Biogeosciences Discuss., https://doi.org/10.5194/bg-2018-189
Manuscript under review for journal Biogeosciences
Discussion started: 9 May 2018

Niche differentiation of ammonia and nitrite oxidizers along a salinity gradient from the Pearl River estuary to the South China Sea

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Abstract

The niche differentiation between ammonia and nitrite oxidizers are controversial because they display disparate patterns in estuarine, coastal, and oceanic regimes. We analyzed ammonia-oxidizing archaea (AOA) and β-proteobacteria (AOB) *amo*A genes, nitrite-oxidizing bacteria (NOB) *nxr*B and 16S rRNA genes, and nitrification rates to identify their niche differentiation along a salinity gradient from the Pearl River estuary to the South China Sea. The archaeal *amo*A genes were generally more abundant than the β-AOB *amo*A genes; however, AOB more clearly attached to particles compared with AOA in the upper reaches of the Pearl River estuary. The NOB *Nitrospira* had higher abundances in the upper and middle reaches of the Pearl River estuary, while *Nitrospina* was dominant in the lower estuary. In addition, AOB and *Nitrospira* could be more active than AOA and *Nitrospina* since significantly positive correlations were observed between their gene abundance and the nitrification rate in the Pearl River estuary. There is a coupling of ammonia and nitrite oxidizers in the hypoxic waters of the estuary, suggesting metabolic interactions between them. Phylogenetic analysis further revealed that the AOA and NOB *Nitrospina* subgroups can be separated into different niches based on their adaptations to substrate levels. Water mass mixing is apparently crucial in regulating the distribution of nitrifiers from the estuary to open ocean. However, when eliminating water mass effect, the substrate availability and the nitrifiers' adaptations to substrate availability via their ecological strategies essentially determine their niche differentiation.

1 Introduction

Nitrification, the biological oxidation of ammonia to nitrate (the largest pool of fixed inorganic nitrogen in water bodies), is a fundamental process in the nitrogen cycle and plays a key role in estuarine and marine ecosystems. Nitrification includes both ammonia and nitrite oxidation, which are catalyzed by different microorganisms who may occupy broad niches in estuarine and marine environments. The first nitrification step, ammonia oxidation, is predominantly carried out by ammonia-oxidizing archaea (AOA) belonging to the phylum *Thaumarchaeota*, and ammonia-oxidizing bacteria (AOB). *Thaumarchaeota* are more adapted to ammonia-limited oligotrophic conditions than AOB (Erguder et al., 2009; Martens-Habbena et al., 2009). The gene coding for ammonia monooxygenase subunit A (*amo*A) has been widely applied as a functional marker gene for ammonia oxidizers (Juretschko et al., 1998; Francis et al., 2005; Leininger et al., 2006; Tourna et al., 2008; Gubry-Rangin et al., 2011; Pester et al., 2012).

In sharp contrast to ammonia oxidation, nitrite oxidation, which is the second step in nitrification, has been investigated less in estuarine and marine ecosystems, despite bacterial nitrite oxidation being the only biochemical reaction known to form nitrate in aerobic conditions. In addition, a considerable fraction of recycled nitrogen or reduced nitrate is re-oxidized back to nitrate via nitrite oxidation in oxygen minimum zones (OMZs; Füssel et al., 2012; Beman et al., 2013; Casciotti et al., 2013; Bristow et al., 2016). Nitrite oxidation is catalyzed by nitrite-oxidizing bacteria (NOB). To date, seven genera of NOB have been described: *Nitrospira*, *Nitrospina*, *Nitrococcus*, *Nitrobacter*, *Nitrolancea*, *Nitrotoga*, and *Candidatus* Nitromaritima (Spieck and Bock 2005; Alawi et al., 2007; Sorokin et al., 2012; Ngugi et al., 2016). Members of the genus *Nitrospira* appear to be the most diverse and widespread in a diverse range of habitats (Daims et al., 2001; Lücker et al., 2010), while *Nitrospina* are reported to be restricted to marine environments (Lücker et al., 2013). *Nitrobacter* and *Nitrococcus* are less abundant and confined mainly to freshwater/estuarine and oceanic settings, respectively (Koops and Pommerening-Roser, 2001; Füssel et al., 2012). *Nitrotoga* has been detected in a marine recirculation aquaculture system (Keuter et al., 2017). *Candidatus* Nitromaritima were recently identified based on metagenomic data in Red Sea brines (Ngugi et al., 2016). The gene encoding subunit beta of nitrite oxidoreductase (*nxr*B) is a functional and phylogenetic marker for NOB (Wertz et al., 2008; Pester et al., 2013; Schwarz, 2013). However, there is a *nxr*B-targeting primer sets coverage limitation, so that the NOB 16S rRNA gene has been used as a useful marker for quantifying the NOB community in various ecosystems (Mincer et al., 2007; Nunoura et al., 2015).

The niche differentiation between ammonia and nitrite oxidizers is controversial because it displays disparate patterns and partnerships in estuarine, coastal, and oceanic regimes. A gradient from an estuary to the ocean, with distinct distribution patterns of various nutrient species, may provide diverse niches for the coexistence of microbial species (Martens-Habbena et al., 2009). It is thus an ideal system to study the niche differentiation of AOA, AOB and NOB and major controlling factors.

The Pearl River is the largest river in southern China. Human activity has seriously affected the regional environment over the past few decades. A persistent oxygen depletion zone was found in the upper reaches of the Pearl River estuary (PRE) (He et al., 2014), which has been attributed to organic matter degradation and nitrification (Dai et al., 2006; 2008; He et al., 2010). The Pearl River drains into the northern part of the tropical oligotrophic South China Sea (SCS), the largest deep (maximum water depth of ~5560 m) semi-enclosed marginal sea in the western Pacific Ocean. Thus, the northern SCS is influenced by large amounts of freshwater and nutrient input from the Pearl River. The Southeast Asia Time-Series Study (SEATS) site, the only active time-series station located in a marginal sea (Wong et al., 2007; Zhang et al., 2014b), is situated in the SCS central basin (18°N, 116°E) at a depth of 3850 m and characterized by low nutrient levels. This environment, spanning the PRE to the SCS, provides a great opportunity to explore the microbial groups driving ammonia and nitrite oxidation within complicated biogeochemical settings.

In this study, the diversity of AOA and AOB *amo*A and NOB *nxr*B genes was investigated by clone libraries, and distributions of AOA and AOB *amo*A and NOB 16S rRNA genes were quantified by quantitative polymerase chain reaction (qPCR) along a salinity gradient from the PRE to the SCS (Fig. 1). Moreover, nitrification rates were determined in the PRE using ^{15}N-labeled ammonium (Sigman et al., 2001). The objectives of this study were to (1) investigate the spatial patterns of diversity and abundance of AOA, AOB, and NOB, (2) explore the niche differentiation and relationship between AOA, AOB, and NOB, and (3) explain the possible environmental parameters governing niche differentiation.

2 Materials and methods

2.1 Study sites and sampling

Twelve sites (P1–P12) along the PRE as well as the SEATS station in the SCS central basin were sampled during two summer research cruises in July–August 2012 and September 2014 (Fig. 1). Both the surface (1 m) and bottom waters (1.5–3.5 m above the seafloor) were sampled at the 12 PRE sites (Table S1); there were exceptions for sites P2, P3 and P4 where only the bottom water was sampled and P6 where only the surface water was sampled. The SEATS site was sampled at 75 m, 200 m, 800 m, and 3000 m water depth. Water samples were collected using a conductivity, temperature, and depth (CTD) rosette sampling system fitted with Go-Flo bottles (SBE 9/17 Plus; SeaBird Inc, USA). A total of 44 samples were subjected to gene analysis. A total of 10 samples from the bottom waters of sites P2–10 and the surface water of site P9 were amended with ^{15}N-labeled ammonium to measure nitrification rates.

2.2 Biogeochemical parameters

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Temperature, salinity, and depth data were obtained from the CTD system. Dissolved oxygen (DO) concentrations were directly measured onboard via the Winkler method (Carpenter, 1965). Water samples for inorganic nutrients such as nitrate, nitrite, phosphate, and silicate were filtered through 0.45 μm cellulose acetate membranes and then analyzed onboard. Ammonium was analyzed by the indophenol blue spectrophotometric method (Pai et al., 2001). Nitrite and nitrate were measured with a four-channel continuous flow Technicon AA3 Auto-Analyzer (Bran-Lube GmbH, Germany) (Han et al., 2012). Water samples for total suspended material (TSM) were filtered on to pre-combusted and pre-weighed glass fiber filter membranes (Whatman), and then stored at -20°C until weighing in the laboratory.

2.3 DNA extraction

One liter of water from each PRE sample was filtered through 3 μm and then 0.22 μm pore-size polycarbonate membranes (47 mm diameter; Millipore) at a pressure of <0.03 MPa to retain the particle-associated (PA) communities (size fraction >3 μm) and free-living (FL) communities (size fraction 0.22–3 μm) for DNA extraction. For the SCS samples, 2 or 4 liter water samples were directly filtered through 0.22 μm pore-size polycarbonate membranes (47 mm diameter; Millipore) for DNA extraction. All of the polycarbonate membranes were flash frozen in liquid nitrogen and then stored at -80°C until further analysis. DNA was extracted using the UltraClean Soil DNA kit (MoBio, San Diego, CA, USA) following the manufacturer's protocols. Concentration and purity of the genomic DNA were checked with a NanoDrop spectrophotometer (Thermo Scientific 2000/2000c) (Johnson, 1994).

2.4 PCR, cloning, sequencing, and phylogenetic analysis

Archaeal and *β*-proteobacterial *amo*A genes were amplified using primer sets Arch-amoAF and Arch-amoAR (Francis et al., 2005), and amoA-34F and amoA-2R (Kim et al., 2008), respectively. *Nitrospira*, *Nitrospina*, *Nitrobacter*, and *Nitrococcus nxr*B genes were amplified. Primer set sequences, PCR reaction mixtures and conditions for each functional gene are listed in Table S2. We designed primers for the *Nitrospina nxr*B gene based on two *nxr*B gene sequences of *Nitrospina gracilis* 3/211 using PREMIER software (Biosoft International, USA). Forward primer nxrBNF (5'-GGG CGA CCA GAT GGA AAC-3') and reverse primer nxrBNR (5'-GGG CCG GAC ATA GAA AGG-3') target the 771–788 and 1237–1254 nucleotide regions, respectively, of the *nxr*B gene in *N. gracilis* 3/211. The specificity of this designed primer pair was tested by BLASTn searches in the GenBank database. The amplified target fragments were purified using an agarose gel DNA purification kit (Takara, Dalian, China), ligated into the pMD18-T vector (Takara), and transformed into competent cells of *Escherichia coli* DH5α. Positive clones were randomly selected for sequencing using an ABI model 3730 automated DNA sequence analyzer with BigDye terminator chemistry (Perkin-Elmer, Applied Biosystems, USA).

All gene sequences were grouped into operational taxonomic units (OTUs) based on a 5%

sequence divergence cutoff (Wankel et al., 2011; Pester et al., 2013; Rani et al., 2017) by using the DOTUR program (Schloss and Handelsman, 2005). Representative nucleotide sequences were analyzed with the BLASTn tool to get the closest reference sequences. Neighbor-joining phylogenetic trees were constructed with MEGA 5 software using a Maximum Composite Likelihood model for archaeal *amo*A gene sequences (Zhang et al., 2014a) and Jukes-Cantor model for *Nitrospira* and *Nitrospina nxr*B gene sequences (Pester et al., 2013). A phylogenetic tree was not constructed for bacterial *amo*A gene and *Nitrobacter nxr*B gene sequences because too few sequences were retrieved. The *Nitrococcus nxr*B gene was not amplified successfully from these samples.

2.5 Quantitative PCR amplification

Abundances of the archaeal and *β*-proteobacterial *amo*A genes, and *Nitrospira* and *Nitrospina* 16S rRNA genes were quantified using a qPCR method and a CFX 96™ (BIO-RAD, Singapore) real-time system. Standard curves were constructed for archaeal and *β*-proteobacterial *amo*A genes using plasmid DNA from clone libraries. For *Nitrospira* and *Nitrospina* 16S rRNA genes, the target DNA fragments of the pure cultured strains were used. Quantitative PCR reactions were performed in triplicate and analyzed against a range of standards (1 to 10^7 copies per μl). Primer pair sequences, qPCR mixtures and conditions for each gene are listed in Table S3. The efficiencies of qPCR amplification ranged from 90% to 104% with R^2 >0.99. The specificity of the qPCR reactions was checked by melting curve analysis and agarose gel electrophoresis. The uncertain products were sequenced to confirm their veracity. Inhibition tests were performed by 2-fold and 5-fold dilutions of all samples and we concluded that our samples were not inhibited.

2.6 ^{15}N-labeled nitrification rate measurements

Nitrification rates (oxidation of ammonia to nitrate) were measured using the stable isotope tracer method described in Hsiao et al. (2014) with minor modifications. Briefly, six 115 mL narrow-necked gas-tight glass bottles were overflowed to more than twice their volume with seawater and sealed without headspace. Then, a syringe was used to replace 1 mL of sample with the $^{15}N\text{-}NH_4^+$ tracer (98% of ^{15}N atoms, Sigma-Aldrich) to attain a final tracer concentration of 1 μmol L^{-1}, which accounted for 1%–10% of total ammonia concentration in the upper PRE (P2–6, *in situ* rates of nitrification can be estimated) and >10% in the middle and lower reaches (P7–10, potential nitrification rates were obtained). Three bottles were filtered immediately after the tracer injection through 0.22 μm polycarbonate filters to represent the initial conditions. The remaining three bottles were kept in the dark for 6 h under *in situ* temperature (±1°C) using a temperature control incubator. The incubations were terminated by filtering through 0.22 μm polycarbonate membranes, and the filtrate was frozen at -20°C until laboratory analysis.

Ammonium, nitrite, and nitrate were detected as described above. The detection limits for ammonium, nitrite and nitrate were 0.16, 0.03 and 0.05 μmol L^{-1}, respectively. The $\delta^{15}N$ of NO_X^- (NO_2^- + NO_3^-) was determined using a bacterial method (Sigman et al., 2001), and gas chromatography (GC; Thermo Finnigan Gasbench, USA) with a cryogenic extraction and purification system interfaced to an isotopic ratio mass spectrometer (IRMS; Thermo Fisher Delta V^{PLUS}, USA). NO_X^- was quantitatively converted to N_2O using the bacterial strain *Pseudomonas aureofaciens* (ATTC no. 13985). The N_2O was then introduced to the GC-IRMS through the on-line N_2O cryogenic extraction and purification system. The $\delta^{15}N$ of NO_X^- was calibrated against nitrate isotope standards (USGS 34, IAEA N3, and USGS 32), which were run after every 10 samples during the run, as well as before and after each run. Accuracy (pooled standard deviation) was better than ±0.2‰ based on analyses of these standards at an injection level of 20 nmol N.

Nitrification rates were primary determined by the accumulation of ^{15}N in the product pool relative to the initial conditions using Eq. (1):

$$NR = d[^{15}N_t]/dt \times ([^{14}NH_4^+] + [^{15}NH_4^+])/[^{15}NH_4^+] \qquad (1)$$

where NR is the nitrification rate, t is the incubation time, $[^{15}N_t]$ is the concentration of ^{15}N in nitrate plus the nitrite pool in the sample at time t, $[^{14}NH_4^+]$ is the observed natural ammonium concentration and $[^{15}NH_4^+]$ is the final tracer concentration after the artificial addition of the stable isotope tracer. The detect limitation of this method is generally better than 0.01 μmol N L^{-1} d^{-1}.

2.7 Statistical analysis

Since normal distribution of the individual data sets was not always met, we used the non-parametric Wilcoxon tests for comparing two variables. Polynomial and exponential growth models (Sigmaplot) were used to determine the relationships between variables. Canonical correspondence analysis (CCA) was used to analyze the variations in the nitrifier communities under the constraint of environmental factors with automatic variable selection procedures in the CANOCO software (version 4.5, Microcomputer Power, USA) (Ter-Braak, 1989). The gene data were normalized as relative abundances. The environmental factors were normalized via Z transformation (Magalhães et al., 2008). The null hypothesis, that the community was independent of environmental parameters, was tested using constrained ordination with a Monte Carlo permutation test (999 permutations).

The standard and partial Mantel tests, which assess the correlations between two matrices controlling for the effects of a third matrix, were run in R (VEGAN) to determine the correlations between environmental factors or nitrification rates and nitrifier population compositions. Dissimilarity matrices of nitrifier communities were based on Bray-Curtis distances between samples, while environmental factors and nitrification rates were based on Euclidean distances between samples. The significance of the Mantel statistics based on Spearman or Kendall's product-moment correlation was obtained after 999 permutations. The results of the statistical tests were assumed to be significant at *P*-values ≤ 0.05.

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3 Results

3.1 Biogeochemical characteristics of the studied transect

According to the geomorphology and geochemical characteristics, the 12 sites in the PRE are situated in the upper (P1–P6), middle (P7 and P8), and lower reaches (P9–P12) of the estuary (Fig. 1). The upper reaches receive a small amount of freshwater, sewage, and industrial effluent discharge. The middle reaches receive about half of the freshwater from the North and West rivers, tributaries of the Pearl River, with little salinity stratification. The lower reaches are controlled mainly by estuarine mixing of freshwater and seawater (Wang et al., 2012). Salinity exhibited consistently low values between 0.12 and 3.82 at sites P1–P6 in the PRE upper reaches, but it sharply increased downstream from 1.23 to 31.92 at sites P7–P12 in the middle and lower reaches of the PRE (Fig. 2a). Temperature varied from 26.34 to 30.14°C and decreased seaward (Fig. 2b). Total suspended material concentrations ranged from 1.78 mg L^{-1} in the surface water of site P12 to 100 mg L^{-1} in the bottom water of site P4 (Fig. 2c). Dissolved oxygen concentrations showed a strong increasing trend seaward from 0.19 to 5.78 mg L^{-1}, with concentrations below 2 mg L^{-1} at sites P1–P6 (Fig. 2d). Accordingly, pH also showed a distinct increasing trend seaward from 7.04 to 8.17 (Fig. 2e). The nutrient (nitrate/nitrite/ammonium, phosphate, and silicate) concentrations showed distinctly decreasing trends seaward (Fig. 2f–j). The ammonium concentrations drastically decreased from 140.1 at site P1 to 9.9 μM at P6 in the upper PRE and had consistently low concentrations (below detection limit to 16.7 μM) in the middle and lower reaches (Fig. 2f). The nitrite concentrations varied from 1.9 μM in the bottom water (2 m above the seafloor) of site P12 to 44.2 μM in the bottom water (3.5 m above the seafloor) of site P4 (Fig. 2g). Overall, the upper PRE was characterized by hypoxic waters containing sufficient nutrients; DO concentrations increased seaward while the nutrient and TSM concentrations distinctly decreased seaward.

Depth profiles of the biogeochemical parameters from SEATS are shown in Fig. S1. Salinity slightly increased from 32.89 to 34.62 with depth. The sea surface temperature was 28.69°C, while the temperature decreased sharply to 2.35°C in the deep waters. The ammonium concentrations varied from below detection limit to 170.75 nM at 140 m depth. The nitrite concentrations ranged from detection limit to 0.63 μM at 55 m. The nitrate concentrations ranged from below detection limit to 39.32 μM along the water column. Phosphate and silicate increased from below detection limit to 2.89 μM and from 2.40 to 145.46 μM, respectively, with increasing water depth.

3.2 Diversity of ammonia and nitrite-oxidizing microbial communities

Archaeal and β-proteobacterial *amo*A and NOB (*Nitrospira*, *Nitrospina*, and *Nitrobacter*) *nxr*B gene clone libraries were constructed for the FL communities from the surface and bottom waters at site P8 and P9 because the most dramatic variations in biogeochemical properties along the PRE transect were present between these two sites (Fig. 2). In addition, archaeal *amo*A gene clone libraries were constructed at 75, 200, 800, and 3000 m water depth from SEATS, while a NOB *Nitrospina nxr*B gene clone library was constructed only at 800 m at SEATS as genes were not amplified successfully at the other three water depths. Rarefaction analyses showed that the diversity of *β*-AOB *amo*A genes observed in the PRE was nearly exhaustive, while the archaeal *amo*A gene libraries were composed of more phylotypes in both the PRE and SCS. Moreover, the richness of archaeal *amo*A genes was higher in the SCS than in the PRE (Fig. S2a). The *nxr*B gene clone libraries might have captured the majority of *Nitrobacter nxr*B gene types in the PRE with the primer sets used, based on the rarefaction curves, but not the *Nitrospira* and *Nitrospina nxr*B genes in the PRE and SCS (Fig. S2b). The same conclusions are supported by the diversity indices (Table S4).

3.3 Phylogenetic analysis of archaeal *amo*A and *Nitrospira* and *Nitrospina nxr*B genes

A total of 519 AOA *amo*A gene sequences were recovered and grouped into five clusters (A, Ba, Bb, D, and E) based on phylogenetic analysis (Fig. 3 and S3). According to the framework of Sintes et al. (2013) for the Atlantic and Arctic oceans, high ammonia clusters (HAC) were present in environments where ammonia concentrations ranged from 20 to 100 nM or even higher; however, low ammonia clusters (LAC) were predominant in environments where ammonia concentrations were frequently below detection limit. About half of the sequences retrieved from the PRE fell into groups A and D and almost all sequences retrieved from SEATS fell into groups Ba and Bb. Groups A and D have been identified as HAC and groups Ba and Bb as LAC by Nunoura et al. (2015) based on a phylogenetic analysis of archaeal *amo*A genes. Another half of the sequences retrieved from the PRE had an 86% to 100% DNA sequence identity with sequences recovered from high ammonia environments, such as lakes, rivers, soil, sewage treatment plants, and biofilters and clustered into group E, which clustered tightly with group D (Fig. 3). Thus, we defined group E as a HAC. The ammonium concentrations at sites where sequences were recovered further confirmed the categorization of groups A, Ba, Bb, D, and E. The sequences falling in groups A, D and E (HAC) were retrieved from sites with ammonium concentrations of 0.032 to 8.09 μM with the exception of four sequences retrieved from 3000 m at SEATS (below detection limit). The sequences falling in group Ba and Bb (LAC) were retrieved from SEATS at depths with ammonium concentrations below detection limit, except for 200 m (0.035 μM) (Fig. 3). Phylogenetic analysis and the relative abundances of each group clearly revealed the distinct distribution of major *amo*A subgroups from the estuary (HAC) to the SCS central basin (LAC) and from the upper water (HAC) to the deep ocean (LAC) (Fig. 3 and S3).

A total of 345 *Nitrospira nxr*B gene sequences were recovered. Phylogenetic analysis (Fig. 4) grouped the sequences into previously described clusters (Pester et al., 2013), except for group H that only contained sequences recovered from the PRE in this study. Despite containing 95% of all of the *Nitrospira nxr*B sequences, groups B, C, D and F all belong to *Nitrospira* Lineage II. Notably, group C was the most dominant branch in the PRE with 92% to 98% DNA sequence identity with *Nitrospira* sp.

enrichment BS10 derived from activated sludge (Spieck et al., 2006). The sequences of group D have 91% to 94% DNA sequence identity with *N. moscoviensis* derived from a heating system (Ehrich et al., 1995), and the sequences of groups B and F are closely related with the *nxr*B sequences from Austrian forest soils (Pester et al., 2013). Around 2% of sequences fell into group A, belonging to *Nitrospira* Lineage I, which could have evolved from an ancestor in *Nitrospira* Lineage II (Pester et al., 2013). The remaining ~2% of sequences were grouped into groups E (*Nitrospira* Lineage V) and G (*Nitrospira* Linage IV). *Nitrospira* Linage IV were reported to contain *N. marina* isolated from the Gulf of Maine (Watson et al., 1986) and sponge-associated *Nitrospira* (Taylor et al., 2007; Off et al., 2010). The *nxr*B gene of *Nitrospira* was not detected at SEATS.

A total of 185 *Nitrospina nxr*B gene sequences were recovered. The phylogenetic tree grouped the sequences into four clusters (Fig. 5). The sequences recovered from SEATS all fell into a single branch (the SCS cluster), which showed high similarity (95% to 99% gene sequence identity) with three sequences belonging to one OTU from the eastern tropical South Pacific (ETSP) OMZ. The sequences retrieved from the PRE fell into three other clusters. Around 9% of total sequences clustered in the ETSP OMZ dominant cluster, and 48% clustered as a unique branch (the PRE cluster), which only contained sequences obtained from this study. Around 23% of total sequences fell in the 3/211 cluster with 88% to 100% gene sequence identity with *N. gracilis* 3/211, which was isolated from ocean surface water (Watson and Waterbury, 1971), and, in this study, was used to design the primers for amplifying the *nxr*B gene of *Nitrospina*. The phylogenetic analysis and relative abundance of each group revealed the distinct distribution of major *Nitrospina nxr*B subgroups from the PRE to the SCS (Fig. 5).

3.4 Abundance distribution of ammonia and nitrite oxidizers and nitrification rates

Abundances of the archaeal and β-proteobacterial *amo*A genes and *Nitrospira* and *Nitrospina* 16S rRNA genes were quantified using the qPCR method at all 12 sites of the PRE for the FL and PA communities in the surface and bottom waters (Table S1). *Nitrobacter* and *Nitrococcus* were not quantified since they were not major NOB groups in either the PRE or SCS sites, as indicated by clone library analysis. Archaeal and β-proteobacterial *amo*A gene abundances varied from below detection limit to 4.54×10^5 copies L^{-1} (PA community in the bottom water of site P9) and from below detection limit to 3.42×10^4 copies L^{-1} (PA community in the bottom water of site P4), respectively. Overall, the archaeal *amo*A genes were significantly more abundant than the β-proteobacterial *amo*A genes (Wilcoxon, $P < 0.01$), but AOB more distinctly attached to particles compared with AOA in the upper reaches of the PRE (sites P1–P6; Fig. 6a and b). *Nitrospira* and *Nitrospina* 16S rRNA gene abundances varied from below detection limit to 2.02×10^6 copies L^{-1} (PA community in the bottom water of site P4) and from 51 to 3.81×10^5 copies L^{-1} (PA community in the bottom water of site P4), respectively. The *Nitrospira* 16S rRNA genes were significantly more abundant than the *Nitrospina* 16S rRNA genes in the upper and middle reaches of the PRE (sites P1–P8, Wilcoxon, $P < 0.01$), whereas the opposite trend was observed in the lower estuary (sites P9–P12, Wilcoxon, $P < 0.01$; Fig. 6c and d). All of the genes were significantly more abundant in the PA than the FL communities (Wilcoxon, $P < 0.01$) (Fig. 6e and f).

Sites P1–P6, located in hypoxic waters that are typically defined when DO concentrations fall below 2 mg L^{-1} (Renaud, 1986), of the PRE upper reaches, have DO concentrations ranging from 0.19 to 1.93 mg L^{-1} (Fig. 7). Generally, the abundance of NOB (sum of *Nitrospira* and *Nitrospina*) 16S rRNA genes was significantly higher than the ammonia-oxidizing microbes (AOM, sum of archaea and *β*-proteobacteria) *amo*A genes in the hypoxic waters (Wilcoxon, $P < 0.01$; Fig. 6g and h). Notably, significant positive relationships were observed between AOM and NOB groups for both the FL (Fig. 8a) and PA (Fig. 8b) communities (eight correlations, $P < 0.05$–0.01, the findings were the same excluding the maximum values), suggesting a coupling between ammonia and nitrite oxidizers in the hypoxic estuarine niche.

The hypoxic zone gradually disappears seaward and the DO concentrations of sites P7–P12 varied from 2.15 to 5.78 mg L^{-1} (Fig. 7). The significant relationship between AOM and NOB collapsed instantly. The abundance of the NOB 16S rRNA genes was comparable with the AOM *amo*A genes (Fig. 6g and h), and archaea and *Nitrospina* became the dominant ammonia and nitrite oxidizers, respectively (Fig. 6b and d–f).

The nitrification rates generally decreased seaward with increasing DO concentrations, ranging from 0.19 μmol L^{-1} day^{-1} in the bottom water (2 m above the seafloor) of site P9 to 75.81 μmol L^{-1} day^{-1} in the bottom water (3.5 m above the seafloor) of site P5 (Fig. 7). Distinctly higher nitrification rates were observed in the hypoxic zone than the middle and lower reaches of the PRE (Wilcoxon rank-sum test, $P < 0.05$).

4 Discussion

4.1 Coverage of the primer pair for *Nitrospina nxr*B genes

The primer pair of nxrBNF and nxrBNR targeting the *Nitrospina nxr*B genes was designed in this study according to two *nxr*B gene sequences of *N. gracilis* 3/211, which is the only isolated *Nitrospina* strain from the oxygenated ocean (Watson and Waterbury, 1971) and the only genome-sequenced *Nitrospina* so far (Lücker et al., 2013). Despite very few reference sequences, phylogenetic analysis of the *Nitrospina nxr*B gene sequences retrieved based on this primer pair indicated diverse phylogenetic taxa, including 12 OTUs and four major phylogenetic clusters. The relative abundances of the four groups showed that 77% of total sequences fell out of the 3/211 cluster (Fig. 5). Feng et al. (2016) and Rani et al. (2017) also designed primer pairs targeting *nxr*B and *nxr*A subunit genes of *Nitrospina*, respectively. However, Feng et al. (2016) did not obtain any *nxr*B target fragments and Rani et al. (2017) focused on the *nxr*A gene in marine sediments.

4.2 Coupling between ammonia and nitrite oxidizers in the estuarine hypoxic niche

The abundance of NOB 16S rRNA genes was significantly higher than the AOM *amo*A gene in PRE hypoxic waters. This is similar to previous observations that NOB can reach high abundances in oceanic OMZs, where *Nitrospina* and *Nitrococcus* are abundant (Füssel et al., 2012; Beman et al., 2013). However, in PRE hypoxic waters, *Nitrospira* and *Nitrospina* were dominant, particularly on the particles. Taken together, distinctly higher nitrification rates in the hypoxic zone and extremely low oxygen concentrations suggests that the PRE system could not supply oxygen fast enough to meet the demands of NOB and thus oxygen may not be the only electron acceptor. It was hypothesized that abundant NOB in a hypoxic zone might benefit from utilizing alternative terminal electron acceptors for nitrite oxidation, such as iodate, Mn(IV) or Fe(III) (Lam and Kuypers, 2011; Casciotti and Buchwald, 2012), which could be more reactive in the particles in hypoxic waters (Hsiao et al., 2014).

Significant positive relationships between AOM and NOB groups in the PRE hypoxic waters for both PA and FL communities suggest a coupling between ammonia and nitrite oxidizers. Similar observations were also found by Mincer et al. (2007) and Santoro et al. (2010) where the distribution profiles of total AOA and *Nitrospina* were correlated in some coastal and open ocean habitats. In Namibian soils, network analysis also indicated that AOA and *Nitrospira* communities were highly correlated (Pester et al., 2013). The tight coupling between ammonia and nitrite oxidizers in abundance and spatial distribution, known as the “nitrification aggregate” (Arp and Bottomley, 2006), could reflect their interactions (Daebeler et al., 2014). The reciprocal feeding (Daims et al., 2016) supports such interactions between nitrifiers. For example, urease-positive (Koch et al., 2015) or cyanase-positive (Starkenburg et al., 2006; Lücker et al., 2010; 2013; Palatinszky et al., 2015) NOB can provide AOM with ammonia from urea and cyanate degradation while NOB obtain nitrite from the AOM. In high particle load environments, such reciprocal feeding interactions might be more prominent than in the open ocean because particles, as well as sludge flocs or biofilms, could provide matrices for the complex interactions of these nitrifiers.

4.3 Succession of dominant nitrifier groups from the estuary to the open ocean

Although the archaeal *amo*A genes were generally more abundant than the β-AOB *amo*A genes, significant positive correlations were observed between the β-AOB *amo*A gene abundance and the nitrification rate (oxidation of ammonia to nitrate) in the PRE ($r = 0.785$, $P < 0.05$; the partial Mantel test controlling for the effects of the NOB abundance: $R = 0.786$, $P < 0.01$). This result suggests that AOB might be more active than AOA, prefer estuarine habitats, and thus dominate the nitrification rate. AOA have been detected in great numbers in coastal and estuarine waters, such as the Columbia River estuary, Monterey Bay, Southern California Bight, San Francisco Bay, Yangtze River estuary and Bering Strait (Crump et al., 2000; Mincer et al., 2007; Beman et al., 2008; Mosier et al., 2008; Zhang et al., 2014a; Damashek et al., 2017), while AOB often comprise less than 0.1% of the microbial community

(Bothe et al., 2000). However, high abundance does not necessarily indicate high turnover rates (Zhang et al., 2014b) and AOB in ammonium-enriched environments might be highly active (Füssel, 2014) and thus substantially contribute to ammonia oxidation despite their low abundance. Similarly, the β-AOB *amo*A gene abundances have been correlated with potential nitrifying activities in the waters of the Seine River estuary (Cébron et al., 2003). In addition, the β-AOB *amo*A gene abundances were found to be significantly correlated to more environmental factors, including nitrite, nitrate, silicate, salinity, TSM, DO, and pH, in the PRE, whereas only one factor (TSM) was correlated to the AOA *amo*A gene (Table S5). We speculate that AOB could be better adapted to the estuarine habitat than AOA.

Nitrospira was more abundant than *Nitrospina* in the upper and middle reaches of the PRE. Moreover, a significant positive correlation was observed between the *Nitrospira* 16S rRNA gene abundance and the nitrification rate in the PRE (r = 0.791, P <0.05; the partial Mantel test controlling for the effects of the *amo*A gene abundance: R = 0.163, P <0.05). These results suggest that *Nitrospira* could be well adapted to eutrophic estuarine environments, with both higher abundance and nitrifying potential. *Nitrospira* is widespread in diverse habitat types and especially abundant in freshwater (Koch et al., 2015) and estuarine (Cébron et al., 2005; Nakamura et al., 2006) environments, but less abundant in marine ecosystems (Hoffmann et al., 2009; Off et al., 2010) despite the fact that the first *Nitrospira* described was isolated from an ocean (Watson et al., 1986).

Archaea and *Nitrospina* became the dominant ammonia and nitrite oxidizers, respectively, along the transect from the PRE to the SCS. This succession of dominant groups can be explained by niche differentiation of these nitrifiers, which involves different adaptations to environmental parameters, ecological strategies, and microbe–microbe interactions. For instance, AOB and *Nitrospira* might be enriched on particles or aggregates (Phillips et al., 1999; Lam et al., 2004; Lebedeva et al., 2008; Haaijer et al., 2013; Ganesh et al., 2014; Zhang et al., 2014a) and play an important role in estuarine ecosystems characterized by high particle densities, whereas AOA and *Nitrospina* might be relatively more adaptable to a FL life strategy (Watson and Waterbury, 1971; Woebken et al., 2007; Ganesh et al., 2014) and thus abundant in low-particle environments.

4.4 Environmental parameters allowing niche differentiation

The CCA analysis based on qPCR data (Fig. 9) revealed that AOB and *Nitrospira* were more adaptable to high nutrient and TSM concentrations; in contrast, AOA and *Nitrospina* FL communities were more adaptable to high salinity, DO, and pH water masses and low nutrient and TSM environments. To some extent, AOA and *Nitrospina* PA communities were positively influenced by TSM. The CCA analysis based on clone libraries (Fig. 10a) further revealed that AOA HAC groups E and D were under the constraint of high nutrient conditions and HAC group A was positively influenced by TSM to an extent. The LAC groups Ba and Bb were under the constraint of high salinity and low temperature water masses. This is consistent with the phylogenetic analysis that indicates niche differentiation of AOA subgroups by adaptation to different ammonia levels. Similarly, the *Nitrospina* SCS cluster was under the constraint of high salinity and low temperature water masses, and other clusters were positively correlated with nutrients or TSM (Fig. 10b). The *Nitrospira* OTU-based ordination was obviously correlated with nutrients, DO, TSM, and salinity in the PRE. Overall, groups d and g were positively correlated with salinity and TSM, and other groups were regulated by nutrients and DO (Fig. 10c). Taken together, these CCA analyses show how environmental parameters allow for the niche differentiation of these nitrifiers.

The environmental factors included three types: water mass parameters (temperature, salinity, and silicate), substrate parameters (ammonia/ammonium, nitrite, and nitrate), and parameters influencing substrate availability (DO, TSM, and pH). AOA have been shown to be adaptable to low ammonia concentrations (<10 nM ammonium threshold, $K_{m(app)}$ = ~3 nM NH_3; Martens-Habbena et al., 2009; Kits et al., 2017), whereas AOB require higher concentrations of ammonia than usually observed in the ocean ($K_{m(app)}$ = 0.25–157.50 µM NH_3; Kits et al., 2017). Therefore, AOA are the major ammonia oxidizers in estuarine, coastal, and oceanic environments (Francis et al., 2005; Lam et al., 2007; Beman et al., 2008; Santoro et al., 2010), and AOB are favored in high ammonium environments (Verhamme et al., 2011). Furthermore, the niche differentiation of AOA subgroups also show their adaptation to different ammonia levels.

Nitrite, a central intermediate compound in nitrification, was positively correlated to NOB 16S

Biogeosciences Discuss., https://doi.org/10.5194/bg-2018-189
Manuscript under review for journal Biogeosciences
Discussion started: 9 May 2018

rRNA and *β*-proteobacterial *amo*A gene abundances (*P* <0.05–0.01, Table S5). *Nitrospira* displays stronger correlations to nitrite than *Nitrospina* in the PRE, suggesting that *Nitrospira* is likely adapted to a higher nitrite flux (Spieck et al., 2006; Lebedeva et al., 2008; Nunoura et al., 2015). Nitrite might be one major factor causing niche differentiation of NOB groups (Both and Laanbroek, 1991). Nitrate, a final product of nitrification, was also significantly positively correlated to *Nitrospira* 16S rRNA and *β*-proteobacterial *amo*A gene abundances (*P* <0.05–0.01, Table S5).

Notably, all genes were significantly positively correlated to TSM concentrations in PA and total communities (*P* <0.05–0.01, Table S5). This is consistent with the observation that all of the genes were significantly more abundant in the PA communities. The suspended particulate microniche could be beneficial to microbial activity because of the vicinal supply of nutrients or substrates from particles (Belser, 1979; Crump et al., 1998; Ouverney and Fuhrman, 2000; Teira et al., 2006; Zhang et al., 2014a). Lower light inhibition could also be a potential reason because of particle protection. The DO concentrations showed a significant negative correlation to the *β*-AOB *amo*A and *Nitrospira* 16S rRNA gene abundances (*P* <0.05, Table S5). Previous studies have shown that ammonia oxidizers are highly abundant under low oxygen conditions because of relatively high ammonia levels (Lam et al., 2007; Beman et al., 2008; Park et al., 2010; Yan et al., 2012), which might benefit the activity of AOB. Accumulations of nitrite under low oxygen conditions would also help NOB *Nitrospira* to oxidize nitrite (Füssel et al., 2012; Beman et al., 2013). pH was also negatively correlated to the *β*-AOB *amo*A and *Nitrospira* 16S rRNA gene abundances (*P* <0.05–0.01, Table S5). This is not consistent with previous studies that showed AOA and AOB *amo*A gene abundances increasing with pH in soils (Gubry-Rangin et al., 2011), sediments (Rani et al., 2017), and the open ocean (Nunoura et al., 2015). It is possible this is related to lower availability of the substrate (ammonia) due to increased ionization to ammonium as pH decreases. However, in an estuary with sufficient nutrients, such as the PRE, negative correlations between gene abundances and pH could in fact be attributed to co-varying of pH with DO concentrations.

In estuarine ecosystems, water mass mixing highly influences the distribution of microbial populations. Both silicate and salinity have been previously recognized as one of the most common indicators to discriminate river water sources in the ocean (Moore, 1986). In this study, silicate concentrations and salinity were found to be positively and negatively correlated, respectively, to the β-AOB *amo*A and *Nitrospira* 16S rRNA gene abundances (*P* <0.05–0.01, Table S5), suggesting that β-AOB and *Nitrospira* recovered in the PRE could partly originate from the Pearl River or upstream.

Partial Mantel tests were further applied to the qPCR dataset and environmental parameters to eliminate the co-varying effect of water mass and substrate availability, and to identify the major process that influences the nitrifier distribution from the estuary to open ocean (Fig. 11). Variations in the distribution of nitrifier populations along the transect were significantly correlated with water mass mixing and substrate availability (standard and partial Mantel tests, *P* <0.05–0.01), except that ammonia-oxidizing populations only correlated to water mass properties (Fig. 11a–i). Notably, however, water mass parameters and those influencing substrate availability significantly controlled variations in the distribution of FL and PA nitrifier populations along the transect (standard and partial Mantel tests, $P < 0.05–0.01$, Fig. 11j–o). This suggests that nitrifiers' life strategies to some extent allow them to be adaptable to substrate availability.

5 Summary

Our work explored the niche differentiation of main nitrifier groups (AOA, β-AOB, NOB *Nitrospira* and *Nitrospina*) from an estuary (PRE) to the open ocean (SCS), and investigated possible environmental parameters allowing this niche differentiation. These environmental factors included water mass parameters (temperature, salinity, and silicate), substrate parameters (ammonia/ammonium, nitrite, and nitrate), and parameters influencing substrate availability (DO, TSM, and pH). We showed that, from the PRE to the SCS, niche differentiation of nitrifier populations is primarily regulated by water mass mixing and the availability of electron donors (substrate availability). Additionally, the nitrifier populations might have specific adaptations to different substrate conditions provided through their ecological/life strategies (e.g. particle-attached). Therefore, the abundance and activity of nitrifiers could reflect a possible substrate, e.g. ammonia/ammonium or nitrite, flux/availability in ecosystems, providing a biogeochemical clue for understanding carbon and nitrogen cycles.

Data availability

The sequences used for this study were deposited in GenBank under accession numbers KY387947–KY388465 and MG025956–MG026485. The qPCR data were available within this paper (Table S1). Other data can be accessed in the form of Excel spreadsheets via the corresponding author.

The Supplement related to this article is available online.

Author contribution

Y.Z. conceived and designed the experiments. L.H., X.X., and X.W. performed the experiments. L.H., X.X., Y.Z., and X.W. analysed the data. Y.Z., L.H., and X.X. wrote the paper. X.W., S.J.K., and N.J. contributed to the interpretation of results and critical revision.

Competing interests

The authors declare no conflicts of interest.

Acknowledgments

We thank Professor Minhan Dai for providing the sampling opportunity during the PRE cruise and nutrient data. We also thank Zuhui Zuo, Zhuoyu Chen, and Duo Zhao for their assistance in DNA/RNA extraction and qPCR measurements. This work was funded by the National Key Research and Development Programs (2016YFA0601400) and NSFC projects (41676125, 41721005, and 91428308). This study is a contribution to the international IMBER project. We thank Kara Bogus, PhD, from Liwen Bianji, Edanz Editing China (www.liwenbianji.cn/ac), for editing the English text of a draft of this manuscript.

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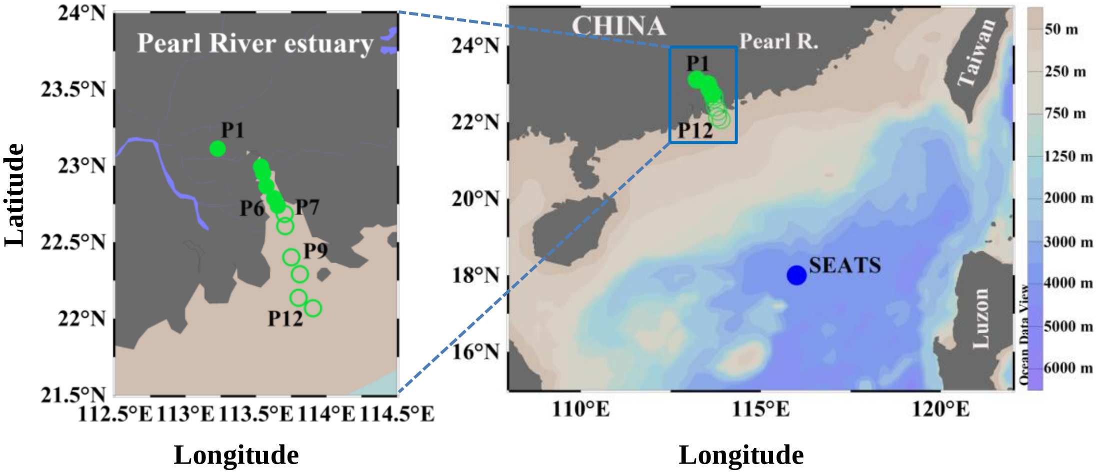

Figure 1. Site locations and bathymetry. The solid green circles indicate hypoxic sites in the PRE, open green circles indicate (low) oxygenated sites in the PRE, and the solid blue circle indicates SEATS in the central basin of the SCS. This figure was produced using Ocean Data View v. 4.6.2 (http://odv.awi.de, 2014). Isobaths are regarded as the background and the color bar indicates depth.

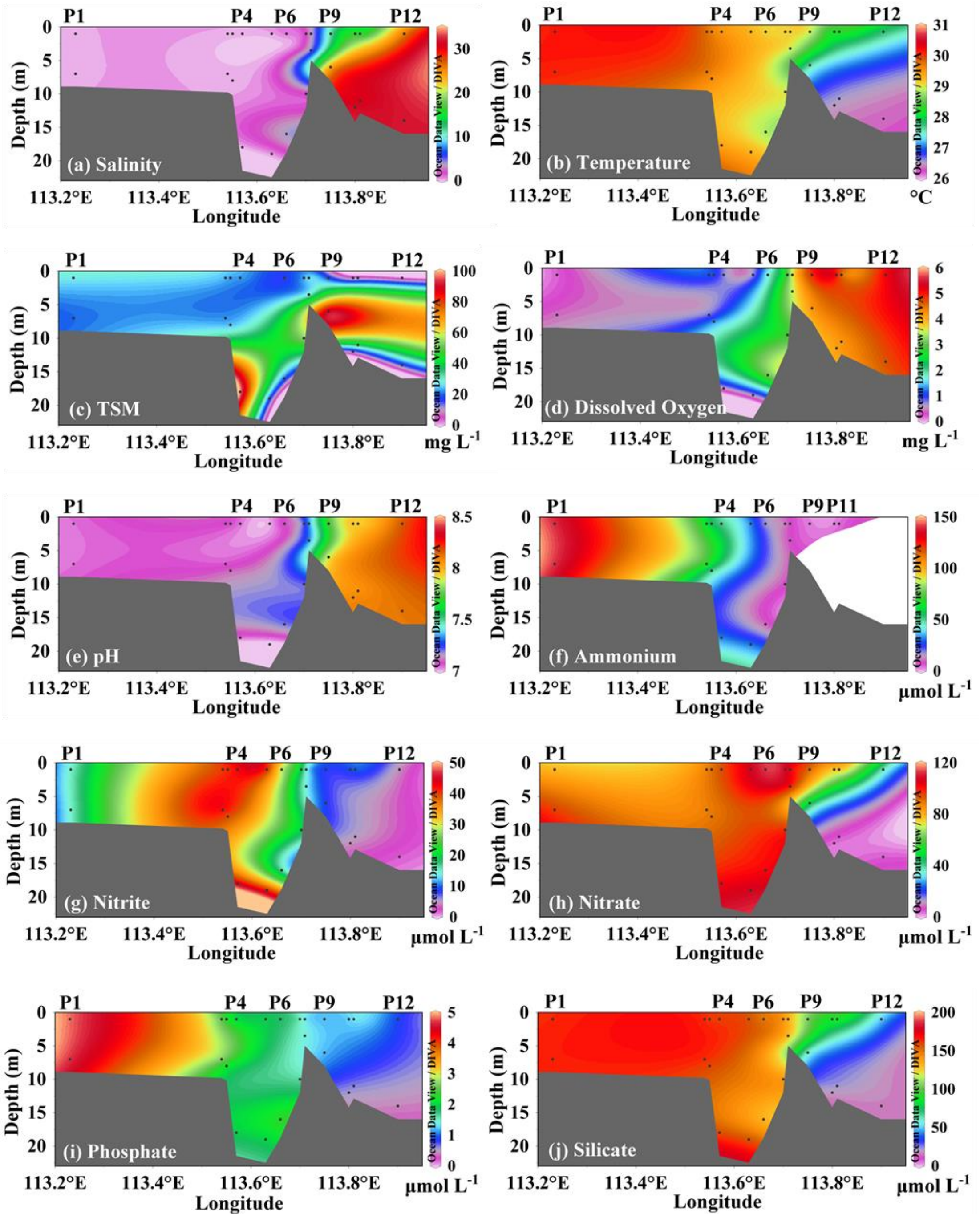

Figure 2. Distributions of biogeochemical factors along the PRE transect. (a) Salinity, (b) temperature, (c) TSM, (d) DO, (e) pH, (f) ammonium, (g) nitrite, (h) nitrate, (i) phosphate, and (j)

silicate concentration. P1–12 indicate PRE sampling sites. Black dots indicate sampling depths.

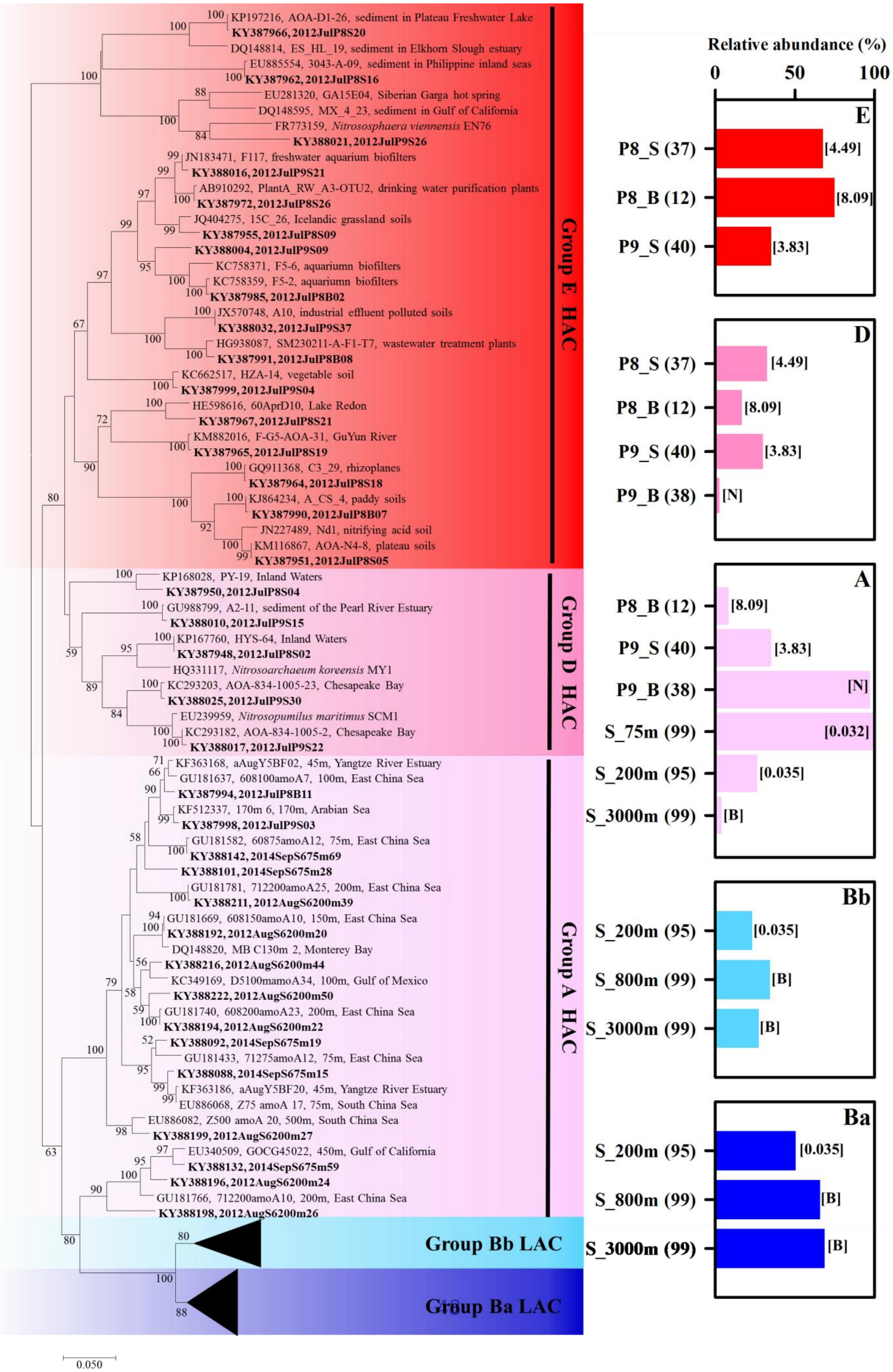
KP197216, AOA-D1-26, sediment in Plateau Freshwater Lake
KY387966, 2012JulP8S20
DQ148814, ES_HL_19, sediment in Elkhorn Slough estuary
EU885554, 3043-A-09, sediment in Philippine inland seas
KY387962, 2012JulP8S16
EU281320, GA15E04, Siberian Garga hot spring
DQ148595, MX_4_23, sediment in Gulf of California
FR773159, Nitrososphaera viennensis EN76
KY388021, 2012JulP9S26
JN183471, F117, freshwater aquarium biofilters
KY388016, 2012JulP9S21
AB910292, PlantA_RW_A3-OTU2, drinking water purification plants
KY387972, 2012JulP8S26
JQ404275, 15C_26, Icelandic grassland soils
KY387955, 2012JulP8S09
KY388004, 2012JulP9S09
KC758371, F5-6, aquariumn biofilters
KC758359, F5-2, aquariumn biofilters
KY387985, 2012JulP8B02
JX570748, A10, industrial effluent polluted soils
KY388032, 2012JulP9S37
HG938087, SM230211-A-F1-T7, wastewater treatment plants
KY387991, 2012JulP8B08
KC662517, HZA-14, vegetable soil
KY387999, 2012JulP9S04
HE598616, 60AprD10, Lake Redon
KY387967, 2012JulP8S21
KM882016, F-G5-AOA-31, GuYun River
KY387965, 2012JulP8S19
GQ911368, C3_29, rhizoplanes
KY387964, 2012JulP8S18
KJ864234, A_CS_4, paddy soils
KY387990, 2012JulP8B07
JN227489, Nd1, nitrifying acid soil
KM116867, AOA-N4-8, plateau soils
KY387951, 2012JulP8S05
KP168028, PY-19, Inland Waters
KY387950, 2012JulP8S04
GU988799, A2-11, sediment of the Pearl River Estuary
KY388010, 2012JulP9S15
KP167760, HYS-64, Inland Waters
KY387948, 2012JulP8S02
HQ331117, Nitrosoarchaeum koreensis MY1
KC293203, AOA-834-1005-23, Chesapeake Bay
KY388025, 2012JulP9S30
EU239959, Nitrosopumilus maritimus SCM1
KC293182, AOA-834-1005-2, Chesapeake Bay
KY388017, 2012JulP9S22
KF363168, aAugY5BF02, 45m, Yangtze River Estuary
GU181637, 608100amoA7, 100m, East China Sea
KY387994, 2012JulP8B11
KF512337, 170m 6, 170m, Arabian Sea
KY387998, 2012JulP9S03
GU181582, 60875amoA12, 75m, East China Sea
KY388142, 2014SepS675m69
KY388101, 2014SepS675m28
GU181781, 712200amoA25, 200m, East China Sea
KY388211, 2012AugS6200m39
GU181669, 608150amoA10, 150m, East China Sea
KY388192, 2012AugS6200m20
DQ148820, MB C130m 2, Monterey Bay
KY388216, 2012AugS6200m44
KC349169, D5100mamoA34, 100m, Gulf of Mexico
KY388222, 2012AugS6200m50
GU181740, 608200amoA23, 200m, East China Sea
KY388194, 2012AugS6200m22
KY388092, 2014SepS675m19
GU181433, 71275amoA12, 75m, East China Sea
KY388088, 2014SepS675m15
KF363186, aAugY5BF20, 45m, Yangtze River Estuary
EU886068, Z75 amoA 17, 75m, South China Sea
EU886082, Z500 amoA 20, 500m, South China Sea
KY388199, 2012AugS6200m27
EU340509, GOCG45022, 450m, Gulf of California
KY388132, 2014SepS675m59
KY388196, 2012AugS6200m24
GU181766, 712200amoA10, 200m, East China Sea
KY388198, 2012AugS6200m26
Group E HAC
Group D HAC
Group A HAC
Group Bb LAC
Group Ba LAC
0.050
Relative abundance (%)
0 50 100
E
P8_S (37) [4.49]
P8_B (12) [8.09]
P9_S (40) [3.83]
D
P8_S (37) [4.49]
P8_B (12) [8.09]
P9_S (40) [3.83]
P9_B (38) [N]
A
P8_B (12) [8.09]
P9_S (40) [3.83]
P9_B (38) [N]
S_75m (99) [0.032]
S_200m (95) [0.035]
S_3000m (99) [B]
Bb
S_200m (95) [0.035]
S_800m (99) [B]
S_3000m (99) [B]
Ba
S_200m (95) [0.035]
S_800m (99) [B]
S_3000m (99) [B]

Figure 3. Unrooted neighbor-joining (NJ) phylogenetic tree of the archaeal *amo*A gene sequences. Clone sequences from this study are shown in bold and sequences sharing 95% DNA identity are grouped. GenBank accession numbers are shown. Groups A, Ba, Bb, and D were defined in Nunoura et al. (2015) and group E is defined in this study. The relative abundance of clones retrieved for each library in the five subgroups is indicated by a bar. Total number of clones for each library is shown in parentheses. Location of sites P8 and P9 (S and B indicate surface and bottom waters, respectively) and SEATS (S) are shown in Fig. 1. Ammonium concentrations are shown in square brackets. Phylogenetic relationships were bootstrapped 1000 times, and bootstrap values greater than 50% are shown. The scale bar indicates 5% estimated sequence divergence. HAC, high ammonia cluster; LAC, low ammonia cluster. N, not measured; B, below detection limit.

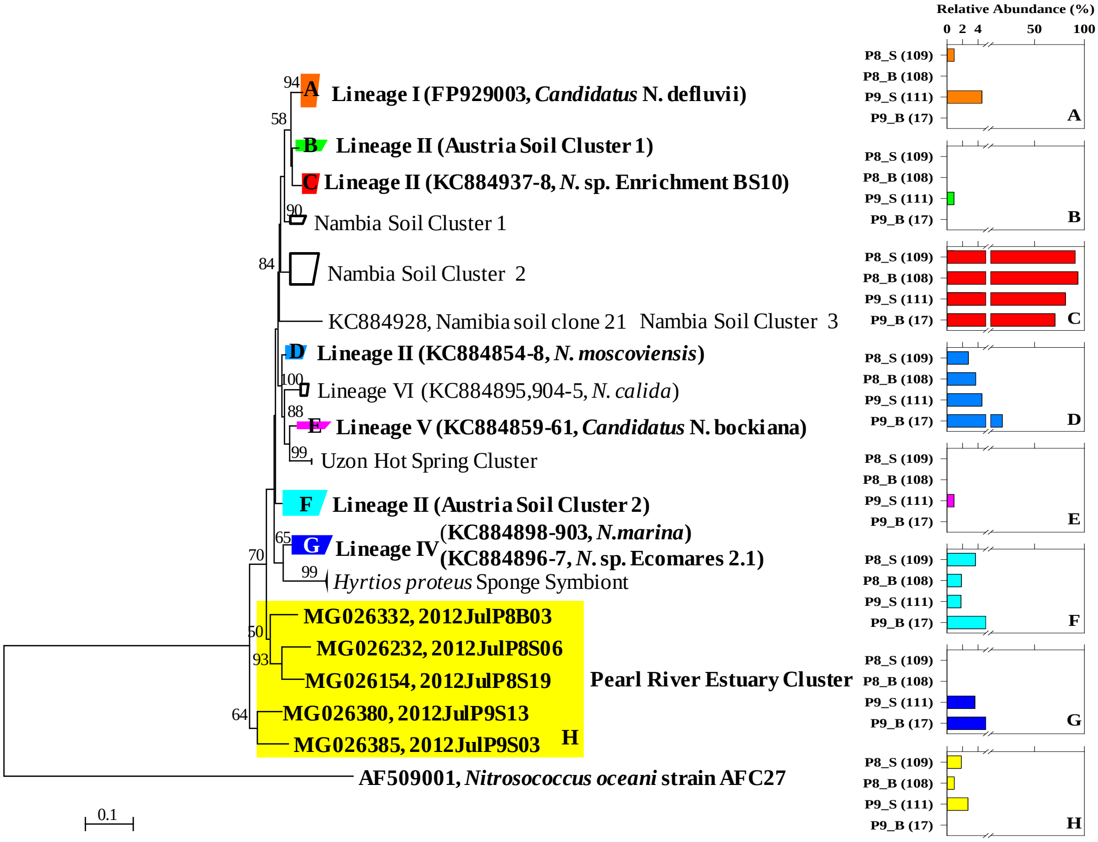

Figure 4. Rooted neighbor-joining (NJ) phylogenetic tree of the *Nitrospira nxr*B gene sequences. Clone sequences from this study are shown in bold and sequences sharing 95% DNA identity are grouped. GenBank accession numbers are shown. Groups a, b, c, d, e, f, and g are defined according to Pester et al. (2013), and Group h (highlighted in yellow) is defined in this study. The relative abundance of clones retrieved for each library in the eight subgroups is indicated by a bar. Total number of clones for each library is shown in parentheses. Location of sites P8 and P9 (S and B indicate surface and bottom waters, respectively) are shown in Fig. 1. Phylogenetic relationships were bootstrapped 1000 times, and bootstrap values greater than 50% are shown. The scale bar indicates 10% estimated

sequence divergence.

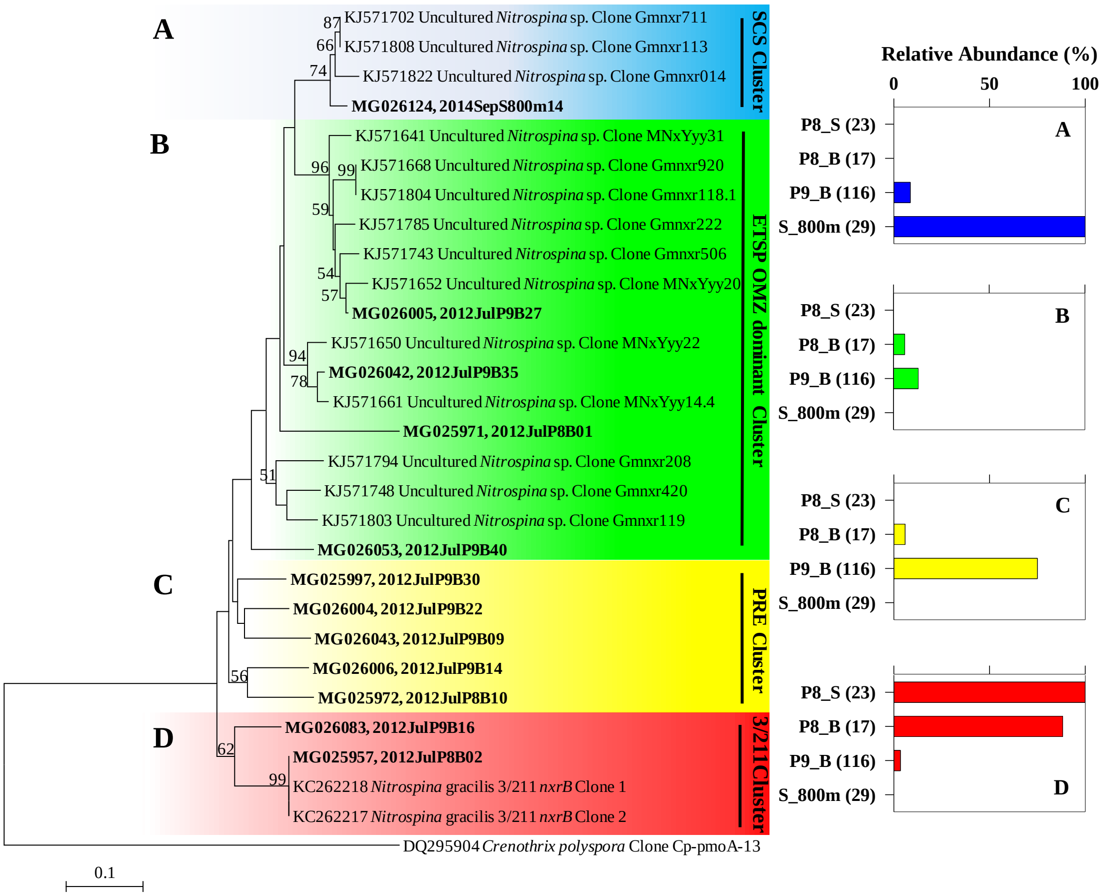

Figure 5. Rooted neighbor-joining (NJ) phylogenetic tree of the *Nitrospina nxr*B gene sequences. Clone sequences from this study are shown in bold and sequences sharing 95% DNA identity are grouped. GenBank accession numbers are shown. Groups A, B, C, and D are defined in this study. The relative abundance of clones retrieved for each library in the four subgroups is indicated by a bar. Total number of clones for each library is shown in parentheses. Location of sites P8 and P9 (S and B indicate surface and bottom waters, respectively) and SEATS (S) are shown in Fig. 1. Phylogenetic relationships were bootstrapped 1000 times, and bootstrap values greater than 50% are shown. The scale bar indicates

10% estimated sequence divergence.

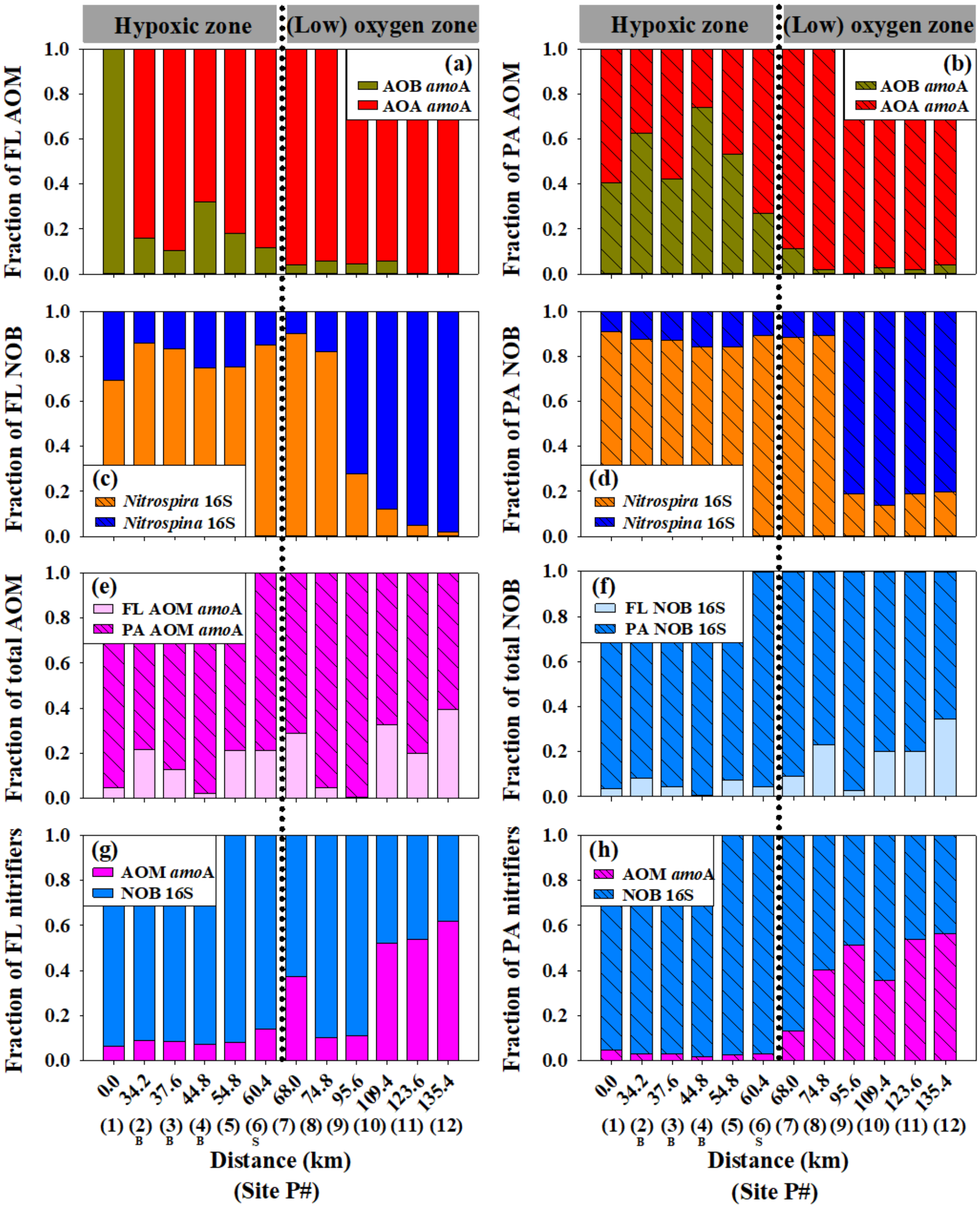

Figure 6. Gene abundance distribution of four nitrifier groups along the PRE transect. (a) Relative abundance of archaeal (AOA) and β-proteobacterial (AOB) *amo*A genes in total FL AOM

(sum of archaea and β-proteobacteria) *amo*A genes. (b) Relative abundance of AOA and AOB *amo*A genes in total PA AOM *amo*A genes. (c) Relative abundance of *Nitrospira* and *Nitrospina* 16S rRNA genes in total FL NOB (sum of *Nitrospira* and *Nitrospina*) 16S rRNA genes. (d) Relative abundance of *Nitrospira* and *Nitrospina* 16S rRNA genes in total PA NOB 16S rRNA genes. (e) Relative abundance of FL and PA AOM *amo*A genes in total *amo*A genes. (f) Relative abundance of FL and PA NOB 16S rRNA genes in total 16S rRNA genes. (g) Relative abundance of AOM *amo*A and NOB 16S rRNA genes in total FL nitrifier genes. (h) Relative abundance of AOM *amo*A and NOB 16S rRNA genes in total PA nitrifier genes. Depth-weighted abundances were used to calculate relative abundances for each site. B, only the bottom water was sampled; S, only the surface water was sampled.

Biogeosciences Discuss., https://doi.org/10.5194/bg-2018-189
Manuscript under review for journal Biogeosciences
Discussion started: 9 May 2018

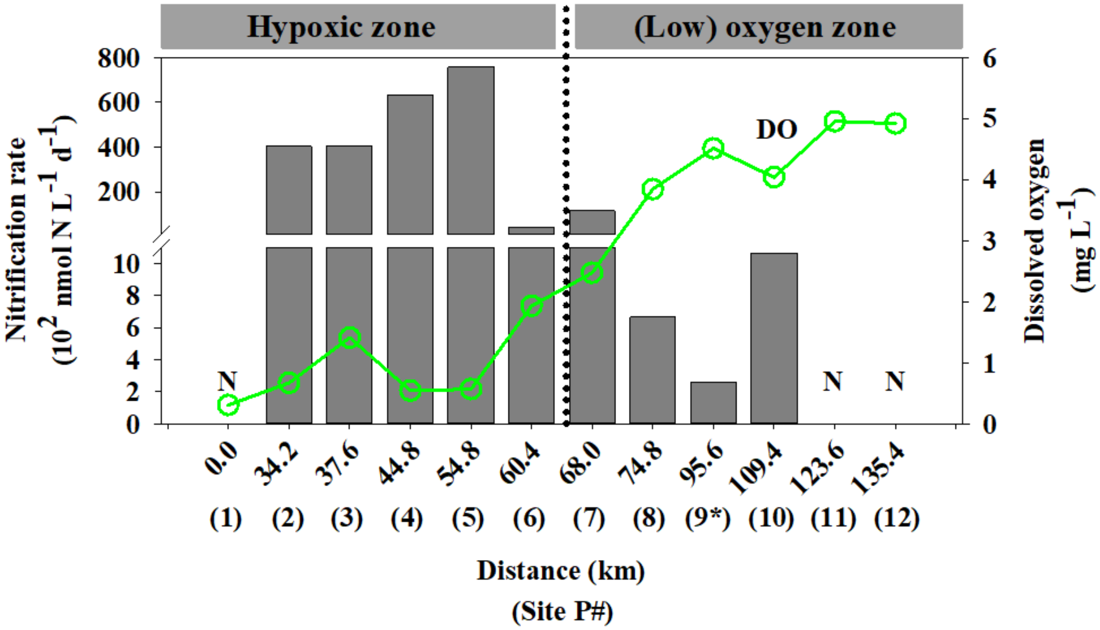

Figure 7. Nitrification rates and DO concentrations along the PRE transect. Nitrification rates were only measured in the bottom waters except for site P9, where rates were measured in both surface and bottom waters. N, not measured; *the depth-weighted value was used.

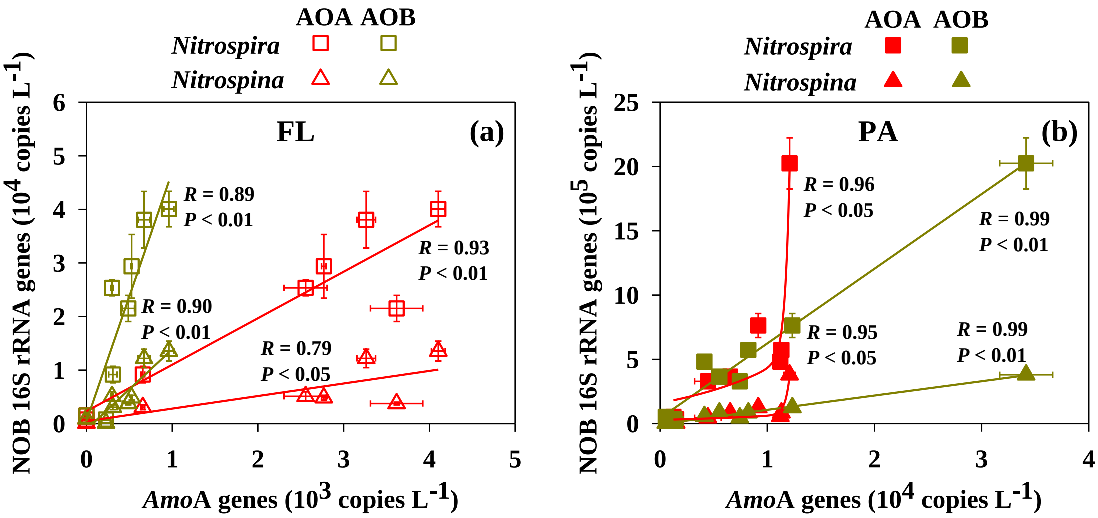

Figure 8. Correlations between ammonia and nitrite oxidizers in the hypoxic zone of the PRE (sites P1–6). There are significant positive correlations (n = 8) between archaeal and β-proteobacterial *amo*A genes and *Nitrospira* and *Nitrospina* 16S rRNA gene abundances in (a) FL and (b) PA communities. Error bars represent standard deviations.

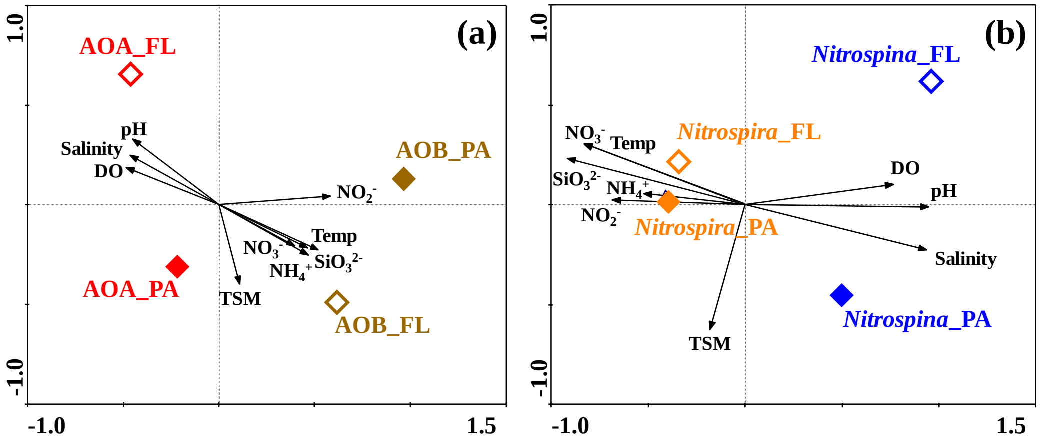

Figure 9. Canonical correspondence analysis. (a) Ammonia and (b) nitrite oxidizers under the constraint of environmental factors. Each diamond represents an individual subgroup. Vectors represent the environmental variables. Temp, temperature.

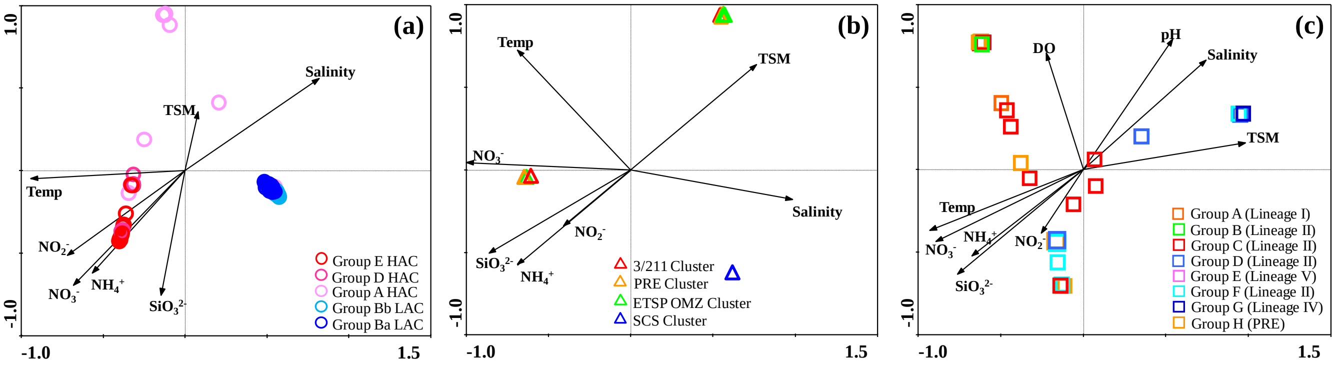

Figure 10. Canonical correspondence analysis (CCA). (a) Ammonia-oxidizing archaea, (b) *Nitrospina*, and (c) *Nitrospira* phylogenetic taxa under the constraint of environmental factors. Each symbol represents an individual OTU. Vectors represent the environmental variables. Temp, temperature. DO and pH were not included in (a) and (b) because they were not measured at SEATS.

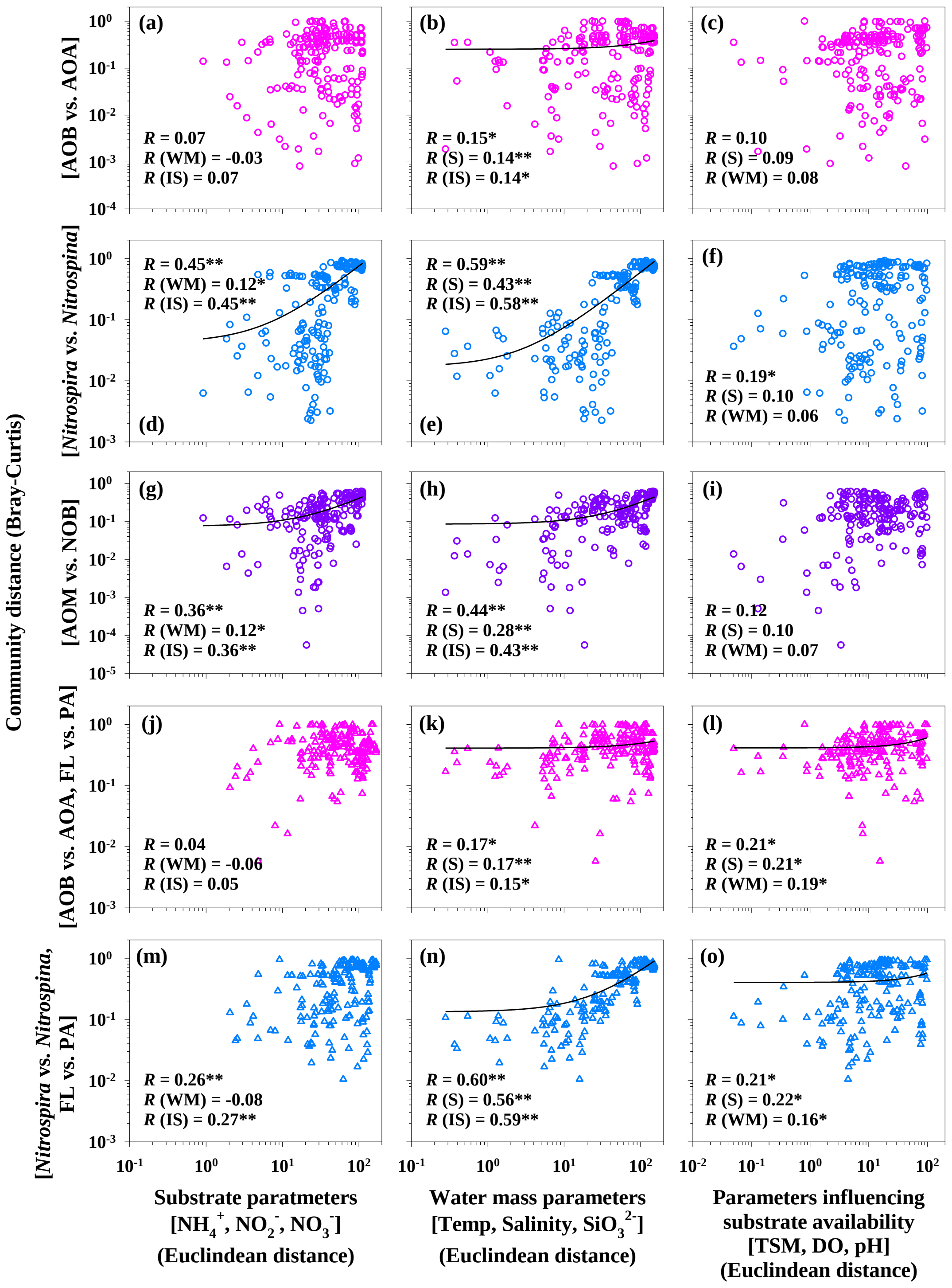
Community distance (Bray-Curtis)
[AOB vs. AOA]
[*Nitrospira* vs. *Nitrospina*]
[AOM vs. NOB]
[AOB vs. AOA, FL vs. PA]
[*Nitrospira* vs. *Nitrospina*, FL vs. PA]
(a)
R = 0.07
R (WM) = -0.03
R (IS) = 0.07
(b)
R = 0.15*
R (S) = 0.14**
R (IS) = 0.14*
(c)
R = 0.10
R (S) = 0.09
R (WM) = 0.08
(d)
R = 0.45**
R (WM) = 0.12*
R (IS) = 0.45**
(e)
R = 0.59**
R (S) = 0.43**
R (IS) = 0.58**
(f)
R = 0.19*
R (S) = 0.10
R (WM) = 0.06
(g)
R = 0.36**
R (WM) = 0.12*
R (IS) = 0.36**
(h)
R = 0.44**
R (S) = 0.28**
R (IS) = 0.43**
(i)
R = 0.12
R (S) = 0.10
R (WM) = 0.07
(j)
R = 0.04
R (WM) = -0.06
R (IS) = 0.05
(k)
R = 0.17*
R (S) = 0.17**
R (IS) = 0.15*
(l)
R = 0.21*
R (S) = 0.21*
R (WM) = 0.19*
(m)
R = 0.26**
R (WM) = -0.08
R (IS) = 0.27**
(n)
R = 0.60**
R (S) = 0.56**
R (IS) = 0.59**
(o)
R = 0.21*
R (S) = 0.22*
R (WM) = 0.16*
10^0
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
10^1
10^2
Substrate paratmeters
[NH_4^+, NO_2^-, NO_3^-]
(Euclindean distance)
Water mass parameters
[Temp, Salinity, SiO_3^{2-}]
(Euclindean distance)
Parameters influencing
substrate availability
[TSM, DO, pH]
(Euclindean distance)

Figure 11. Correlations between nitrifier community composition and water mass parameters (temperature, salinity, and silicate), substrate parameters (ammonia/ammonium, nitrite, and nitrate), or parameters influencing substrate availability (TSM, DO, and pH). Standard and partial Mantel tests were run to measure the correlation between two matrices. Dissimilarity matrices of nitrifier communities were based on Bray-Curtis distances; environmental factors were based on Euclidean distances between samples. Spearman or Kendall's correlation coefficient (*R*) values are shown for standard (first value) and partial Mantel (second and third) tests. The *P*-values were calculated using the distribution of the Mantel test statistics estimated from 999 permutations. $^{*}P < 0.05$; $^{**}P < 0.01$. Matrix of the nitrifier community was calculated according to (a–c) ammonia-oxidizing archaeal and bacterial abundances (AOB vs. AOA), (d–f) *Nitrospira* and *Nitrospina* abundances (*Nitrospira* vs. *Nitrospina*), (g–i) ammonia and nitrite-oxidizing microbial abundance (AOM vs. NOB), (j–l) FL and PA ammonia-oxidizing archaeal and bacterial abundances (AOB vs. AOA, FL vs. PA), and (m–o) FL and PA *Nitrospira* and *Nitrospina* abundances (*Nitrospira* vs. *Nitrospina*, FL vs. PA). (a, d, g, j, and m) Matrix of substrate parameters included NH_4^+, NO_2^-, and NO_3^- concentrations, (b, e, h, k, and n) matrix of water mass parameters included temperature (Temp), salinity, and SiO_3^{2-}, and (c, f, i, l, and o) matrix of parameters influencing substrate availability included TSM, DO, and pH.