge interrestrial input (most commonly, dissolved inorganic nitrogen, DIN) from about 20 rivers of the coastal zone [30, 31]. 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 N₂O emission, and contribution of anammox to N₂ 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 1,237 km²; 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 km², receiving extensive agricultural and industrial discharges. The Di river is small sewage river (23 km long, catchment area 119 km²), 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 (NO₃⁻-N), nitrite (NO₂⁻-N), and ammonium (NH₄⁺-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 1M HCl [32], and concentrations of eight heavy metals (As, Co, Cd, Cr, Cu, Ni, Pb and Zn) were determined with an ELAN DRC II plasma mass spectrometry (ICP-MS) (PerkinElmer, Hong Kong).

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 quantitative PCR (qPCR). Primers used were as listed in Table 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 a 25 μl volume 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 2x (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, 40s 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 more than 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 2 layers, 9 sites in 2 seasons. Environmental factors during the samplings are shown in the Fig. S1. Water temperature ranged from 7.6°C 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 psu (practical salinity units) to 50.3 psu, there was no statistically significant difference between the seasons or locations ($P>0.05$; Fig. S1). A similar situation occurred for other environmental factors such as pH (5.97 – 8.61), dissolved oxygen (228.1 – 361.3 μ M), 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 μ m, 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 [39], concentrations of heavy metals As (0.84-3.91mg/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) [31]. Nevertheless, among the three tidal flats, JL generally had the most high levels of heavy metals. Significant differences in concentrations of Cd, Cu, Ni, Pb and Zn were only found between BL and Di ($P<0.05$, 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 ($P<0.05$, Fig. S1). In addition, there was no significant effect of sediment

layer on heavy metal concentrations (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 ($\rho = 0.74$, $P < 0.01$), $\text{NH}_4\text{-N}$ ($\rho = 0.61$, $P < 0.05$) 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 are Table 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; Table S1), which was between 0.5 to 26.6 times the level of AOB-*amoA* found in the samples (Table 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 (Table 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 (Table 2).

3.3 Correlation Analysis

In order to explore the relationship between gene abundances and environmental variables, Spearman's correlations were performed (Table 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 $\text{NO}_2\text{-N}$ ($\rho = -0.74$, $P < 0.01$). 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 (Table 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 $\text{NO}_2\text{-N}$ ($\rho = -0.85$, $P < 0.01$), $\text{NO}_3\text{-N}$ ($\rho = -0.58$, $P < 0.05$) 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 N₂ 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 NH₄-N ($\rho=0.59$, $P<0.05$). In addition, a positive correlation was identified between *AMB/nosZ* and As ($\rho=0.61$, $P<0.05$) (Table 3).

Correlations between different genes in samples cross locations and seasons were analyzed (Table 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 AOA and *nosZ* or *AMB* were observed from the dataset for both layer samples, but not the upper layer samples (Table 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 (Table 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 [19], 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 [40]. 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 those reported for other estuaries [41-44].

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 [41, 45-47]. 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 [19, 43, 48, 49]. Our further analysis indicated a negative correlation between AOA/AOB ratio and pH (Table 3), which is consistent with previous notion that AOA outnumbers AOB in soils with lower pH [50]. 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 (Table 3), contrasting to the study in the San Francisco Bay estuary, where Ni and Pd showed strong correlations with abundance of AOA [19]. 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, 10000 mg/kg; Pb, 200 mg/kg; Cu, 1600-2400 mg/kg) [51, 52], 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 [10], but contrast with several reports that *nirS*-type dominated in estuarine sediments [21, 22, 53]. This is consistent with the idea that *nirS*- and *nirK*-type denitrifiers have different habitat preferences, as suggested by several studies [9, 54]. 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 [55], whereas *nirS* was highest in the more marine sites and *nirK* abundance was highest in riverine sites [22]. 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 [24], 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 [22]; 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 [53, 56]; 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 [57]. Nevertheless, a positive correlation between *nirS* and Pb was also observed [22]. 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 (Table 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 N₂O intermediary [10]. Therefore, the value of *nirS+nirK-nosZ* or the ratio of (*nirS+nirK*)/*nosZ* could be used as predictors of the potential for N₂O emission. The seasonal effect on *nirS+nirK-nosZ* was significant (t-test, $P<0.01$; Fig. 2D), predicting a greater N₂O emission potential in winter than in summer in the LZB estuarine tidal flats. This seasonal

pattern of N₂O emission has long been observed [58], although the N₂O 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 N₂O production was stimulated with the progressive increase of metals (Cu, Zn, Cr and Cd) to estuarine sediments [53]. 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 [17], and nitrite concentration are significantly correlated with abundance of anammox bacteria [59]. 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 [60]. 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 [60]. 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 [61], which could supply more NO₂⁻ 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 N₂ production (%R_{amx}). The rationale is that both the AMB and the *nosZ*-type denitrifiers are responsible for N₂ 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 ¹⁵N 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 NH₄-N in sediments, and negatively with dissolved oxygen in the overlying water (Table 3). In agreement, the %R_{amx} was positively correlated with both the organic carbon content of the sediment and the concentration of NO₃⁻ in the overlying water from intertidal flats in southeast England [62]. 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 NO₃⁻ concentrations are consistently high [63]. 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} [17]. Therefore, the usefulness of the abundance ratio of AMB 16S rRNA/*nosZ* as an indicator of AMB's contribution to N₂ 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 N₂ 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 N₂ 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 N₂O 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.

Acknowledgments

This work was supported by the CAS Knowledge Innovation Project (No. KZCX2-YW-JC203), the Natural Science Foundation of China (No. 41206155), the CAS Scientific Innovation Program - Interdisciplinary Field, the One Hundred Talents Program of CAS, and International Partnership Program for Creative Research Teams. Thanks are due to Ms. Xiaohong Guo, Beibei Xing and Drs. Kexin Zhao and Xiaobo Liu for helps in sampling and laboratory assistance.

Conflict of Interest

The authors have declared no conflict of interest.

5 References

- [1] X. A. Alvarez-Salgado, G. Roson, E. F. Perez, F. G. Figueiras, Y. Pazos, Nitrogen cycling in an estuarine upwelling system, the Ria de Arousa (NW Spain). I. Short-time-scale patterns of hydrodynamic and biogeochemical circulation, *Mar. Ecol. Prog. Ser.* 1996, 135, 259–273.
- [2] K. L. Spencer, C. L. MacLeod, Distribution and partitioning of heavy metals in estuarine sediment cores and implications for the use of sediment quality standards, *Hydrol. Earth Syst. Sc.* 2002, 6(6), 989–998.
- [3] R. W. Howarth, R. Marino, Nitrogen as the limiting nutrient for eutrophication in coastal marine ecosystems: Evolving views over three decades, *Limnol. Oceanogr.* 2006, 51(1, part 2), 364–376.
- [4] L. A. Codispoti, J. A. Brandes, J. P. Christensen, A. H. Devol, S. W. A. Naqvi, H. W. Paerl, T. Yoshinari, The oceanic fixed nitrogen and nitrous oxide budgets: moving targets as we enter the Anthropocene? *Scientia Marina* 2001, 65(Suppl. 2), 85–105.
- [5] C. A. Francis, K. J. Roberts, J. M. Beman, A. E. Santoro, B. B. Oakley, Ubiquity and diversity of ammonia-oxidizing archaea in water columns and sediments of the ocean, *Proc. Natl. Acad. Sci. USA* 2005, 102(41), 14683–14688.
- [6] W. G. Zumft, Cell biology and molecular basis of denitrification, *Microbiol. Mol. Biol. Rev.* 1997, 61(4), 533–616.
- [7] C. A. Francis, J. M. Beman, M. M. Kuypers, New processes and players in the nitrogen cycle: the microbial ecology of anaerobic and archaeal ammonia oxidation, *ISME J.* 2007, 1(1), 19–27.
- [8] Z. F. Zhou, Y. M. Zheng, J. P. Shen, L. M. Zhang, J. Z. He, Response of denitrification genes *nirS*, *nirK*, and *nosZ* to irrigation water quality in a Chinese agricultural soil, *Environ. Sci. Pollut. Res. Int.* 2011, 18(9), 1644–1652.
- [9] S. Hallin, C. M. Jones, M. Schlöter, L. Philippot, Relationship between N-cycling communities and ecosystem functioning in a 50-year-old fertilization experiment, *ISME J.* 2009, 3(5), 597–605.
- [10] A. García-Lledó, A. Vilar-Sanz, R. Trias, S. Hallin, L. Bañeras, Genetic potential for N₂O emission from the sediment of a free water surface constructed wetland, *Water Research* 2011, 45(17), 5621–5632.
- [11] D. G. Petersen, S. J. Blazewicz, M. Firestone, D. J. Herman, M. Turetsky, M. Waldrop, Abundance of microbial genes associated with nitrogen cycling as indices of biogeochemical process rates across a vegetation gradient in Alaska, *Environ. Microbiol.* 2012, 14(4), 993–1008.
- [12] L. F. Dong, C. J. Smith, S. Papaspyrou, A. Stott, A. M. Osborn, D. B. Nedwell, Changes in benthic denitrification, nitrate ammonification, and anammox process rates and nitrate and nitrite reductase gene abundances along an estuarine nutrient gradient (the Colne Estuary, United Kingdom), *Appl. Environ. Microbiol.* 2009, 75(10), 3171–3179.
- [13] S. E. Morales, T. Cosart, W. E. Holben, Bacterial gene abundances as indicators of greenhouse gas emission in soils, *ISME J.* 2010, 4(6), 799–808.
- [14] M. M. M. Kuypers, G. Lavik, D. Woebken, M. Schmid, B. M. Fuchs, R. Amann, B. B. Jørgensen, et al. Massive nitrogen loss from the Benguela upwelling system through anaerobic ammonium oxidation, *Proc. Natl. Acad. Sci.* 2005, 102(18), 6478–6483.

- [15] P. Lam, M. M. Jensen, G. Lavik, D. F. McGinnis, B. Müller, C. J. Schubert, R. Amann, et al. Linking crenarchaeal and bacterial nitrification to anammox in the Black Sea, *Proc. Natl. Acad. Sci.* 2007, 104(17), 7104–7109.
- [16] M. C. Schmid, N. Risgaard-Petersen, J. V. de Vossenberg, M. M. M. Kuypers, G. Lavik, J. Petersen, S. Hulth, et al. Anaerobic ammonium-oxidizing bacteria in marine environments: widespread occurrence but low diversity, *Environ. Microbiol.* 2007, 9(6), 1476–1484.
- [17] O. R. Dale, C. R. Tobias, B. Song, Biogeographical distribution of diverse anaerobic ammonium oxidizing (anammox) bacteria in Cape Fear River Estuary, *Environ. Microbiol.* 2009, 11(5), 1194–1207.
- [18] J. M. Beman, C. A. Francis, Diversity of ammonia-oxidizing archaea and bacteria in the sediments of a hypernutrified subtropical estuary: Bahía del Tóbari, Mexico, *Appl. Environ. Microbiol.* 2006, 72(12), 7767–7777.
- [19] A. C. Mosier, C. A. Francis, Relative abundance and diversity of ammonia-oxidizing archaea and bacteria in the San Francisco Bay estuary, *Environ. Microbiol.* 2008, 10(11), 3002–3016.
- [20] A. E. Santoro, C. A. Francis, N. R. de Sieyes, A. B. Boehm, Shifts in the relative abundance of ammonia-oxidizing bacteria and archaea across physicochemical gradients in a subterranean estuary, *Environ. Microbiol.* 2008, 10(4), 1068–1079.
- [21] G. C. J. Abell, A. T. Revill, C. Smith, A. P. Bissett, J. K. Volkman, S. S. Robert, Archaeal ammonia oxidizers and nirS-type denitrifiers dominate sediment nitrifying and denitrifying populations in a subtropical macrotidal estuary, *ISME J.* 2010, 4(2), 286–300.
- [22] A. C. Mosier, C. A. Francis, Denitrifier abundance and activity across the San Francisco Bay estuary, *Environ. Microbiol. Rep.* 2010, 2(5), 667–676.
- [23] C. Teixeira, C. Magalhães, S. B. Joye, A. A. Bordalo, Potential rates and environmental controls of anaerobic ammonium oxidation in estuarine sediments, *Aquat. Microb. Ecol.* 2012, 66(1), 23–32.
- [24] F. M. Morel, N. M. Price, The biogeochemical cycles of trace metals in the oceans, *Science* 2003, 300(5621), 944–947.
- [25] R. Yu, B. Lai, S. Vogt, K. Chandran, Elemental profiling of single bacterial cells as a function of copper exposure and growth phase, *PloS One* 2011, 6(6), e21255.
- [26] M. Nojiri, Y. Xie, T. Inoue, T. Yamamoto, H. Matsumura, K. Kataoka, Deligeer, K Yamaguchi, et al. Structure and function of a hexameric copper-containing nitrite reductase, *Proc. Natl. Acad. Sci.* 2007, 104(11), 4315–4320.
- [27] T. E. Freitag, J. I. Prosser, Community structure of ammonia-oxidizing bacteria within anoxic marine sediments, *Appl. Environ. Microbiol.* 2003, 69(3), 1359–1371.
- [28] R. J. G. Mortimer, S. J. Harris, M. D. Krom, T. E. Freitag, J. I. Prosser, J. Barnes, P. Anschutz, et al. Anoxic nitrification in marine sediments, *Mar. Ecol. Prog. Ser.* 2004, 276, 37–52.
- [29] C. Rooks, M. C. Schmid, W. Mehsana, M. Trimmer, The depth-specific significance and relative abundance of anaerobic ammonium-oxidizing bacteria in estuarine sediments (Medway Estuary, UK), *FEMS Microbiol. Ecol.* 2012, 80(1), 19–29.

- [30] Z. F. Zhang, X. He, Z. Zhang, G. C. Han, Y. Wang, L. J. Wang, Eutrophication status, mechanism and its coupling effect with algae blooming in Bohai. *Mar. Environ. Sci.* 2012, 31(4), 465-483. (in Chinese with English abstract)
- [31] X. Luo, R. Zhang, J. Yang, R. Liu, W. Tang, Q. Yan, Distribution and pollution assessment of heavy metals in surface sediment in Laizhou Bay, *Ecol. Environ. Sci.* 2010, 19(2), 262-269. (in Chinese with English abstract)
- [32] S. Mccready, G. F. Birchard, S. E. Taylor, Extraction of heavy metals in Sydney Harbour sediments using 1M HCl and 0.05M EDTA and implications for sediment-quality guidelines, *Aust. J. Earth Sci.* 2003, 50(2), 249-255.
- [33] C. Wuchter, B. Abbas, M. J. L. Coolen, L. Herfort, J. van Bleijswijk, P. Timmers, M. Strous, et al. Archaeal nitrification in the ocean, *Proc. Natl. Acad. Sci. U.S.A.* 2006, 103(33), 12317-12322.
- [34] J. H. Rotthauwe, K. P. Witzel, W. Liesack, The ammonia monooxygenase structural gene *amoA* as a functional marker: molecular fine-scale analysis of natural ammonia-oxidizing populations, *Appl. Environ. Microbiol.* 1997, 63(12), 4704-4712.
- [35] R. Hornek, A. Pommerening-Roser, H. P. Koops, A. H. Farnleitner, N. Kreuzinger, A. Kirschner, R. L. Mach, Primers containing universal bases reduce multiple *amoA* gene specific DGGE band patterns when analysing the diversity of beta-ammonia oxidizers in the environment, *J. Microbiol. Methods* 2006, 66(1), 147-155.
- [36] J. Geets, M. de Cooman, L. Wittebolle, K. Heylen, B. Vanparys, P. De Vos, W. Verstraete, et al. Real-time PCR assay for the simultaneous quantification of nitrifying and denitrifying bacteria in activated sludge, *Appl. Microbiol. Biotechnol.* 2007, 75(1), 211-221.
- [37] F. Schreiber, P. Stief, A. Gieseke, I. M. Heisterkamp, W. Verstraete, D. De Beer, et al. Denitrification in human dental plaque, *BMC Biol.* 2010, 8(1), 24.
- [38] S. Melero, A. Pérez-de-Mora, J. M. Murillo, F. Buegger, K. Kleinedam, S. Kublik, K. Vanderlinden, et al. Denitrification in a vertisol under long-term tillage and no-tillage management in dryland agricultural systems: Key genes and potential rates, *Appl. Soil Ecol.* 2011, 47(3), 221-225.
- [39] SEPA (State Environmental Protection Administration of China), *Marine Sediment Quality (GB 18668-2002)*, Standards Press of China, Beijing, 2002.
- [40] A. E. Bernhard, Z. C. Landry, A. Blevins, J. R. de la Torre, A. E. Giblin, D. A. Stahl, Abundance of ammonia-oxidizing archaea and bacteria along an estuarine salinity gradient in relation to potential nitrification rates, *Appl. Environ. Microbiol.* 2010, 76(4), 1285-1289.
- [41] T. Jin, T. Zhang, L. Ye, O. O. Lee, Y. H. Wong, P. Y. Qian, Diversity and quantity of ammonia-oxidizing Archaea and Bacteria in sediment of the Pearl River Estuary, China, *Appl. Microbiol. Biotechnol.* 2011, 90(3), 1137-1145.
- [42] H. Dang, J. Li, R. Chen, L. Wang, L. Guo, Z. Zhang, MG Klotz, Diversity, abundance, and spatial distribution of sediment ammonia-oxidizing Betaproteobacteria in response to environmental gradients and coastal eutrophication in Jiaozhou Bay, China, *Appl. Environ. Microbiol.* 2010, 76(14), 4691-4702.

- [43] A. E. Bernhard, T. Donn, A. E. Giblin, D. A. Stahl, Loss of diversity of ammonia-oxidizing bacteria correlates with increasing salinity in an estuary system, *Environ. Microbiol.* 2005, 7(9), 1289–1297.
- [44] D. Woebken, B. M. Fuchs, M. M. Kuypers, R. Amann, Potential interactions of particle-associated anammox bacteria with bacterial and archaeal partners in the Namibian upwelling system, *Appl. Environ. Microbiol.* 2007, 73(14), 4648–4657.
- [45] J. M. Caffrey, N. Bano, K. Kalanetra, J. T. Hollibaugh, Ammonia oxidation and ammonia-oxidizing bacteria and archaea from estuaries with differing histories of hypoxia, *ISME J.* 2007, 1(7), 660–662.
- [46] S. J. Park, B. J. Park, S. K. Rhee, Comparative analysis of archaeal 16S rRNA and amoA genes to estimate the abundance and diversity of ammonia-oxidizing archaea in marine sediments, *Extremophiles* 2008, 12(4), 605–615.
- [47] J. M. Beman, C. A. Francis, Diversity of ammonia-oxidizing archaea and bacteria in the sediments of a hypernutrified subtropical estuary: Bahia del Tobari, Mexico, *Appl. Environ. Microbiol.* 2006, 72(12), 7767–7777.
- [48] E. Sahan, G. Muyzer, Diversity and spatio-temporal distribution of ammonia-oxidizing archaea and bacteria in sediments of the Westerschelde estuary, *FEMS Microbiol. Ecol.* 2008, 64(2), 175–186.
- [49] G. W. Nicol, D. Tscherko, L. Chang, U. Hammesfahr, J. I. Prosser, Crenarchaeal community assembly and microdiversity in developing soils at two sites associated with deglaciation, *Environ. Microbiol.* 2006, 8(8), 1382–1393.
- [50] J. I. Prosser, G. W. Nicol, Relative contributions of archaea and bacteria to aerobic ammonia oxidation in the environment, *Environ. Microbiol.* 2008, 10(11), 2931–2941.
- [51] X. Li, Y.G. Zhu, T.R. Cavagnaro, M. Chen, J. Sun, X. Chen, M. Qiao, Do ammonia-oxidizing archaea respond to soil Cu contamination similarly as ammonia-oxidizing bacteria? *Plant Soil* 2009, 324(1-2), 209–217.
- [52] J. Ollivier, N. Wanat, A. Austruy, A. Hitmi, E. Joussein, G. Welzl, J. C. Munch, et al. Abundance and diversity of ammonia-oxidizing prokaryotes in the root–rhizosphere complex of *Miscanthus x giganteus* grown in heavy metal-contaminated soils, *Microb. Ecol.* 2012, 64(4), 1038–1046.
- [53] C. Magalhães, J. Costa, C. Teixeira, A. A. Bordalo, Impact of trace metals on denitrification in estuarine sediments of the Douro River estuary, Portugal, *Mar. Chem.* 2007, 107(3), 332–341.
- [54] P. Junier, O. S. Kim, K. P. Witzel, J. F. Imhoff, O. Hadas, Habitat partitioning of denitrifying bacterial communities carrying *nirS* or *nirK* genes in the stratified water column of Lake Kinneret, Israel, *Aquat. Microb. Ecol.* 2008, 51(2), 129–140.
- [55] C. J. Smith, D. B. Nedwell, L. F. Dong, A. M. Osborn, Diversity and abundance of nitrate reductase genes (*narG* and *napA*), nitrite reductase genes (*nirS* and *nrfA*) and their transcripts in estuarine sediments, *Appl. Environ. Microbiol.* 2007, 73(11), 3612–1622.
- [56] C. M. Magalhães, A. Machado, P. Matos, A. A. Bordalo, Impact of copper on the diversity, abundance and transcription of nitrite and nitrous oxide reductase genes in an urban European estuary, *FEMS Microbiol. Ecol.* 2011, 77(2), 274–284.
- [57] Y. Cao, P. G. Green, P. A. Holden, Microbial community composition and denitrifying enzyme

activities in salt marsh sediments, *Appl. Environ. Microbiol.* 2008, 74(24), 7585–7595.

- [58] W. M. Kieskamp, L. Lohse, E. Epping, W. Helder, Seasonal variation in denitrification rates and nitrous oxide fluxes in intertidal sediments of the western Wadden Sea, *Mar. Ecol. Prog. Ser.* 1991, 72, 145–151.
- [59] H. Dang, R. Chen, L. Wang, L. Guo, P. Chen, Z. Tang, F. Tian, et al. Environmental factors shape sediment anammox bacterial communities in hypernutrified Jiaozhou Bay, China, *Appl. Environ. Microbiol.* 2010, 76(21), 7036–7047.
- [60] R. C. Jin, G. F. Yang, J. J. Yu, P. Zheng, The inhibition of the Anammox process: a review, *Chem. Eng. J.* 2012, 197, 67–79.
- [61] N. Labbé, S. Parent, R. Villemur, Addition of trace metals increases denitrification rate in closed marine systems, *Water Res.* 2003, 37(4), 914–920.
- [62] J. C. Nicholls, M. Trimmer, Wide spread occurrence of the anammox reaction in estuarine sediments, *Aquat. Microb. Ecol.* 2009, 55(2), 105–113.
- [63] J. J. Rich, O. R. Dale, B. Song, B. B. Ward, Anaerobic ammonium oxidation (anammox) in Chesapeake Bay sediments, *Microb. Ecol.* 2008, 55(2), 311–320.

Table 1. Primers for target genes used in this study.

Target gene	Primer	Sequence (5' -3')	Length of amplicon (bp)	Reference
AOA- <i>amoA</i>	Arch- <i>amoA</i> -for	CTGAYTGGGCTTGGACATC	256	[33]
AOB- <i>amoA</i>	Arch- <i>amoA</i> -rev	TTCTTCTTTGTTGCCAGTA	490	[34, 35]
	<i>amoA</i> 1F	GGGGTTTCTACTGGTGGT		
<i>nirS</i>	<i>amoA</i> r new	CCCCTCBGSAAAVCCTTCTTC	425	[36]
	Cd3aF	GTSAAACGTSAAAGGARACSGG		
<i>nirK</i>	R3cd	GASTTCGGRTGSGTCTTGA	472	[37]
	F1aCu	ATCATGGTSCTGCCGCG		
<i>nosZ</i>	R3Cu	GCCTCGATCAGRTTGTGGTT	267	[38]
	<i>nosZ</i> 2F	CGCRACGGCAASAAGGTSMSST		
Anammox bacterial 16S rRNA	<i>nosZ</i> 2R	CAKRTGCAKSGCRTGGCAGAA	232	[17]
	AMX808F	ARCYGTAAACGATGGGCACTAA		
	AMX1040R	CAGCCATGCAACACCTGTRATA		

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
<i>nirK/nirS</i>	2.6	2.2	1.7	4.4	1.8	2.0	2.3	0.2	1.2	0.7	0.2	0.2
<i>nirK/nosZ</i>	13.4	12.3	21.2	16.2	121.7	16.1	47.2	16.7	255.3	66.5	0.2	0.3
<i>nirS/nosZ</i>	5.2	5.6	12.5	3.7	67.6	8.2	20.9	68.0	217.7	90.6	1.0	1.4
<i>(nirK+nirS)/nosZ</i>	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/ <i>nosZ</i>	0.1	0.1	4.3	4.1	1.7	0.4	7.3	11.6	2.9	1.2	0.1	2.2

*AOB = bacterial *amoA* gene; AMB = anammox 16S rRNA gene; AOA = archaeal *amoA* gene; L = lower layer; U = upper layer.

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

	As	Cd	Cu	C/N	DO	Grain	NH ₄ -N	NO ₂ -N	NO ₃ -N	pH	Salinity	Temp
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
<i>nirS</i>	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	-0.75
<i>nirK</i>	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	-0.91
<i>nosZ</i>	ns	0.63	ns	ns	ns	ns	ns	-0.74	ns	ns	ns	-0.76
<i>nirS+nirK-nosZ</i>	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
<i>nirK/nirS</i>	ns	0.87	ns	ns	ns	ns	ns	-0.85	-0.58	-0.71	ns	ns
<i>nirK/nosZ</i>	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
<i>nirS/nosZ</i>	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
<i>(nirS+nirK)/nosZ</i>	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
AMB/ <i>nosZ</i>	0.61	ns	ns	0.78	-0.68	ns	0.59	ns	ns	ns	ns	0.70

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

*AOA = archaeal *amoA* gene; AOB = bacterial *amoA* gene; AMB = anammox 16S rRNA gene; DO = dissolved oxygen; Grain = sediment grain size; ns = not significant; Temp = temperature.

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

	AOB	AOA	<i>nirS</i>	<i>nirK</i>	<i>nosZ</i>	AMB
AOB	-	ns	0.80	0.63	ns	ns
AOA	ns	-	0.51	0.59	ns	ns
<i>nirS</i>	0.63	0.38	-	0.92	0.50	ns
<i>nirK</i>	0.54	ns	0.93	-	0.67	ns
<i>nosZ</i>	0.42	ns	0.67	0.78	-	0.49
AMB	ns	0.34	ns	ns	0.35	-

**Only the significant correlations ($P < 0.05$) are shown, and the highly significant correlations ($P < 0.01$) are highlighted in bold. AOA=archaeal *amoA* gene; AOB=bacterial *amoA* gene; AMB=anammox bacterial 16S rRNA gene; ns = not significant.

Figure Legends

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

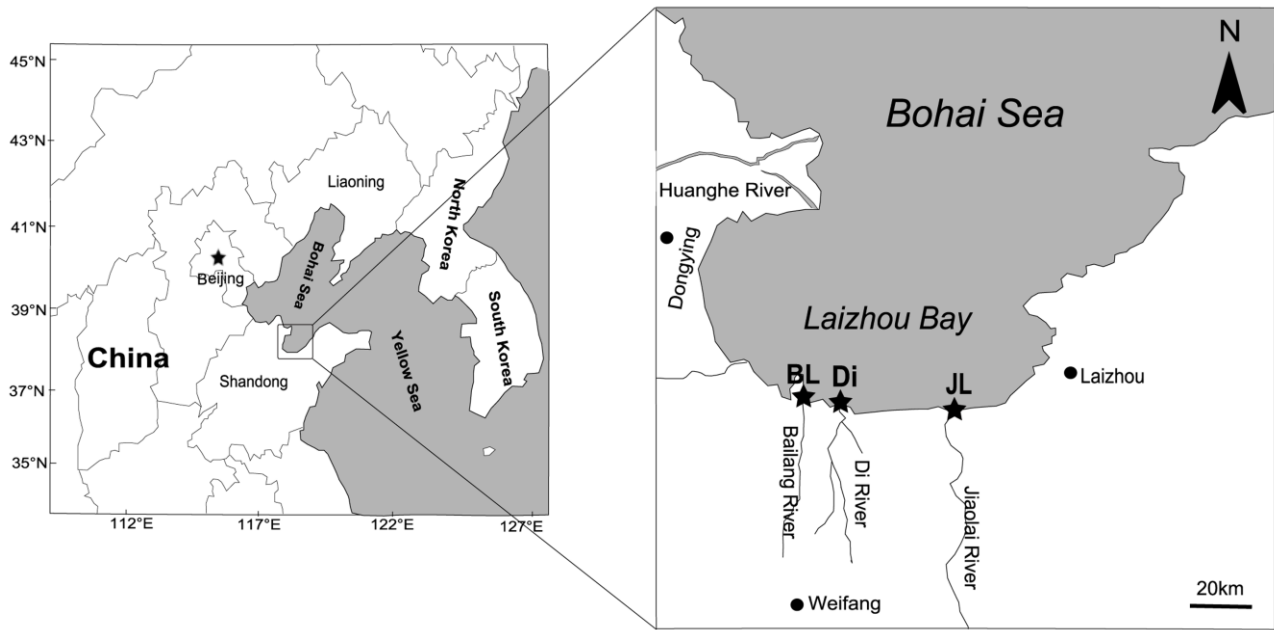
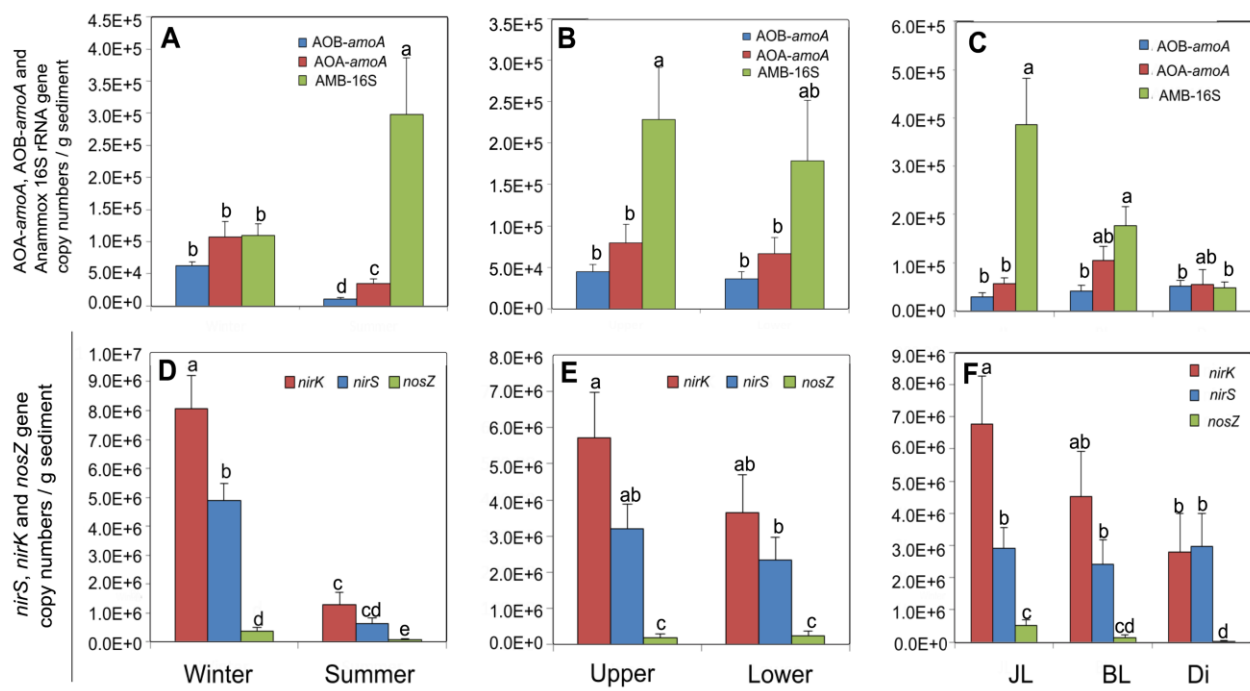


Figure 2. Copy numbers of anammox bacterial 16S rRNA and AOB-*amoA*, AOA-*amoA* (A–C), *nirS*, *nirK* and *nosZ* genes (D–F) determined for sediment samples (g^{-1} wet wt) from Laizhou Bay estuaries. Standard errors of the mean are indicated. Different letters above the bars indicate significant differences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



Supporting Information

Table S1. Copy numbers (mean \pm standard error) of anammox bacterial 16S rRNA and AOB-*amoA*, AOA-*amoA*, *nirS*, *nirK* and *nosZ* genes determine for sediment samples (g^{-1} dry wt) from the estuarine tidal flats of Laizhou Bay*.

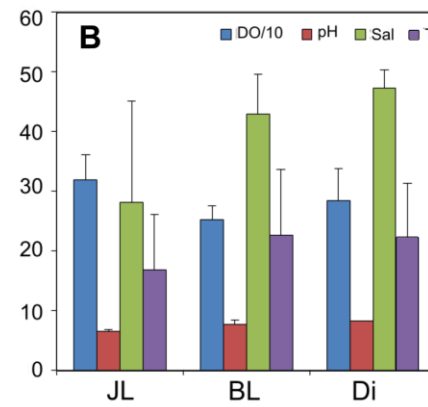
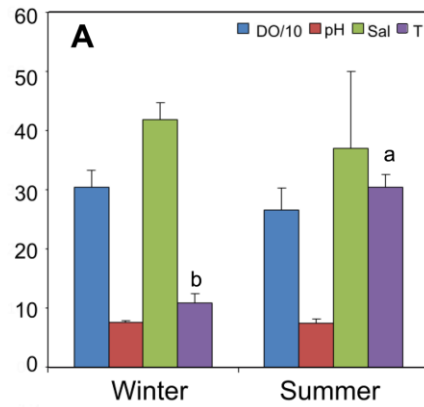
Sample	JL-U-W	JL-L-W	BL-U-W	BL-L-W	D-U-W	D-L-W	JL-U-S	JL-L-S	BL-U-S	BL-L-S	D-U-S	D-L-S
AMB 16S rRNA ($\times 10^4$)	10.0 \pm 3.4 ^{bc}	7.5 \pm 1.5 ^c	15.6 \pm 8.7 ^{bc}	17.0 \pm 5.4 ^b	7.6 \pm 4.2 ^{bc}	7.7 \pm 1.5 ^{bc}	66.1 \pm 18.4 ^a	70.8 \pm 29.7 ^a	36.5 \pm 10.7 ^a	1.2 \pm 0.5 ^d	1.0 \pm 0.5 ^d	2.9 \pm 2.2 ^d
AOB- <i>amoA</i> ($\times 10^3$)	56.0 \pm 21.9 ^a	28.2 \pm 4.6 ^b	69.0 \pm 8.4 ^a	66.7 \pm 9.6 ^a	71.3 \pm 10.7 ^a	83.4 \pm 19.0 ^a	24.8 \pm 4.1 ^b	8.2 \pm 2.5 ^b	2.8 \pm 0.3 ^e	3.4 \pm 0.6 ^e	14.8 \pm 0.3 ^c	7.0 \pm 1.2 ^d
AOA- <i>amoA</i> ($\times 10^4$)	5.5 \pm 2.6 ^b	9.3 \pm 4.3 ^b	25.5 \pm 4.4 ^a	6.8 \pm 3.1 ^b	3.6 \pm 2.7 ^b	13.8 \pm 8.9 ^{ab}	2.2 \pm 0.3 ^c	5.4 \pm 0.6 ^b	7.3 \pm 2.2 ^b	2.2 \pm 1.9 ^{bc}	1.3 \pm 0.2 ^c	0.5 \pm 0.2 ^c
<i>nirS</i> ($\times 10^4$)	419.3 \pm 74.7 ^{ab}	493.6 \pm 121.9 ^{ab}	621.4 \pm 63.8 ^a	332.5 \pm 192.0 ^{ab}	577.2 \pm 237.0 ^{ab}	596.6 \pm 125.0 ^{ab}	192.9 \pm 64.2 ^b	63.1 \pm 42.7 ^b	104.0 \pm 42.0 ^b	7.2 \pm 2.3 ^c	7.7 \pm 3.2 ^c	1.8 \pm 1.0 ^c
<i>nirK</i> ($\times 10^4$)	1077.4 \pm 277.2 ^{ab}	1086.5 \pm 249.9 ^a	1118.9 \pm 161.4 ^a	649.1 \pm 199.0 ^{ab}	677.0 \pm 355.7 ^{ab}	438.0 \pm 156.2 ^b	327.7 \pm 106.7 ^b	279.4 \pm 47.8 ^b	235.2 \pm 118.7 ^b	1.8 \pm 0.8 ^{cd}	1.9 \pm 0.6 ^c	0.3 \pm 0.1 ^d
<i>nosZ</i> ($\times 10^4$)	80.5 \pm 48.2 ^a	88.3 \pm 47.3 ^a	9.2 \pm 1.5 ^a	40.4 \pm 33.7 ^a	2.7 \pm 1.0 ^b	6.6 \pm 3.8 ^{ab}	15.4 \pm 7.6 ^{ab}	17.2 \pm 14.6 ^a	5.0 \pm 1.5 ^a	0.1 \pm 0.04 ^d	7.6 \pm 0.1 ^{ab}	1.3 \pm 0.1 ^c

*Sample labels: BL, Bailang tidal flat; D, Di tidal flat; JL, Jiaolai tidal flat; L, lower layer; S, summer; U, upper layer; W, winter.

Different letters in superscript represent significant differences at the 95% confidence level.

Figure S1. Environmental parameters of the overlying water (A–B) and sediment samples; mean values of dissolved oxygen (DO, μ M), pH, salinity (Sal, psu), temperature (T, $^{\circ}$ C), heavy metals (Cr, Co, Ni, Cu, Zn, Cd, Pb, As, mg/kg), nitrate ($\text{NO}_3\text{-N}$, mg/kg), nitrite ($\text{NO}_2\text{-N}$, mg/kg), ammonium ($\text{NH}_4\text{-N}$, mg/kg), ratio of organic carbon to organic nitrogen (C/N), and sediment grain size ($d_{0.5}$, μ m). Significant differences were tested at $P<0.05$. Letters indicate significant difference. Error bars show standard deviation. Samples from all sites were subjected to seasonal or layer comparisons.

Environmental parameters
of the overlying water
see legend for units



Environmental parameters
of sediment samples
see legend for units

