Manuscript Number: bg-2018-189

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

Response to Editor

Comments to the Author:

Dear Dr. Hou

Your manuscript has now been seen by two referees. Although both reports are positive, one of the Referee # 1 has made some significant technical issues that need to be addressed by you. Although s/he has recommended minor revision, I would still send the revised manuscript to her/him for approval. Please take care of the comments. I look forward to receiving the revision at an early date.

Best regards

Wajih Naqvi

Dear Editor,

Thank you for taking the time to handle our manuscript and your assessment. We have carefully addressed each comment from two referees and tried our best to improve the manuscript according to their suggestions.

For some technical issues mentioned by Reviewer #1, please refer to our responses below for details. According to Reviewer #1's comment, we carried out qPCR using the pairs of primers Arch-amoA-for/Arch-amoA-rev (WuchterAOA) from Wuchter et al. (2006) for all samples of the PRE, and Arch-amoAFA/Arch-amoAR (BemanAOA) from Beman et al. (2008) for six samples at the upper reaches, middle reaches, lower reaches of the PRE. We replaced the data (using FranAOA primer set) at the lower reaches sites P9–12 with the new abundance data (using WuchterAOA primer set) of archaeal *amo*A gene in the revised manuscript, and also revised the related statements.

Our responses to all comments are listed below. We welcome any further comments. Thank you again for your time and kind efforts.

Best wishes

Yao Zhang

We greatly thank the reviewers for the valuable comments, useful suggestions and careful revisions, based on which we have revised the manuscript. And the point-by-point responses to the comments are in blue colour as follows.

Response to Reviewer #1

T. NUNOURA

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Received and published: 5 June 2018

The authors characterized the diversity, abundance and activity of nitrifiers associated with waters from the Pearl River estuary to the South China Sea. The data set provides novel insights into the niche separation and interactions of ammonia and nitrite oxidizers. However, I found technical issues in the quantification of archaeal ammonia oxidizers in the current version of manuscript as shown below.

In this study, archaeal *amo*A genes were used for a molecular marker for archaeal ammonia oxidizers, and there are technical issues in both diversity analysis and quantitative PCR. 1. The composition of archaeal *amo*A diversity is highly biased in PCR amplification comparing to the SSU rRNA gene analysis as reported previously (Meinhardt et al., 2015; Nunoura et al., 2015). In addition, it would lead inappropriate selection of sequences used to obtain standard curves in qPCR.

Response:

Meinhardt et al. (2015) showed that the archaeal *amo*A diversity retrieved from soil using their new-designed GenAOA primer set was more similar to that from the metagenomic data, compared with using FranAOA primer set, which was used in our manuscript. However, all the mainly different clades (*Nitrosotalea* 1.1, *Nitrososphaera* 1 (non-54d9), *Nitrososphaera* 1.1 and *Nitrososphaera* 54d9) between FranAOA clone library and metagenomic data were not adapted to the marine habitats.

Nunoura et al. (2015) showed that the distribution and abundance patterns of four subgroups of archaeal *amo*A genes (Group A, Ba, Bb, and D) in the Challenger Deep measured by qPCR were more similar to the MGI SSU rRNA gene clone library community structure, rather than the archaeal *amo*A gene clone library (using the FranAOA primer set). However, more specifically, above the Abyssal zone (4000–6000 m), the qPCR abundance patterns of the archaeal *amo*A subgroups were similar

to the archaeal *amo*A gene clone library analysis; the inconsistent pattern was present in the hadal zone (below 6000 m). Our clone library analysis of archaeal *amo*A gene was carried out at two estuary sites and in the South China Sea basin at 75 m, 200 m, 800 m, and 3000 m water depth. Our qPCR measurements of archaeal *amo*A gene were only performed in the estuary with the depths of ≤19 m. Thus, it is plausible to select the most dominant Group A (high ammonia cluster, HAC) OTU sequence (KY387998) to construct standard curves for qPCR measurements in the estuary.

2. Thus, the authors should mention about the possibility that the interpretation of the niche separation among AOA subgroups may be influenced by PCR bias in this manuscript.

Response:

Agree. We added the statement in the paragraph of AOA clone library analysis in 3.3 section. — "*Although the niche separation among AOA subgroups may be influenced by some bias during PCR amplification, overall distribution of HAC and LAC subgroups are plausible*." (Page 18, Line 7–8)

(HAC, high ammonia cluster; LAC, low ammonia cluster)

3. The authors obtained archaeal *amo*A gene sequences in the clone analysis and then used the selected sequences to obtain standard curves in the following qPCR. However, qPCR primer set for archaeal *amo*A genes was identical to a primer set used for the conventional clone analysis. This generally allows complete match between archaeal *amo*A clone sequences and primer sequences in PCR reaction for obtaining standard curves. In contrast, the presence of few mismatch residues is expected between environmental *amo*A gene sequences and primer sequences in qPCR. The gap would be a reason for underestimation of archaeal *amo*A genes in environmental samples. In qPCR of archaeal *amo*A, a primer set, Wuchter et al. 2006, or other primer set that does not overlap the annealing regions in the initial clone analysis is recommended.

Response:

Primers are designed always based on existing sequences. So, there is always few mismatches between environmental sequences and primer sequences even if we use

different primer sets for qPCR and PCR, which would lead to underestimation of abundance. We admit it, but it is a normal issue in the field.

As for standards, selecting a most dominant OTU sequence in the studied region for standard curves construction should be the most reasonable selection.

Our qPCR measurements of archaeal *amo*A gene were only performed in the estuary (Peal River Estuary, PRE) in this manuscript. According to the reviewer's suggestion, we carried out qPCR using the pairs of primers Arch-amoA-for/Arch-amoA-rev (WuchterAOA, targeting 'high-ammonia concentration' archaeal *amo*A, HAC) from Wuchter et al. (2006) for all samples of the PRE, and Arch-amoAFA/Arch-amoAR (BemanAOA, targeting 'shallow' clades, group A) from Beman et al. (2008) for six samples at sites P6 (upper reaches), P8 (middle reaches), P11 and P12 (lower reaches) of the PRE.

(1) The results show that the abundances of archaeal *amo*A gene measured by BemanAOA primer set were similar to those measured by FranAOA primer set used in our manuscript (Fig. R1).

(2) However, WuchterAOA primer set cannot detect archaeal *amo*A gene for the samples from hypoxic zone (the upper reaches of the PRE), obtained similar abundance in the middle reaches of the PRE, and retrieved higher abundance of archaeal *amo*A gene from the samples in the lower reaches of the PRE (Table R1). Standard curves were shown in Fig. R2. Thus, we replaced the data (using FranAOA primer set) at the lower reaches sites P9–12 with the new abundance data (using WuchterAOA primer set) of archaeal *amo*A gene in the revised manuscript. We also revised the related statements. Overall, the conclusions based on the new data set are consistent with the previous ones.

Detailed revisions are listed below:

2.6 Quantitative PCR amplification:

"*Standard curves were constructed for archaeal and β-proteobacterial amoA genes using plasmid DNA (accession numbers KY387998 (targeted by the primers Arch-amoAF and Arch-amoAR) and MH638327 (targeted by the primers Arch-amoA-for and Arch-amoA-rev) for AOA and MH458281 for AOB) from clone libraries.*" (Page 11, Line 1–3)

3.4 Abundance distribution of ammonia and nitrite oxidizers and nitrification rates:

"*Archaeal and β-proteobacterial amoA gene abundances varied from below detection limit to* 6.82×10^5 *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.*" (Page 20, Line 5)

"*All of the genes were significantly more abundant in the PA than the FL communities (Wilcoxon, P <0.05–0.01) (Fig. 6e and f).*" (Page 20, Line 16, and Page 58)

"*The abundance of the NOB 16S rRNA genes rapidly decreased and the AOM amoA genes increased (Fig. 6g and h), and archaea and Nitrospina became the dominant ammonia and nitrite oxidizers, respectively (Fig. 6a–f).*" (Page 21, Line 10–12, and Page 58)

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

Deleted "*In addition, the β-AOB amoA 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 amoA gene (Table S5). We speculate that AOB could be better adapted to the estuarine habitat than AOA.*" (Page 25, Line 7)

4.4 Environmental parameters allowing niche differentiation:

Added "*Both nitrite and nitrate concentrations were negatively correlated to archaeal amoA gene abundance in the estuary (P <0.05–0.01, Table S5), which is consistent with the observations from the present study and previous studies that AOA are more dominant in oligotrophic environments (Wuchter et al., 2006; Newell et al., 2013).*" (Page 28, Line 5–8, and Supplement Page 9)

"*Notably, all genes were significantly positively correlated to TSM concentrations in PA communities (P <0.05–0.01, Table S5).*" (Page 28, Line 9–10, and Supplement Page 9)

Deleted "*This is consistent with the observation that all of the genes were significantly more abundant in the PA communities.*" (Page 28, Line 10)

Added "*pH was also negatively correlated to the β-AOB amoA and Nitrospira 16S rRNA gene abundances, but positively correlated to the archaeal amoA gene (P <0.05–0.01, Table S5). A similar observation was found by Li et al. (2011) in mangrove sediments at the northwestern corner of the New Territories of Hong Kong.*

However, AOA and AOB amoA gene abundances were both previously found increasing with pH in soils (Gubry-Rangin et al., 2011) and the open ocean (Nunoura et al., 2015). This is probably related to lower availability of the substrate (ammonia) due to increased ionization to ammonium as pH decreases. 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." (Page 29, Line 1–9, and Supplement Page 9)

"*In this study, silicate concentrations and salinity were found to be positively and negatively correlated, respectively, to the β-AOB amoA and Nitrospira 16S rRNA gene abundances; the opposite correlations were observed in archaeal amoA gene abundance (P <0.05–0.01, Table S5). These results suggest that β-AOB and Nitrospira recovered in the PRE could partly originate from the Pearl River or upstream and AOA could partly originate from the SCS.*" (Page 29, Line 14–17, and Supplement Page 9)

The revised figures and tables include Fig. 6, Fig. 9, Fig.11, Table S1, Table S3, and Table S5. Please see the revised manuscript and supplement. (Page 58, Page 62, Page 64, Supplement Page 1–2, Supplement Page 5 and Supplement Page 9)

Table R1. Archaeal *amo*A gene copies using FranAOA primer set versus WuchterAOA primer set.

Station	Water Depth (m)	Sampling Depth (m)	Archaeal *amo*A (copies L^{-1}) FranAOA				Archaeal *amo*A (copies L^{-1}) Wuchter AOA			
			FL	SD	PA	SD	FL	SD	PA	SD
P1	8.9	1	0		1.50×10^{3}	4.00×10	ND		ND	
		7	0		1.25×10^{3}	5.72×10	ND		ND	
P2	9.8	1	NS				NS			
		7	2.77×10^{3}	2.69×10	4.46×10^{3}	1.24×10^{3}	ND		ND	
P3	10.2	1	NS				NS			
		8	2.56×10^{3}	2.51×10^{2}	1.13×10^{4}	8.45×10	ND		ND	
P4	21.5	1	NS				NS			
		18	6.57×10^{2}	2.18×10	1.21×10^{4}	5.16×10^{2}	ND		ND	
P5	22.5	1	4.10×10^{3}	8.00×10	6.54×10^{3}	3.00×10	ND		ND	
		19	3.26×10^{3}	1.09×10^{2}	9.16×10^{3}	3.27×10^{2}	ND		ND	
P6	18.8	1	3.62×10^{3}	3.05×10^{2}	1.12×10^{4}	2.41×10^{2}	ND		ND	
		16	NS				NS			
P7	12	1	4.07×10^{4}	2.18×10^{3}	1.09×10^{5}	4.43×10^{3}	ND		ND	
		10	1.02×10^{4}	2.23×10^{3}	8.27×10^{3}	6.77×10^{2}	1.25×10^{4}	3.69×10^{2}	1.41×10^{4}	1.67×10^{3}
P8	5	1	2.61×10^{3}	4.72×10	6.44×10^{4}	3.10×10^{3}	ND		6.86×10^{4}	8.14×10^{3}
		3.5	2.90×10^{3}	2.72×10^{2}	4.95×10^{4}	4.52×10^{3}	ND		ND	
P9	8	1	1.02×10^{3}	5.51×10	2.39×10^{4}	1.72×10^{3}	2.04×10^{4}	1.10×10^{3}	3.54×10^{4}	2.54×10^{3}
		6	5.01×10^{2}	2.19×10	4.54×10^{5}	1.67×10^{4}	1.01×10^{4}	4.42×10^{2}	6.82×10^{5}	2.51×10^{4}
P10	12.9	1	1.11×10^{3}	2.75×10	7.30×10^{2}	2.31×10^{2}	7.20×10^{4}	2.10×10^{4}	2.55×10^{4}	4.63×10^{3}
		11	3.68×10^{3}	1.60×10^{2}	9.48×10^{3}	1.63×10^{3}	1.13×10^{5}	4.92×10^{3}	1.86×10^{5}	3.20×10^{4}
P11	14.2	1	0		0		1.44×10^{4}	2.52×10^{3}	7.75×10^{2}	8.12×10
		12	4.71×10^{3}	4.35×10^{2}	1.83×10^{4}	1.14×10^{3}	1.30×10^{5}	6.30×10^{3}	1.37×10^{5}	1.48×10^{4}
P12	16	1	7.14×10^{2}	5.22×10	0		1.21×10^{4}	1.92×10^{3}	4.72×10^{3}	9.84×10^{2}
		14	2.18×10^{4}	1.73×10^{3}	3.30×10^{4}	3.98×10^{3}	3.02×10^{5}	7.81×10^{4}	2.41×10^{5}	4.90×10^{3}

FL, free-living; PA, particle-associated; NS, no sample; ND, not detected (We tried various optimization strategies for qPCR.)

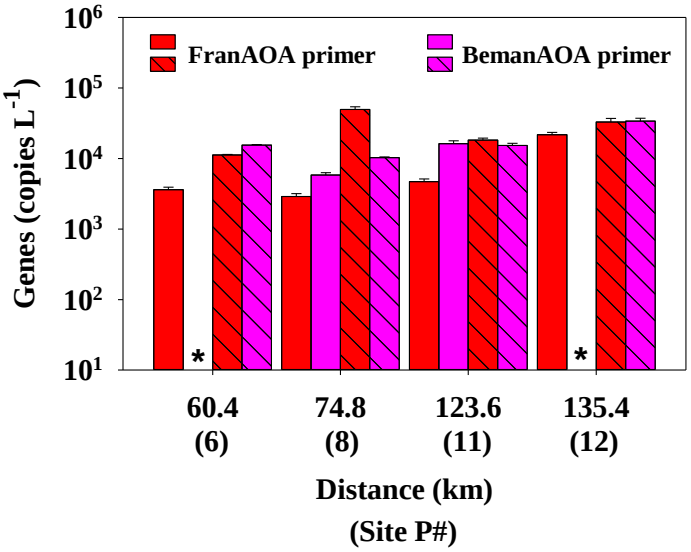


Figure R1. The archaeal *amo*A gene copies from six samples at sites P6 (upper reaches), P8 (middle reaches), P11 and P12 (lower reaches) of PRE using the two primer sets. *, No DNAs.

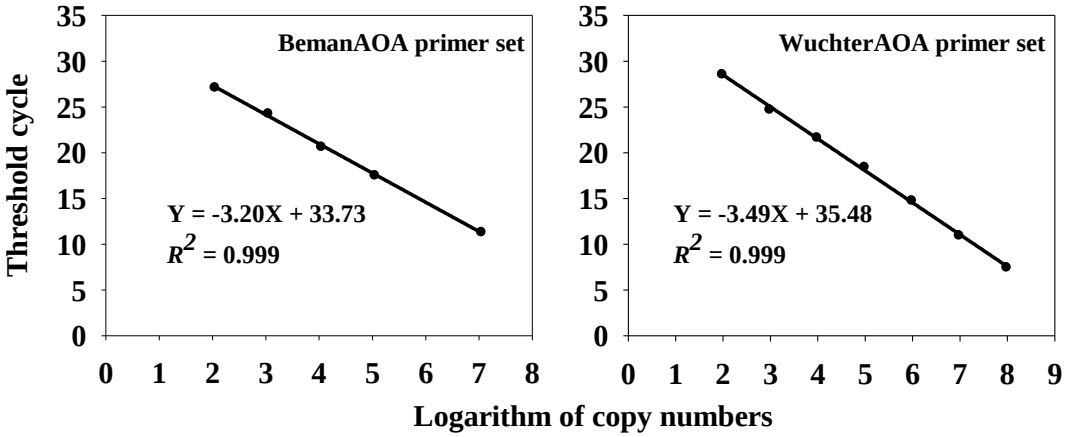


Figure R2. Standard curves for the BemanAOA and WuchterAOA primer sets qPCR measurements.

P2, L3: We analyzed diversity and abundance of ammonia-oxidizing archaea (AOA) and betaproteobacteria (AOB), nitrite-oxidizing bacteria (NOB), and nitrification rates to

Response:

Revised as suggested. (Page 2, Line 3–4)

P2, L6-7: AOA were generally more abundant than betaproteobacterial AOB, however,

Response:

Revised as suggested. (Page 2, Line 6)

P2, L12: What does "a coupling of ammonia and nitrite oxidizers" mean?

Response:

Sorry for the unclear sentence. We revised as "*There is a significant positive correlation between ammonia and nitrite oxidizer abundances in the hypoxic waters of the estuary, suggesting a possible coupling through metabolic interactions between them.*" (Page 2, Line 11–13)

P4, L2-14: Please insert a sentence to present the close relationship between *Nitrospina* and "*Ca.* Nitromaritima". Sequences belong to "*Ca.* Nitromaritima" had been reported as a group in *Nitrospina* until the definition of "*Ca.* Nitromaritima". Thus, the discussion in Lucker et al. 2013 includes both *Nitrospina* and "*Ca.* Nitromaritima".

Response:

Thanks for the reviewer's suggestion. We supplied this information in the revised version. — "*Candidatus* Nitromaritima were recently identified based on metagenomic data in Red Sea brines (Ngugi et al., 2016), which were previously reported as a group in *Nitrospina*." (Page 4, Line 10–11)

P4, L6: Information from Pachiadaki et al. 2017 should be referred through the manuscript.

Response:

We added the citation of "*Pachiadaki et al. 2017*" in the revised manuscript. (Page 4, Line 6)

P6, L5: Strains and/or genomic DNA from the public repositories used in this study should be summarized as the first paragraph in Materials and methods.

Response:

Thanks for the reviewer's suggestion. We added this paragraph in Materials and methods. Please see the below.

2.1 Strains and genomic DNAs

We obtained strains Candidatus Nitrospira defluvii A17 and Nitrospina gracilis 3/211 and their genomic DNAs from the University of Hamburg, Germany. The full-length 16S rRNA gene fragments were used as the standards for construction of standard curves during qPCR amplification. (Page 6, Line 18 and Page 7, Line 1–3)

P8, L18-: Did the authors determine OTUs for each library or among the libraries obtained in this study ?

Response:

We revised this sentence as "*all sequences among the libraries for each gene were grouped into operational taxonomic units (OTUs) based on a 5% sequence divergence cutoff* ." (Page 10, Line 2–4)

P8, L18-: Did the authors conduct any chimera check programs? It has been reported that more than 10% of the archaeal *amo*A gene sequences in the public database are chimera sequences (Eloy Alves et al. 2018).

Response:

Thanks for the reviewer's suggestion. We did chimera check through Bellerophon and manual BLASTp analysis. We added this statement in Materials and methods. Please see the below.

"*All sequences were analyzed with Bellerophon program (http://comp-bio.anu.edu.au/bellerophon/bellerophon.pl) to detect chimeric sequences in multiple sequences alignments (Huber et al., 2004). The putative chimeras were further checked manually through BLASTp analysis to verify whether these were chimeras.*" (Page 9, Line 17–18 and Page 10, Line 1–2)

P9, L13: Names of the sequence used to obtain standard curves for the qPCR should be presented.

Response:

We added the accession numbers for the sequences used to obtain standard curves. — "*Standard curves were constructed for archaeal and β-proteobacterial amoA genes using plasmid DNA (accession numbers KY387998 (targeted by the primers Arch-amoAF and Arch-amoAR) and MH638327 (targeted by the primers Arch-amoA-for*

and Arch-amoA-rev) for AOA and MH458281 for AOB) from clone libraries." (Page 11, Line 1–3)

P9, L14: How did the authors obtain DNA fragments?

Response:

Genomic DNAs of Can*didatus* Nitrospira defluvii A17 and *Nitrospina gracilis* 3/211 were obtained from Professor Eva Spieck from the University of Hamburg, Germany. We added a paragraph on strains and genomic DNAs in Materials and methods. Please see the below.

2.1 Strains and genomic DNAs

We obtained strains Candidatus Nitrospira defluvii A17 and Nitrospina gracilis 3/211 and their genomic DNAs from the University of Hamburg, Germany. The full-length 16S rRNA gene fragments were used as the standards for construction of standard curves during qPCR amplification. (Page 6, Line 18 and Page 7, Line 1–3)

P11, L5: *Pseudomonas chlororaphis* subsp. *aureofaciens* (ATCC 13985)

Response:

Thanks. Revised as suggested. (Page 12, Line 12–13)

P13, L3-: Did the authors obtain data of turbidity or light intensity during the sampling?

Response:

We did not obtain data of turbidity and light intensity. But we showed the data of total suspended material (TSM) concentrations, which can reflect the turbidity and light intensity.

P15, L11-: Please clarify how many clone libraries constructed for each gene. Supplementary tables presenting distribution of OTUs will help readers to understand the results.

Response:

We described clone libraries constructed for each gene in 3.2 section. — "*Archaeal and β-proteobacterial amoA and NOB (Nitrospira, Nitrospina, and Nitrobacter) nxrB 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 amoA gene clone libraries were constructed at 75, 200, 800, and 3000 m water depth from SEATS, while a NOB Nitrospina nxrB gene clone library was constructed only at 800 m at SEATS as genes were not amplified successfully at the other three water depths."

According to the reviewer's suggestion, we added the number of the clone libraries for each gene in Table S4. (Supplement Page 8)

P18, L10: Please present the values of detection limits in each qPCR if possible.

Response:

The values of detection limits of all genes we measured were 2-3 copies L^{-1}. Supplied these values in Table S3 as suggested. (Supplement Page 5)

P19, L6: As I know, the abundance of ammonia oxidizers is generally higher than nitrite oxidizers in aquatic environments. I am afraid that the result was influenced by the technical issues described above.

Response:

The abundance of ammonia oxidizers is generally higher than nitrite oxidizers in the oxygenated oceanic water column. However, in oxygen-deficient waters, NOB can reach high abundances exceeding ammonia oxidizers. For example, Füssel et al. (2012) and Beman et al. (2013) observed highly abundant *Nitrospina* and *Nitrococcus* in oceanic OMZs. We discussed this content in 4.2 section (Coupling between ammonia and nitrite oxidizers in the estuarine hypoxic niche).

For the technical issues, please see the response above. We also verified qPCR results using additional two published primer sets. Please see Table R1 and Figure R1 above.

P21, L5: dominant NOB.

Response:

Revised as suggested. (Page 23, Line 4)

P21, L5-: Information from Hawley et al. 2014 should be integrated in this discussion.

Response:

We supplied the information from Hawley et al. 2014 in the revised manuscript. —"*With metaproteomic analysis, Hawley et al. (2014) reported higher expression of*

NXR from NOB Nitrospira and Nitrospina than that of Amo from Thaumarchaeota in an oxygen-deficient water column, Saanich Inlet, British Columbia." (Page 23, Line 5–7)

P25, L17: "availability of ammonia" or "ammonia concentration/flux" would be better than "ammonia levels".

Response:

We revised "*ammonia levels*" as " *ammonia concentration/flux*". (Page 27, Line 16)

P25, L17: Appropriate references should be provided.

Response:

Added. Please see below. (Page 27, Line 16)

Sintes, E., Bergauer, K., De Corte, D., Yokokawa, T., and Herndl, G. J.: Archaeal *amo*A gene diversity points to distinct biogeography of ammonia-oxidizing *Crenarchaeota* in the ocean, Environ. Microbiol., 15, 1647–1658, 2013. (Page 45, Line 4–6)

Sintes, E., De Corte, D., Haberleitner, E., and Herndl, G. J.: Geographic distribution of archaeal ammonia oxidizing ecotypes in the Atlantic Ocean, Front. Microbiol., 7, 1–14, 2016. (Page 45, Line 7–8)

Nunoura, T., Takaki, Y., Hirai, M., Shimamura, S., Makabe, A., Koide, O., Kikuchi, T., Miyazaki, J., Koba, K., Yoshida, N., Sunamura, M., and Takai, K.: Hadal biosphere: insight into the microbial ecosystem in the deepest ocean on Earth, Proc. Natl. Acad. Sci. USA, 112, E1230–E1236, 2015. (Page 42, Line 12–14)

P26, L12: Appropriate references for light inhibition on the growth of nitrifiers should be provided.

Response:

We added two citations. Please see below. (Page 28, Line 13–14)

Lomas, M. W., and Lipschultz, F.: Forming the primary nitrite maximum: Nitrifiers or phytoplankton?, Limnol. Oceanogr., 51, 2453–2467, 2006. (Page 40, Line 15–16)

Merbt, S. N., Stahl, D. A., Casamayor, E. O., Mart ́ı E., Nicol, G. W., and Prosser, J. I.: Differential photoinhibition of bacterial and archaeal ammonia oxidation, FEMS Microbiol. Lett., 327, 41–46, 2012. (Page 41, Line 11–13)

References: Proc. Natl. Acad. Sci. USA instead of P. Natl. Acad. Sci. USA

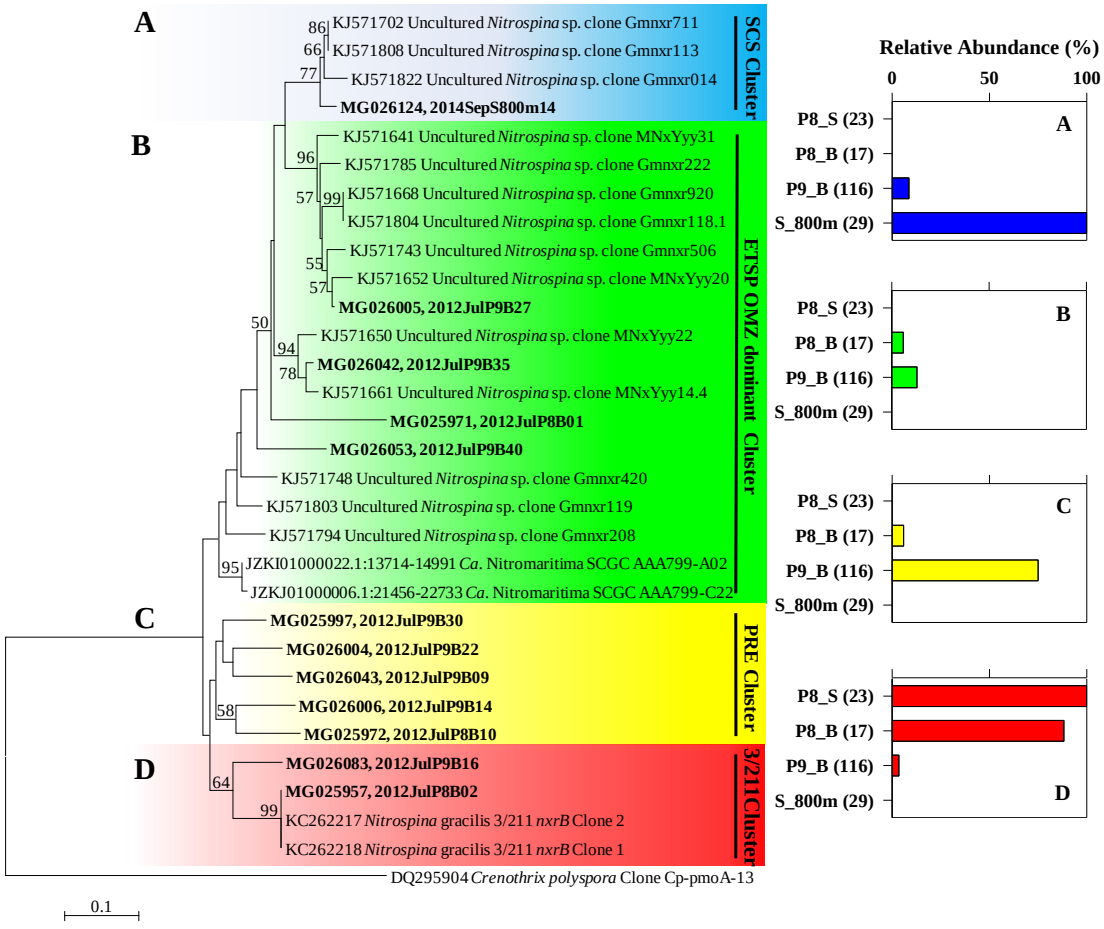
Response:

Revised throughout the references list. (Page 33, Line 17; Page 36, Line 6; Page 36, Line 16; Page 39, Line 9; Page 39, Line 17; Page 41, Line 1; Page 42, Line 14; Supplement Page 4, Line 4)

Fig. 5: Did the Nitromaritima sequence excluded in this phylogenetic analysis?

Response:

Thanks for the reviewer's suggestion. We reconstructed the phylogenetic tree of *Nitrospina*, in which two nitrite oxidoreductase beta subunits (*nxr*B) gene sequences of *Candidatus* Nitromaritima were included. Please see the revised Figure 5 (below). (Page 56)



References:

Beman, J. M., Popp, B. N., and Francis, C. A.: Molecular and biogeochemical evidence for ammonia oxidation by marine *Crenarchaeota* in the Gulf of California, ISME J., 2, 429–441, 2008.

Beman, J. M., Shih, J. L., and Popp, B. N.: Nitrite oxidation in the upper water column and oxygen minimum zone of the eastern tropical North Pacific Ocean, ISME J., 7, 2192–2205, 2013.

Füssel, J., Lam, P., Lavik, G., Jensen, M. M., Holtappels, M., Günter, M., and Kuypers, M. M.: Nitrite oxidation in the Namibian oxygen minimum zone, ISME J., 6, 1200–1209, 2012.

Gubry-Rangin, C., Hai, B., Quince, C., Engel, M., Thomson, B. C., James, P., Schloter, M., Griffiths, R. I., Prosser, J. I., and Nicol, G. W.: Niche specialization of terrestrial archaeal ammonia oxidizers, Proc. Natl. Acad. Sci. USA, 108, 21206–21211, 2011.

Hawley, A. K., Brewer, H. M., Norbeck, A. D., Paša-Tolić, L., and Hallam, S. J.: Metaproteomics reveals differential modes of metabolic coupling among ubiquitous oxygen minimum zone microbes, Proc. Natl. Acad. Sci. USA, 111, 11395–11400, 2014.

Huber, T., Faulkner, G., and Hugenholtz, P.: Bellerophon: a program to detect chimeric sequences in multiple sequence alignments, Bioinformatics, 20, 2317–2319, 2004.

Li, M., Cao, H., Hong, Y., and Gu, J. D.: Spatial distribution and abundances of ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB) in mangrove sediments, Appl. Microbiol. Biotechnol., 89, 1243–1254, 2011.

Lomas, M. W., and Lipschultz, F.: Forming the primary nitrite maximum: Nitrifiers or phytoplankton?, Limnol. Oceanogr., 51, 2453–2467, 2006. (Page 58, Line 4–5)

Meinhardt, K. A., Bertagnolli, A., Pannu, M. W., Strand, S. E., Brown, S. L., and Stahl, D. A.: Evaluation of revised polymerase chain reaction primers for more inclusive quantification of ammonia - oxidizing archaea and bacteria, Env. Microbiol. Rep., 7, 354–363, 2015.

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Response to Reviewer #2

Anonymous Referee #2

Received and published: 28 June 2018

Hou et al. investigated the distribution of Ammonia and Nitrite oxidizers in a subtropical estuary of China by using the functional gene-based clone library and qPCR analyses as well as the determination of nitrification rates. The main conclusion of this work is that substrate affinity/preference of Ammonia and Nitrite oxidizers may play an important role in determining their distribution patterns in estuarine-ocean gradient. Some small comments are provided for improving this manuscript.

1. page 2, Line 2, and page 4, Line 15, I think "between" should be changed into "of";

Response:

Revised as suggested. (Page 2, Line 2, and Page 4, Line 16)

2. page 4, Line 15-18, Please add some background information related to niche differentiation of ammonia and nitrite oxidizers. This may facilitate readers to get a quick view of the current research status.

Response:

Thanks for the reviewer's suggestion. We added some background information related to niche differentiation of ammonia and nitrite oxidizers. — "*For example, both AOA and AOB are frequently found together in estuarine and coastal regimes and share the same ecosystem function (Bernhard et al., 2010; Zhang et al., 2014a), but in many situations, only AOA or AOB are predominant (Cébron et al., 2003; Hollibaugh et al., 2011; Li et al., 2014) as their physiological responses to environmental stressors may be different. Similarly, Nitrospira, Nitrospina, Nitrococcus, and/or Nitrobacter are frequently found together in estuarine and marine regimes, but there is no a consistent distribution pattern between them (Cébron et al., 2005; Füssel et al., 2012; Nunoura et al., 2015; Pachiadaki et al., 2017), suggesting that niche partitioning and niche specialization support the coexistence of sympatric NOB. Moreover, between ammonia and nitrite oxidizers, there is a coupling in abundance and distribution in Monterey Bay and the North Pacific Subtropical Gyre (Mincer et al., 2007) or decoupling in Gulf of Mexico (Bristow et al., 2015).*" (Page 4, Line 17–18 and Page 5, Line 1–9)

3. page 8, Line 9, could you provide a coverage information about this primer set you designed?

Response:

Thanks for the reviewer's suggestion. There were only two *nxr*B gene sequences (from *Nitrospina gracilis* 3/211) in the NCBI database when the primer pair of nxrBNF and nxrBNR was designed, and the coverage is 100%. We discussed the coverage of this primer pair in Discussion 4.1 section and added the coverage analysis. — "*Among 23 sequences of Nitrospina nxrB genes available in the databases, only seven sequences could not be targeted by the primers nxrBNF and nxrBNR due to >3 mismatching bases for either primer, indicating a ~70% coverage of the primers (100% if allowing 5 mismatching bases).*" (Page 22, Line 10–13)

4. page 13, Line 6, reference citations? or based on your results?

Response:

Sorry for the unclear sentence. The reference citation on the characteristics of the upper, middle and lower reaches of the PRE is Wang et al. (2012). We revised this sentence as "*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).*" (Page 14, Line 12–16)

5. page 16, Line 5, I think the group E of AOA belong to the typical Soil/Sediment cluster, while other groups you defined belong to the typical Water/Sediment cluster. Actually, the HAC and LAC clusters were defined on the basis of the later one, especially for the Marine cluster within the Water/Sediment cluster. If you want to define the members within Soil/Sediment cluster, like group E, please provide more supporting evidence/cited references.

Response:

Many thanks for the reviewer's suggestion. Indeed, group E belongs to Soil/sediment cluster. We added the related statement in 3.3 section — "*According to the framework of Francis et al. (2005), groups A, Ba, and Bb were defined as Water column cluster, group D was defined as Sediments cluster, and group E was defined as Soil/sediment*

cluster." (Page 17, Line 2–4) We also added the cluster information in Figure 3 and S3. (Page 52, and Supplement Page 12)

According to the framework of Sintes et al. (2013), there is a rough range of ammonia concentration for HAC (20 to 100 nM or even higher) and LAC (frequently below detection limit). Our field data on ammonia concentration confirmed the categorization of groups A (HAC), Ba and Bb (LAC), D (HAC), and E (HAC). We also added a reference citation to support that group E can be defined as HAC. — "*Tourna et al. (2011) and Hatzenpichler et al. (2008) have reported that two ammonia-oxidizing archaea Nitrososphaera viennensis and Nitrososphaera gargensis belonging to group E (crenarchaeal group I. 1b) tolerate high ammonia concentrations (1–15 mM and 0.14–3.08 mM, respectively).*" (Page 17, Line 14–16)

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Pachiadaki, M. G., Sintes, E., Bergauer, K., Brown, J. M., Record, N. R., Swan, B. K., Mathyer, M. E., Hallam, S. J., Lopez-Garcia, P., Takaki, Y., Nunoura, T., Woyke, T., Herndl, G. J., and Stepanauskas, R.: Major role of nitrite-oxidizing bacteria in dark ocean carbon fixation, Science, 358, 1046–1051, 2017.

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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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[†]Contributed equally

Abstract

The niche differentiation of ammonia and nitrite oxidizers are controversial because they display disparate patterns in estuarine, coastal, and oceanic regimes. We analyzed diversity and abundance of ammonia-oxidizing archaea (AOA) and *β*-proteobacteria (AOB), nitrite-oxidizing bacteria (NOB), and nitrification rates to identify their niche differentiation along a salinity gradient from the Pearl River estuary to the South China Sea. AOA were generally more abundant than *β*-AOB; 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 significant positive correlation between ammonia and nitrite oxidizer abundances in the hypoxic waters of the estuary, suggesting a possible coupling through 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.

Comment [YZ1]: Revised as suggested. (RC2)

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Deleted: *nxr*B and 16S rRNA genes

Comment [YZ2]: Revised as suggested. (RC1)

Comment [YZ3]: Revised as suggested. (RC1)

Deleted: The archaeal *amo*A genes

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Deleted: *amo*A genes

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Comment [YZ4]: Made this sentence more clear. (RC1)

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; Pachiadaki et al., 2017). *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), which were previously reported as a group in *Nitrospina*. 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 of ammonia and nitrite oxidizers is controversial because it displays disparate patterns and partnerships in estuarine, coastal, and oceanic regimes. For example, both AOA and AOB are frequently found together in estuarine and coastal regimes and share the same ecosystem

Comment [YZ5]: Added as suggested. (RC1)

Comment [YZ6]: We supplied the information related to the close relationship between *Nitrospina* and *Ca.* Nitromaritima in the revised version. (RC1)

Comment [YZ7]: Revised as suggested. (RC2)

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function (Bernhard et al., 2010; Zhang et al., 2014a), but in many situations, only AOA or AOB are predominant (Cébron et al., 2003; Hollibaugh et al., 2011; Li et al., 2014) as their physiological responses to environmental stressors may be different. Similarly, *Nitrospira*, *Nitrospina*, *Nitrococcus*, and/or *Nitrobacter* are frequently found together in estuarine and marine regimes, but there is no a consistent distribution pattern between them (Cébron et al., 2005; Füssel et al., 2012; Nunoura et al., 2015; Pachiadaki et al., 2017), suggesting that niche partitioning and niche specialization support the coexistence of sympatric NOB. Moreover, between ammonia and nitrite oxidizers, there is a coupling in abundance and distribution in Monterey Bay and the North Pacific Subtropical Gyre (Mincer et al., 2007) or decoupling in Gulf of Mexico (Bristow et al., 2015). A gradient from an estuary to the ocean, with various environmental gradients and 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.

Comment [YZ8]: We added some background information related to the niche differentiation of ammonia and nitrite oxidizers. (RC2)

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 Strains and genomic DNAs

We obtained strains *Candidatus* Nitrospira defluvii A17 and *Nitrospina gracilis* 3/211 and their genomic DNAs from the University of Hamburg, Germany. The full-length 16S rRNA gene fragments were used as the standards for construction of standard curves during qPCR amplification.

Comment [YZ9]: We added the information related to strains and/or genomic DNA used in this study in Materials and methods 2.1 section. (RC1)

2.2 Study sites and sampling

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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.

2.3 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 ℃ until weighing in the laboratory.

2.4 DNA extraction

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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 ℃ 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).

2.5 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 *N. 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 sequences were analyzed with Bellerophon program (http://comp-bio.anu.edu.au/bellerophon/bellerophon.pl) to detect chimeric sequences in multiple

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sequences alignments (Huber et al., 2004). The putative chimeras were further checked manually through BLASTp analysis to verify whether these were chimeras. After removing chimeric sequences, all sequences among the libraries for each gene 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). Rarefaction, non-parametric coverage, and phylotype richness estimators (Chao 1, Shannon, and Simpson) were calculated. 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.

2.6 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

Comment [YZ10]: We did chimera check through Bellerophon and manual BLASTp analysis. We added this statement in Materials and methods. (RC1)

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Comment [YZ11]: We determined OTUs among the libraries for each gene. (RC1)

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DNA (accession numbers KY387998 (targeted by the primers Arch-amoAF and Arch-amoAR) and MH638327 (targeted by the primers Arch-amoA-for and Arch-amoA-rev) for AOA and MH458281 for AOB) 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.

Comment [YZ12]: We added the accession numbers for the sequences used to obtain standard curves. (RC1)

2.7 ^{15}N-labeled nitrification rate measurements

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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 ℃) using a temperature control incubator. The incubations were terminated by filtering through 0.22 μm polycarbonate membranes, and the filtrate was frozen at -20 ℃ 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 chlororaphis* subsp. *aureofaciens* (ATCC 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

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to the initial conditions using Eq. (1):

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

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}.

2.8 Statistical analysis

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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.

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

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Comment [YZ14]: The reference citation on the characteristics of the upper, middle and lower reaches of the PRE is Wang et al. (2012). We revised it for clarification. (RC2)

26.34 to 30.14 ℃ and decreased seaward (Fig. 2b). TSM 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). DO 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 ℃, while the temperature decreased sharply to 2.35 ℃ 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

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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 three clusters (five groups A, Ba, Bb, D, and E) based on phylogenetic analysis (Fig. 3 and S3). According to the framework of Francis et al. (2005), groups A, Ba, and Bb were defined as Water column cluster, group D was defined as Sediments cluster, and group E was defined as Soil/sediment cluster. 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 fell into group E and had an 86% to 100% DNA sequence identity with sequences recovered from high ammonia environments, such as soil, sediment, biofilters, rivers, lakes, and water treatment plants (Fig. 3). Tourna et al. (2011) and Hatzenpichler et al. (2008) have reported that two ammonia-oxidizing archaea *Nitrososphaera viennensis* and *Nitrososphaera gargensis* belonging to group E (crenarchaeal group I. 1b) tolerate high ammonia concentrations (1–15 mM and 0.14–3.08 mM, respectively). 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,

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Comment [YZ15]: Group E belongs to Soil/sediment cluster. We added the related statement. (RC2)

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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). Although the niche separation among AOA subgroups may be influenced by some bias during PCR amplification, overall distribution of HAC and LAC subgroups are plausible.

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 *Nitrospira 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.,

Comment [YZ17]: We added the statement to mention about the possibility that the interpretation of the niche separation among AOA subgroups may be influenced by PCR bias. (RC1)

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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 *Nitrospira 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.

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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 6.82 $\times 10^5$ copies L^{-1} (PA community in the bottom water of site P9) and from below detection limit to 3.42 $\times 10^4$ 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 $\times 10^6$ copies L^{-1} (PA community in the bottom water of site P4) and from 51 to 3.81 $\times 10^5$ 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 <0.05–0.01) (Fig. 6e and f).

Sites P1–P6, located in hypoxic waters that are typically defined when DO concentrations fall

Comment [YZ18]: We revised the maximum value of archaeal *amo*A gene abundance after replacing the data (using FranAOA primer set) at the lower reaches sites P9–12 with the new abundance data (using WuchterAOA primer set) of archaeal *amo*A gene in the revised manuscript. (RC1)

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Comment [YZ19]: We reanalyzed the correlation after replacing the data (using FranAOA primer set) at the lower reaches sites P9–12 with the new abundance data (using WuchterAOA primer set) of archaeal *amo*A gene in the revised manuscript. (RC1)

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 rapidly decreased and the AOM *amo*A genes increased (Fig. 6g and h), and archaea and *Nitrospina* became the dominant ammonia and nitrite oxidizers, respectively (Fig. 6a–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).

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Comment [YZ20]: We revised the related statement after replacing the data (using FranAOA primer set) at the lower reaches sites P9–12 with the new abundance data (using WuchterAOA primer set) of archaeal *amo*A gene in the revised manuscript. (RC1)

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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). Among 23 sequences of *Nitrospina nxr*B genes available in the databases, only seven sequences could not be targeted by the primers nxrBNF and nxrBNR due to >3 mismatching bases for either primer, indicating a ~70% coverage of the primers (100% if allowing 5 mismatching bases). 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.

Comment [YZ21]: We discussed the coverage of the primer pair of nxrBNF and nxrBNR designed in this study. (RC2)

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 NOB, particularly on the particles. With metaproteomic analysis, Hawley et al. (2014) reported higher expression of NXR from NOB *Nitrospira* and *Nitrospina* than that of Amo from *Thaumarchaeota* in an oxygen-deficient water column, Saanich Inlet, British Columbia. 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

Comment [YZ22]: Revised as suggested. (RC1)

Comment [YZ23]: We supplied the information from Hawley et al. 2014 in the revised manuscript. (RC1)

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).

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

Comment [YZ24]: We reanalyzed the correlations and updated the statements after replacing the data (using FranAOA primer set) at the lower reaches sites P9–12 with the new abundance data (using WuchterAOA primer set) of archaeal *amo*A gene in the revised manuscript. (RC1)

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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 concentration/flux (Sintes et al., 2013; 2016; Nunoura et al., 2015).

Nitrite, a central intermediate compound in nitrification, was positively correlated to NOB 16S rRNA and *β*-proteobacterial *amo*A gene abundances (P <0.05–0.01, Table S5). *Nitrospira* displays

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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). Both nitrite and nitrate concentrations were negatively correlated to archaeal *amo*A gene abundance in the estuary (P <0.05–0.01, Table S5), which is consistent with the observations from the present study and previous studies that AOA are more dominant in oligotrophic environments (Wuchter et al., 2006; Newell et al., 2013).

Notably, all genes were significantly positively correlated to TSM concentrations in PA communities (P <0.05–0.01, Table S5). 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 (Lomas et al., 2006; Merbt et al., 2012). 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

Comment [YZ27]: We reanalyzed the correlations and updated the related statements after replacing the data (using FranAOA primer set) at the lower reaches sites P9–12 with the new abundance data (using WuchterAOA primer set) of archaeal *amo*A gene in the revised manuscript. (RC1)

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Comment [YZ28]: We added appropriate references for light inhibition on the growth of nitrifiers. (RC1)

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, but positively correlated to the archaeal *amo*A gene (*P* <0.05–0.01, Table S5). A similar observation was found by Li et al. (2011) in mangrove sediments at the northwestern corner of the New Territories of Hong Kong. However, AOA and AOB *amo*A gene abundances were both previously found increasing with pH in soils (Gubry-Rangin et al., 2011), and the open ocean (Nunoura et al., 2015). This is probably related to lower availability of the substrate (ammonia) due to increased ionization to ammonium as pH decreases. 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; the opposite correlations were observed in archaeal *amo*A gene abundance (*P* <0.05–0.01, Table S5). These results suggest that *β*-AOB and *Nitrospira* recovered in the PRE could partly originate from the Pearl River or upstream and AOA could partly originate from the SCS.

Partial Mantel tests were further applied to the qPCR dataset and environmental parameters to

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Comment [YZ29]: We reanalyzed the correlations and revised the related statements after replacing the data (using FranAOA primer set) at the lower reaches sites P9–12 with the new abundance data (using WuchterAOA primer set) of archaeal *amo*A gene in the revised manuscript. (RC1)

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Comment [YZ30]: We reanalyzed the correlations and revised the related statements after replacing the data (using FranAOA primer set) at the lower reaches sites P9–12 with the new abundance data (using WuchterAOA primer set) of archaeal *amo*A gene in the revised manuscript. (RC1)

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), National Programme on Global Change and Air-Sea Interaction (GASI-03-01-02-03), 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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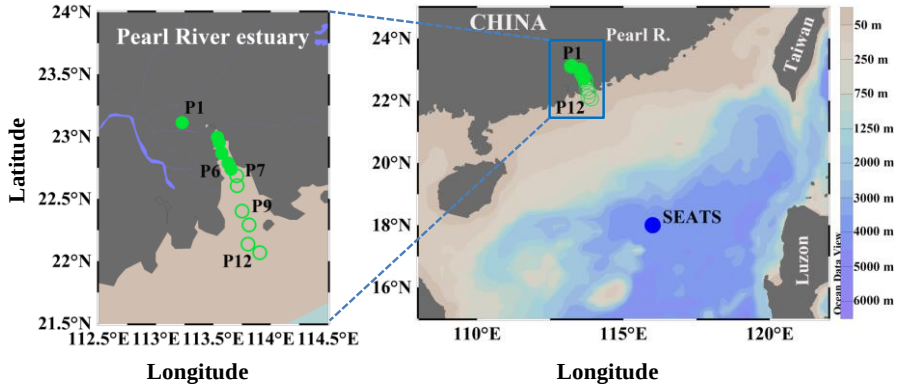


Figure 1. Site locations and bathymetry. The solid green circles indicate hypoxic sites in the PRE, open green circles indicate (low) oxygenated sites in the PRE, and the solid blue circle indicates SEATS in the central basin of the SCS. This figure was produced using Ocean Data View v. 4.6.2 (http://odv.awi.de,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

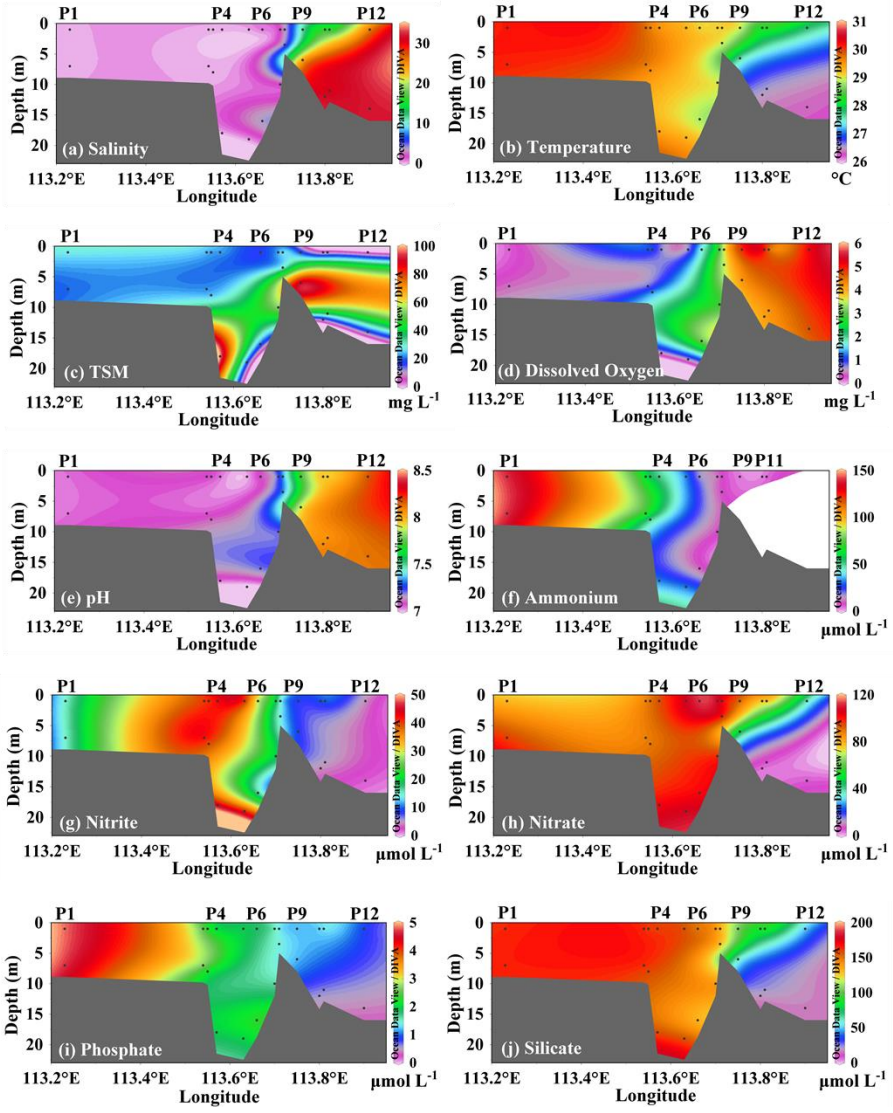
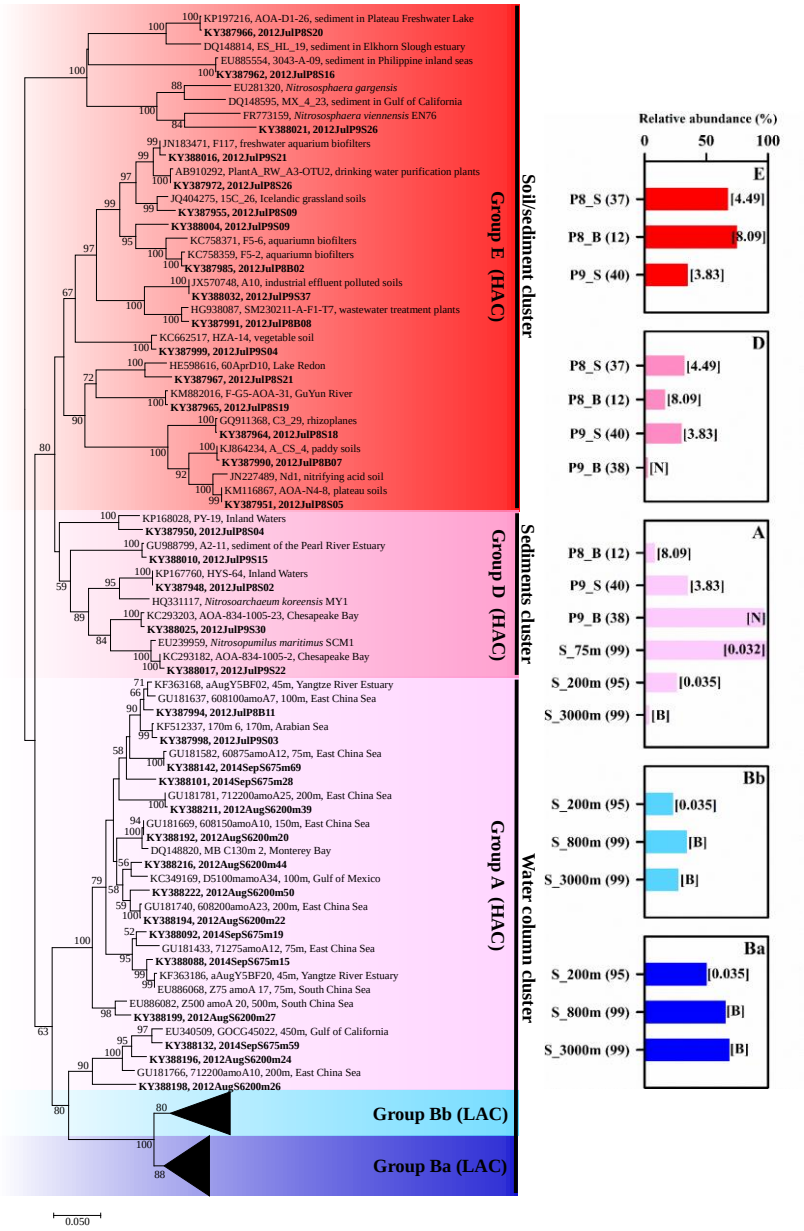


Figure 2. Distributions of biogeochemical factors along the PRE transect. (a) Salinity, (b) temperature, (c) TSM, (d) DO, (e) pH, (f) ammonium, (g) nitrite, (h) nitrate, (i) phosphate, and (j)

1 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, Nitrososphaera gargensis
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
Group E (HAC)
Soil/sediment cluster
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
Group D (HAC)
Sediments cluster
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 A (HAC)
Water column cluster
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]

1

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.

Comment [YZ38]: Group E belongs to Soil/sediment cluster. We added the cluster information in Figure 3. (RC2)

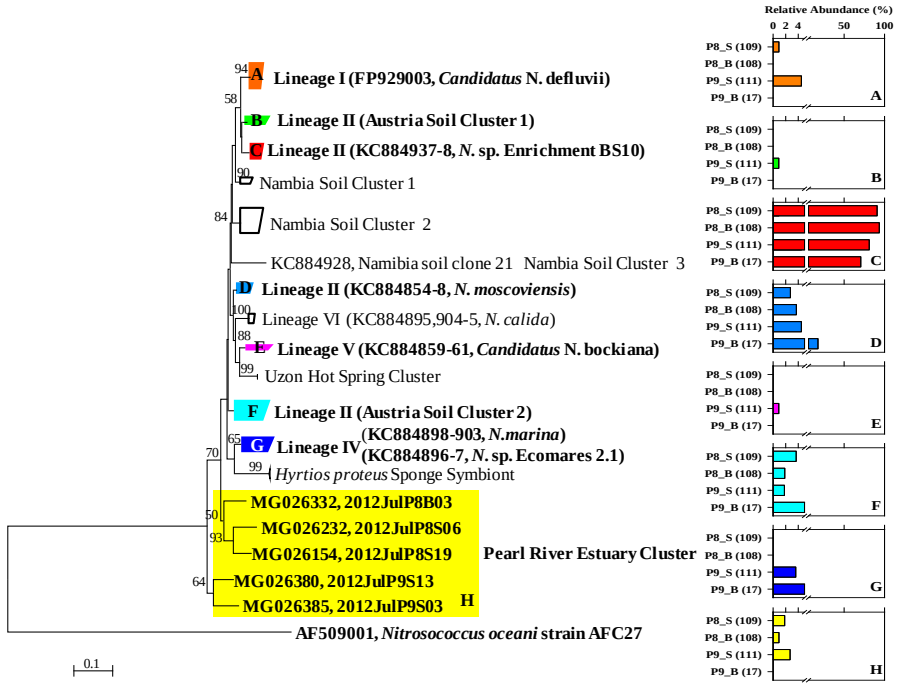


Figure 4. Rooted neighbor-joining (NJ) phylogenetic tree of the *Nitrospira nxr*B gene sequences. Clone sequences from this study are shown in bold and sequences sharing 95% DNA identity are grouped. GenBank accession numbers are shown. Groups A, B, C, D, E, F, and G are defined according to Pester et 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

Deleted: a
Deleted: b
Deleted: c
Deleted: d
Deleted: e
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Deleted: g
Deleted: h

sequence divergence.

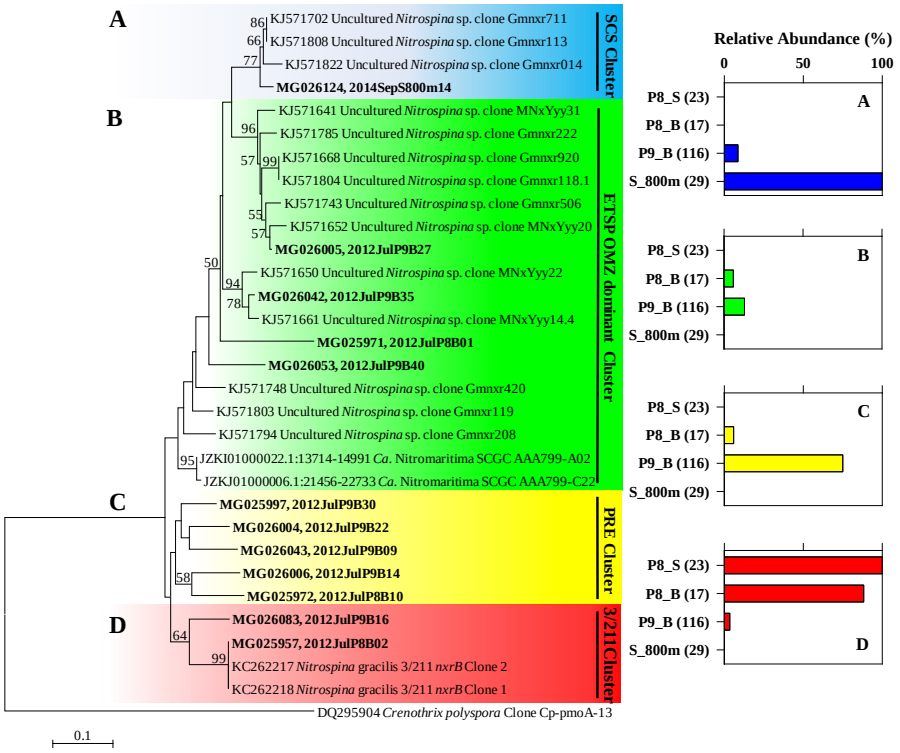

Figure 5. Rooted neighbor-joining (NJ) phylogenetic tree of the *Nitrospina nxr*B gene sequences. Clone sequences from this study are shown in bold and sequences sharing 95% DNA identity are grouped. GenBank accession numbers are shown. Groups A, B, C, and D are defined in this study. The relative abundance 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

Comment [YZ39]: We reconstructed the phylogenetic tree of *Nitrospina*, in which two nitrite oxidoreductase beta subunits (*nxr*B) gene sequences of *Candidatus* Nitromaritima were included. (RC1)

10% estimated sequence divergence.

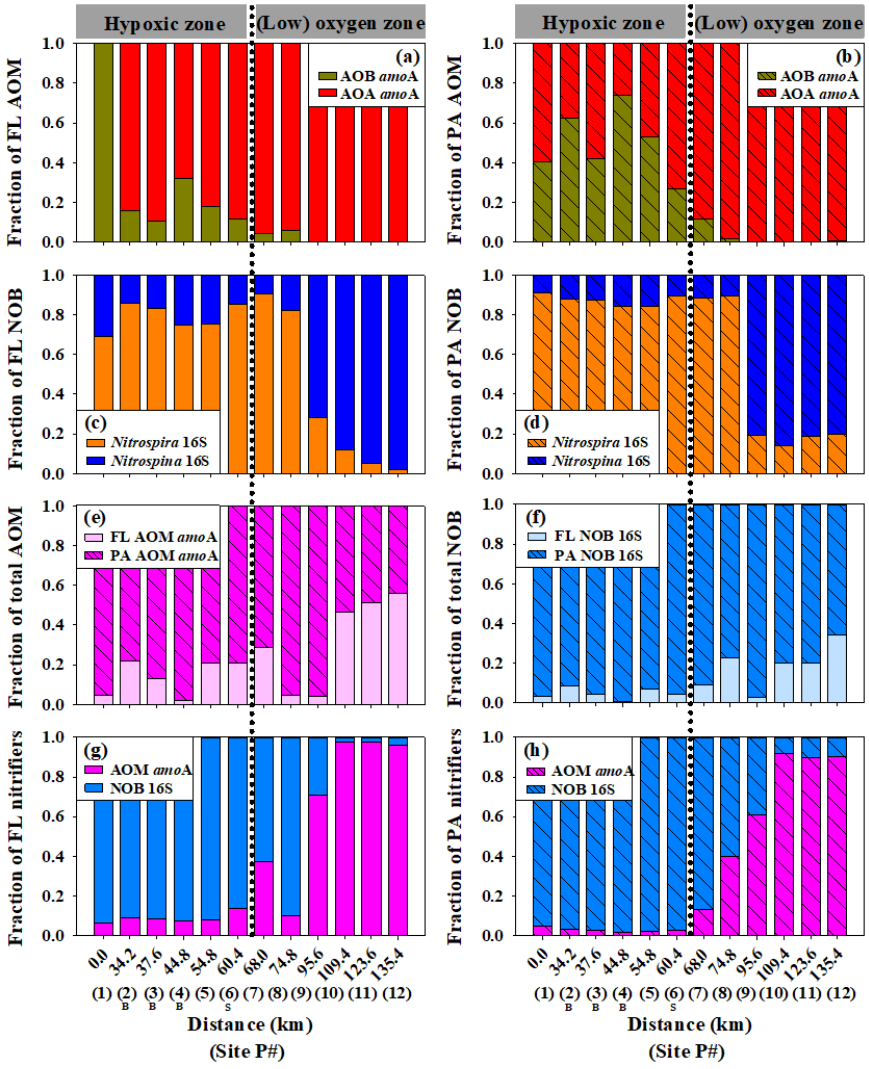

Figure 6. Gene abundance distribution of four nitrifier groups along the PRE transect. (a) Relative abundance of archaeal (AOA) and *β*-proteobacterial (AOB) *amo*A genes in total FL AOM

Comment [YZ40]: We replotted the Figure 6 after replacing the data (using FranAOA primer set) at the lower reaches sites P9–12 with the new abundance data (using WuchterAOA primer set) of archaeal *amo*A gene in the revised manuscript. (RC1)

(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.

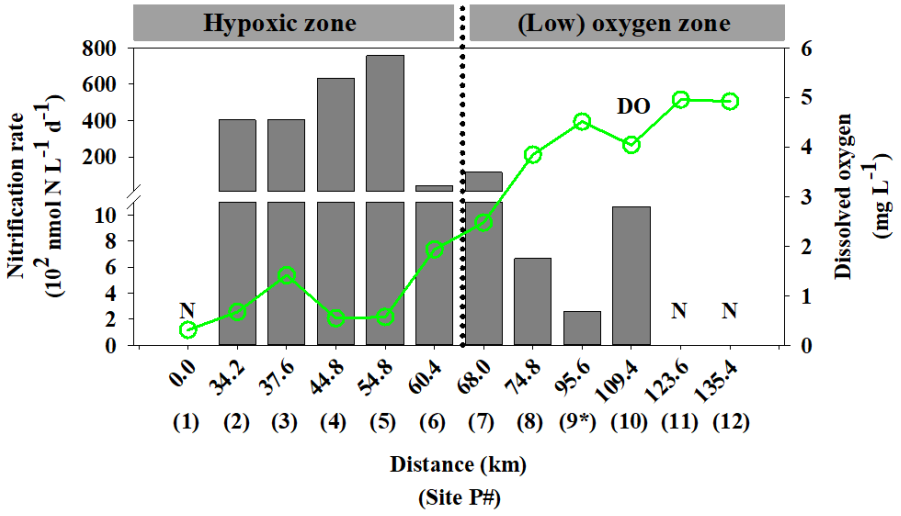

Figure 7. Nitrification rates and DO concentrations along the PRE transect. Nitrification rates were only measured in the bottom waters except for site P9, where rates were measured in both surface and bottom waters. N, not measured; *the depth-weighted value was used.

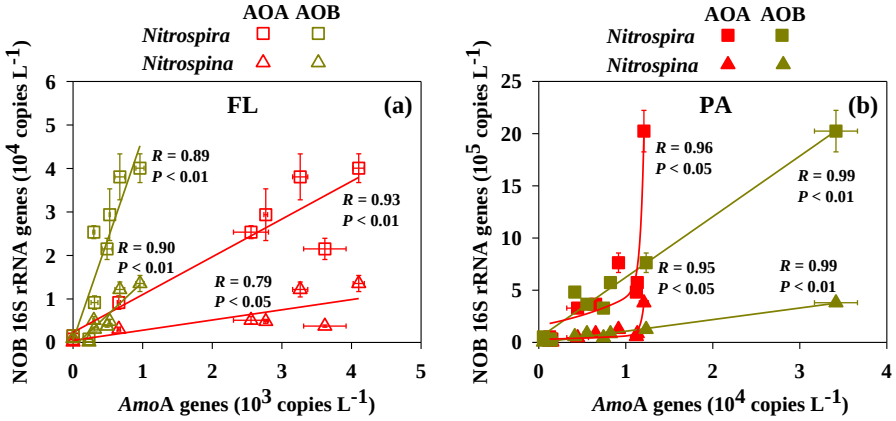

Figure 8. Correlations between ammonia and nitrite oxidizers in the hypoxic zone of the PRE (sites P1–6). There are significant positive correlations (n = 8) between archaeal and *β*-proteobacterial *amo*A genes and *Nitrospira* and *Nitrospina* 16S rRNA gene abundances in (a) FL and (b) PA communities.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

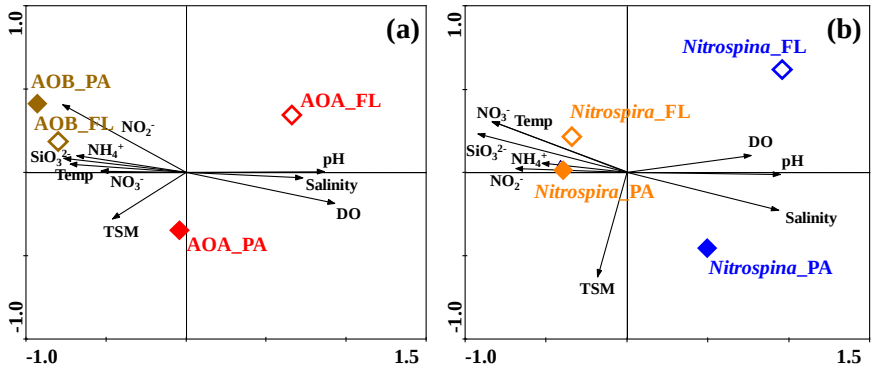

Figure 9. Canonical correspondence analysis. (a) Ammonia and (b) nitrite oxidizers under the constraint of environmental factors. Each diamond represents an individual subgroup. Vectors represent the environmental variables. Temp, temperature.

Comment [YZ41]: We replotted the Figure 9 after replacing the data (using FranAOA primer set) at the lower reaches sites P9–12 with the new abundance data (using WuchterAOA primer set) of archaeal *amo*A gene in the revised manuscript. (RC1)

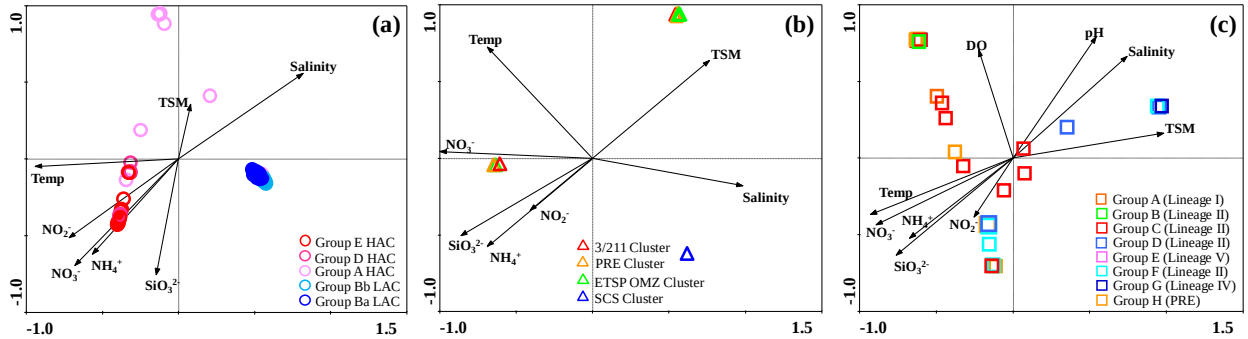

Figure 10. Canonical correspondence analysis (CCA). (a) Ammonia-oxidizing archaea, (b) *Nitrospina*, and (c) *Nitrospira* phylogenetic taxa under the constraint of environmental factors. Each symbol represents an individual OTU. Vectors represent the environmental variables. Temp, temperature. DO and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

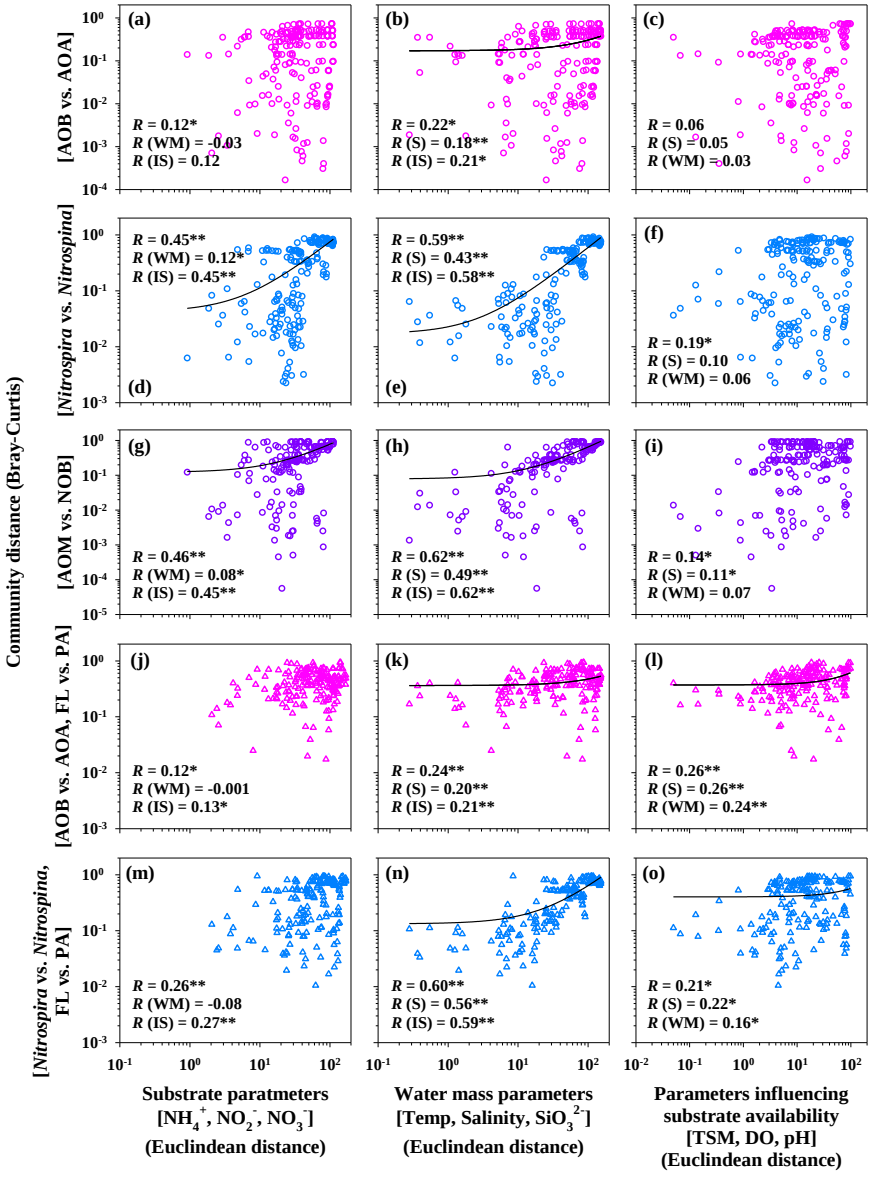
Community distance (Bray-Curtis)
[AOB vs. AOA]
[Nitrospira vs. Nitrospina]
[AOM vs. NOB]
[AOB vs. AOA, FL vs. PA]
[Nitrospira vs. Nitrospina, FL vs. PA]
(a) R = 0.12* R (WM) = -0.03 R (IS) = 0.12
(b) R = 0.22* R (S) = 0.18** R (IS) = 0.21*
(c) R = 0.06 R (S) = 0.05 R (WM) = 0.03
(d) R = 0.45** R (WM) = 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 = 0.46** R (WM) = 0.08* R (IS) = 0.45**
(h) R = 0.62** R (S) = 0.49** R (IS) = 0.62**
(i) R = 0.14* R (S) = 0.11* R (WM) = 0.07
(j) R = 0.12* R (WM) = -0.001 R (IS) = 0.13*
(k) R = 0.24** R (S) = 0.20** R (IS) = 0.21**
(l) R = 0.26** R (S) = 0.26** R (WM) = 0.24**
(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*
Substrate paratmeters [NH4+, NO2-, NO3-] (Euclindean distance)
Water mass parameters [Temp, Salinity, SiO32-] (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.

Comment [YZ42]: We replotted the Figure 11 after replacing the data (using FranAOA primer set) at the lower reaches sites P9–12 with the new abundance data (using WuchterAOA primer set) of archaeal *amo*A gene in the revised manuscript. (RC1)

Table S1. Abundances of the archaeal and *β*-proteobacterial *amo*A genes and *Nitrospira* and *Nitrospina* 16S rRNA genes in the PRE.

Station	Water Depth (m)	Sampling Depth (m)	Archaeal *amo*A (copies L^{-1})				*β*-proteobacterial *amo*A (copies L^{-1})				*Nitrospira* 16S rRNA (copies L^{-1})				*Nitrospina* 16S rRNA (copies L^{-1})			
			FL[a]	SD	PA[b]	SD	FL[a]	SD	PA[b]	SD	FL[a]	SD	PA[b]	SD	FL[a]	SD	PA[b]	SD
P1	8.9	1	0		1501	40	0		1338	799	1533	446	33025	525	909	747	4537	691
		7	0		1248	57	228	56	528	17	776	76	53287	1086	121	6	3902	179
P2	9.8	1	NS[c]				NS[c]				NS[c]				NS[c]			
		7	2768	27	4462	1243	526	6	7441	562	29374	5945	328697	15430	4851	447	45997	2792
P3	10.2	1	NS[c]				NS[c]				NS[c]				NS[c]			
		8	2556	251	11321	85	298	14	8239	482	25360	1478	573425	12573	5103	850	85027	9576
P4	21.5	1	NS[c]				NS[c]				NS[c]				NS[c]			
		18	657	22	12080	516	308	49	34158	2469	9175	1541	2024263	198739	3068	441	380537	14232
P5	22.5	1	4104	80	6535	30	961	58	5532	428	40070	3306	365741	18556	13556	1852	84860	3967
		19	3263	109	9162	327	672	70	12341	292	38076	5273	763345	93318	12186	1715	125427	10142
P6	18.8	1	3617	305	11219	241	488	71	4136	208	21516	2437	482519	13994	3763	250	56974	2244
		16	NS[c]				NS[c]				NS[c]				NS[c]			
P7	12	1	40742	2180	108877	4425	2012	245	14259	3443	69806	5991	735150	42882	7820	652	95572	4870
		10	10212	2234	8267	677	221	11	974	171	11393	3342	46220	3334	950	103	7540	399
P8	5	1	2614	47	64350	3095	150	40	1024	72	16111	1427	70874	15050	3368	539	7479	625
		3.5	2904	272	49549	4515	201	117	1228	92	26179	3334	83656	10345	5913	668	11065	1838
P9	8	1	20355	1102	35409	2540	70		553	127	3536	475	40278	3435	8309	1501	75342	10147
		6	10081	442	681539	25091	0		1392	64	0		47889	10565	769	139	296757	20048
P10	12.9	1	72002	20991	25516	4630	0		121		506	128	1663	946	1814	98	2033	214
		11	113345	4922	185761	31978	298	71	165	29	63	23	973	292	2340	256	14241	2378
P11	14.2	1	14384	2520	775	81	0		172	118	43		495	135	412	151	51	

Comment [YZ1]: We replaced the data (using FranAOA primer set) at the lower reaches sites P9–12 with the new abundance data (using WuchterAOA primer set) of archaeal *amo*A gene in the revised manuscript. (RC1)

		12	130393	6302	137480	14835	0	224	14	154	101	2542	750	3400	315	12913	2894
P12	16	1	12087	1917	4724	984	0	0		68		0		6945	3360	362	
		14	302349	78106	240640	4899	0	1423	74	227	110	5294	807	6635	851	20858	530

1 [a], Free-living; [b], Particle-associated; [c], No sample

Table S2. Primer set sequences, PCR reaction mixtures and conditions for each gene.

Target gene	Primer	Sequence (5'-3')	PCR mixture	PCR conditions	References
β-proteobacterial *amo*A	amoA-34F	GCGGCRAAAATGCCGCCGGAAGCG	50 μL reaction mixture: Failsafe Premix F (Epicentre Biotechnologies, Madison, WI, U.S.A.) 25 μL, primers 0.5 μM, plantium Taq DNA polymerase (Invitrogen, Carlsbad, CA, U.S.A.) 1 U, DNA template 1 μL	95 ℃ for 2 min; hot start at 80 ℃; and 25 x (95 ℃ for 30 s, 57 ℃ for 30 s, 73 ℃ for 3 min).	Kim et al., 2008; Hu et al., 2010
	amoA-2R	CCCCTCKGSAAAGCCTTCTTC			
Archaeal *amo*A	Arch-amoAF	STAATGGTCTGGCTTAGACG	50 μL reaction mixture: Failsafe Premix F (Epicentre Biotechnologies) 25 μL, primers 0.5 μM, plantium Taq DNA polymerase (Invitrogen) 1 U, DNA template 1 μL	95 ℃ for 5 min; 30 x (94 ℃ for 45 s, 53 ℃ for 60 s, and 72 ℃ for 60 s); and 72 ℃ for 15 min.	Francis et al., 2005; Hu et al., 2010
	Arch-amoAR	GCGGCCATCCATCTGTATGT			
Nitrobacter and *Nitrococcus nxr*B	nxrBF706	AAGACCTAYTTCAACTGGTC	50 μL reaction mixture: Ex Taq DNA polymerase 0.25 μL (TaKaRa), 10×Buffer 5 μL, dNTP 4 μL, Mg^{2+} 4 μL, primers 1 μM, BSA (20 μg/uL) 0.125 μL, DNA template 2 μL	95 ℃ for 5 min; 35 x (95 ℃ for 40 s, 56 ℃ 30 s, 72 ℃ 30 s), 72 ℃ for 10 min.	Koch, 2009
	nxrBR1431	CGCTCCATCGGYGGAACMAC			
*Nitrospira nxr*B	nxrB169F	TACATGTGGTGGAACA	25 μL reaction mixture: Platinum Taq DNA polymerase 0.1 μL (Invitrogen), 10×Buffer 2.5 μL, dNTP 2 μL, Mg^{2+} 4 μL, primers 1 μM, BSA (200 ng/μL) 2.5 μL, DNA template 1 μL	95 ℃ for 5 min; 35 x (95 ℃ for 40 s, 56.2 ℃ 40 s, 72 ℃ 90 s), 72 ℃ for 10 min.	Modified from Pester et al., 2013
	nxrB638R	CGGTTCTGGTCRATCA			
*Nitrospina nxr*B	nxrBNF	GGGCGACCAGATGGAAAC	25 μL reaction mixture: LA Taq DNA polymerase 0.25 μL (TaKaRa), 10×Buffer 2.5 μL, dNTP 1 μL, Mg^{2+} 5 μL, primers 1 μM, BSA (200 ng/μL) 2.5 μL, DNA template 1 μL	95 ℃ for 5 min; 35 x (95 ℃ for 40 s, 56.2 ℃ 40 s, 72 ℃ 90 s), 72 ℃ for 10 min.	This study
	nxrBNR	GGGCCGGACATAGAAAGG			

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Comment [YZ2]: Revised as suggested. (RC1)

Table S3. Primer pair sequences, qPCR mixtures and conditions for each gene.

Target gene	Primer	Sequence (5'-3')	PCR mixture	PCR conditions	Efficiency	Detection limits	References
β-proteobacterial *amo*A	amoA-1F	GGGGHTTYTACTGGTGGT	25 μL reaction mixture: SYBR® Premix Ex Taq™ (TakaRa, Dalian, China) 12.5 μL, BSA 5 μg, primers 0.4 μM, DNA template 1 μL	94 ℃ for 30 s; 45 × (94 ℃ for 15 s, 60 ℃ for 60 s, and 72 ℃ for 90 s).	96-104 %	2 copies μl^{-1}	Rotthauwe et al., 1997; Hu et al., 2[illegible] Mincer et al., 2007
	amoA-2R	CCCCTCKGSAAAGCCTTCTTC					
Archaeal *amo*A	Arch-amoAF	STAATGGTCTGGCTTAGACG	25 μL reaction mixture: SYBR® Premix Ex Taq™ (TakaRa) 12.5 μL, BSA 5 μg, primers 0.4 μM, DNA template 1 μL	95 ℃ for 30 s; 40 × (95 ℃ for 30 s, 53 ℃ for 60 s, and 72 ℃ for 45 s).	91-98%	3 copies μl^{-1}	Francis et al., 2005; Hu et al., 2011
	Arch-amoAR	GCGGCCATCCATCTGTATGT					
Archaeal *amo*A[a]	Arch-amoA-for	CTGAYTGGGCYTGGACATC	25 μL reaction mixture: SYBR® Premix Ex Taq™ (TakaRa) 12.5 μL, BSA 10 μg, primers 1 μM, DNA template 1 μL	95 ℃ for 30 s; 41 × (95 ℃ for 30 s, 58.5 ℃ for 40 s, and 72 ℃ for 30 s and 80 ℃ for 25 s).	94-99%	2 copies μl^{-1}	Wuchter et al., 20[illegible] Bergauer et al., 2[illegible]
	Arch-amoA-rev	TTCTTCTTTGTTGCCCAGTA					
Nitrospira 16S rRNA	Nspra-675f	GCGGTGAAATGCGTAGAKATCG	25 μL reaction mixture: SYBR® Premix Ex Taq™ (TakaRa) 12.5μL, BSA 15 μg, primers 0.2 μM, DNA template 1 μL	95 ℃ for 10 min; 45 × (94 ℃ for 30 s, 64 ℃ for 30 s, 72 ℃ for 60 s).	92-98 %	2 copies μl^{-1}	Graham et al., 2007; Attard et al., 2010
	Nspra-746r	TCAGCGTCAGRWAYGTTCCAGAG					
Nitrospina 16S rRNA	NitSSU_130F	GGGTGAGTAACACGTGAATAA	25 μL reaction mixture: SYBR® Premix Ex Taq™ (TakaRa) 12.5μL, BSA 1 μg, primers 0.4 μM, DNA template 1 μL	94 ℃ for 15 min; 45 × (94 ℃ for 15 s, 57.5 ℃ for 15 s, 72 ℃ for 30 s, 77 ℃ for 1 s).	90-100 %	3 copies μl^{-1}	Mincer et al., 2007
	NitSSU_282R	TCAGGCCGGCTAAMCA					

[a], The primer set was used in the samples from the lower reaches (sites P9–12) of the estuary.

Comment [YZ3]: We supplied the values of detection limits in each gene qPCR. (RC1)

Comment [YZ4]: qPCR mixtures and conditions for WuchterAOA primer set. (RC1)

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Table S4. Diversity indices of AOA and *β*-AOB *amo*A, *Nitrospira*, *Nitrospina*, and *Nitrobacter nxr*B genes based on 5% nucleic acid sequences cutoff.

Genes	No. of Libraries	n	No. of OTUs	C (%)	H'	1/D	Chao1
AOA *amo*A (SCS)	4/4	392	60	0.94	3.04	10.64	49.46
AOA *amo*A (PRE)	4/4	127	23	0.90	2.13	4.87	42.5
β-AOB *amo*A (PRE)	2/4	26	3	0.96	0.43	1.28	3
*Nitrospira nxr*B (PRE)	4/4	345	29	0.96	1.79	3.57	42
*Nitrospina nxr*B (PRE & SCS)	4/8	185	12	0.98	1.79	4.83	12.75
*Nitrobacter nxr*B (PRE)	2/4	48	3	0.98	0.78	2.13	3

n, number of sequences; OTU, operational taxonomic unit; C, coverage; H', Shannon-Wiener Index; 1/D, Simpson's diversity Index; SCS, South China Sea; PRE, Pearl River estuary. Numbers before slash indicate successful libraries; numbers after slash indicate all amplified samples.

Comment [YZ5]: We added the number of the clone libraries for each gene. (RC1)

Table S5. *r* values for the relationship between gene abundances of nitrifiers and environmental parameters in the PRE.

Gene	Community	Water mass parameters			Substrate parameters			Parameters influencing substrate availability		
		Temperature (n = 20)	Salinity (n = 20)	SiO_3^{2-} (n = 20)	NH_4^+ (n = 15)	NO_2^- (n = 20)	NO_3^- (n = 20)	TSM (n = 19)	DO (n = 20)	pH (n = 20)
AOB *amo*A	FL[a]	0.302	−0.441	0.439	−0.108	0.527*	0.759**	−0.053	−0.425	−0.512*
	PA[b]	0.332	−0.474*	0.475*	−0.048	0.706**	0.464*	0.520*	−0.525*	−0.496*
	FL+PA	0.341	−0.471*	0.487*	−0.053	0.718**	0.491*	0.504*	−0.536*	−0.513*
AOA *amo*A	FL[a]	−0.754**	0.691**	−0.709**	−0.376	−0.461*	−0.728**	−0.203	0.412	0.585**
	PA[b]	−0.528*	0.539*	−0.524*	−0.407	−0.361	−0.486*	0.498*	0.348	0.434
	FL+PA	−0.717**	0.703**	−0.697**	−0.468	−0.470*	−0.673**	0.330	0.441	0.577**
Nitrospira 16S rRNA	FL[a]	0.426	−0.580**	0.537*	−0.205	0.643**	0.772**	−0.099	−0.464*	−0.625**
	PA[b]	0.356	−0.474*	0.491*	−0.073	0.730**	0.518*	0.504*	−0.541*	−0.524*
	FL+PA	0.367	−0.475*	0.503*	−0.080	0.743**	0.539*	0.493*	−0.550*	−0.540*
Nitrospina 16S rRNA	FL[a]	0.097	−0.167	0.158	−0.268	0.436	0.253	−0.315	−0.190	−0.230
	PA[b]	0.108	−0.134	0.162	−0.105	0.453*	0.173	0.822**	−0.276	−0.221
	FL+PA	0.111	−0.140	0.167	−0.115	0.468*	0.182	0.811**	−0.282	−0.229

[a], Free-living; [b], Particle-associated; *, $P < 0.05$; **, $P < 0.01$; TSM, Total suspended material; DO, Dissolved oxygen

Comment [YZ6]: We revised the correlations between archaeal *amo*A gene abundance and various environmental factors after replacing the data (using FranAOA primer set) at the lower reaches sites P9–12 with the new abundance data (using WuchterAOA primer set) of archaeal *amo*A gene in the revised manuscript. (RC1)

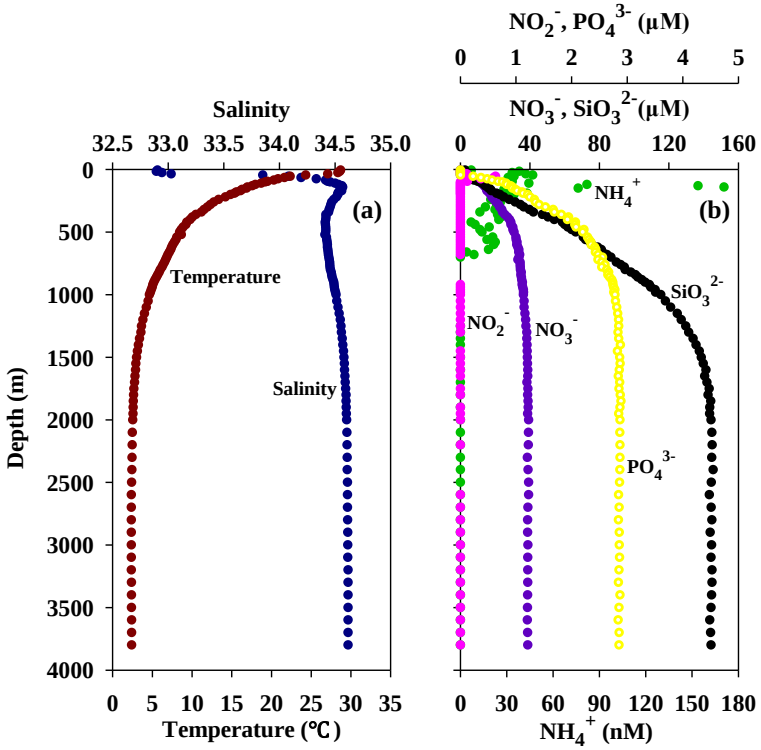

Figure S1. Depth profiles of biogeochemical parameters at SEATS.

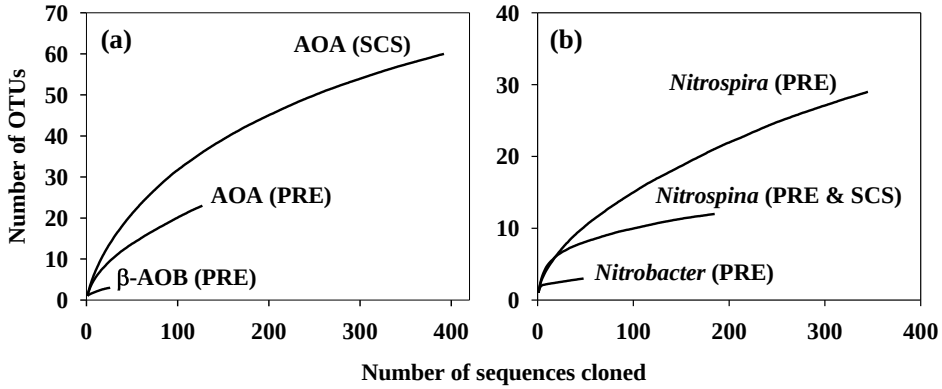

Figure S2. Rarefaction curves of (a) AOA and *β*-AOB *amo*A gene sequences and (b) *Nitrospira*, *Nitrospina*, and *Nitrobacter nxr*B gene sequences. The curves were generated at 95% DNA sequence identity.

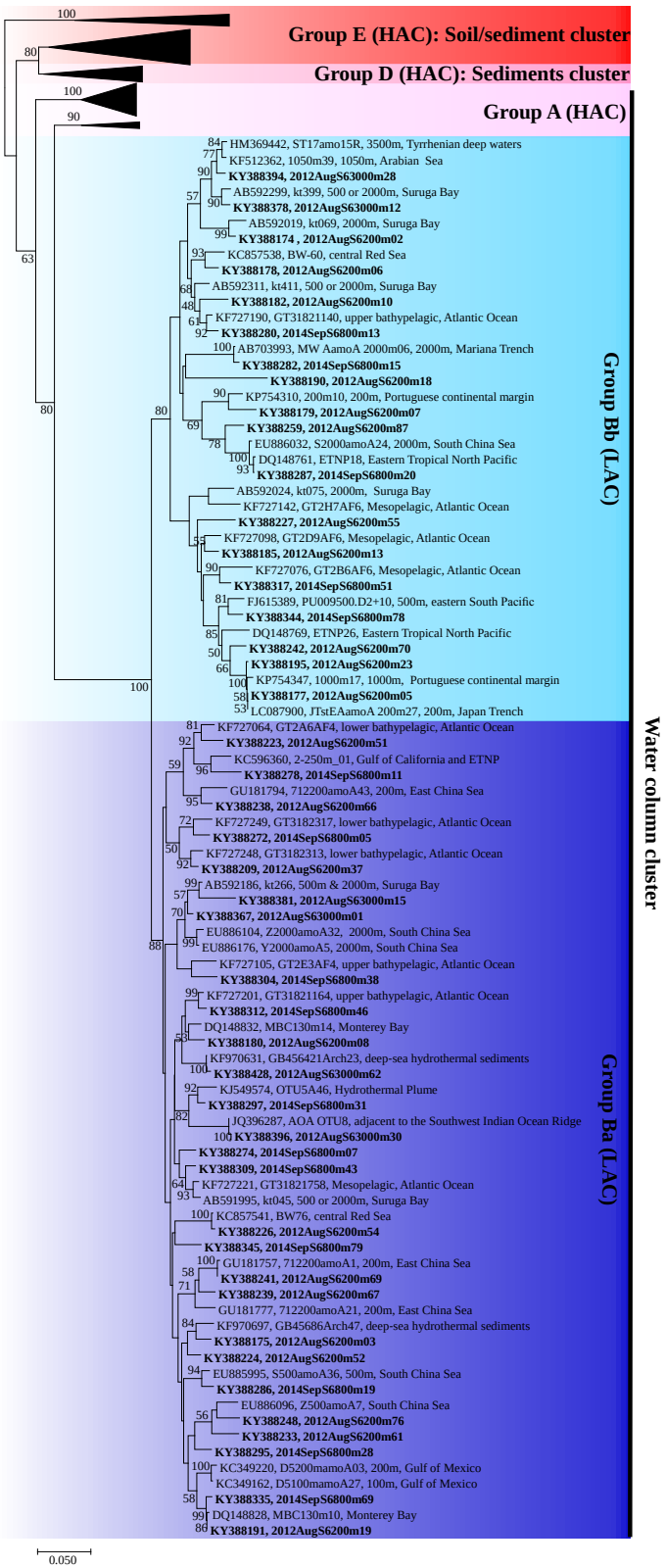
Group E (HAC): Soil/sediment cluster
Group D (HAC): Sediments cluster
Group A (HAC)
Group Bb (LAC)
Group Ba (LAC)
Water column cluster
HM369442, ST17amo15R, 3500m, Tyrrhenian deep waters
KF512362, 1050m39, 1050m, Arabian Sea
KY388394, 2012AugS63000m28
AB592299, kt399, 500 or 2000m, Suruga Bay
KY388378, 2012AugS63000m12
AB592019, kt069, 2000m, Suruga Bay
KY388174 , 2012AugS6200m02
KC857538, BW-60, central Red Sea
KY388178, 2012AugS6200m06
AB592311, kt411, 500 or 2000m, Suruga Bay
KY388182, 2012AugS6200m10
KF727190, GT31821140, upper bathypelagic, Atlantic Ocean
KY388280, 2014SepS6800m13
AB703993, MW AamoA 2000m06, 2000m, Mariana Trench
KY388282, 2014SepS6800m15
KY388190, 2012AugS6200m18
KP754310, 200m10, 200m, Portuguese continental margin
KY388179, 2012AugS6200m07
KY388259, 2012AugS6200m87
EU886032, S2000amoA24, 2000m, South China Sea
DQ148761, ETNP18, Eastern Tropical North Pacific
KY388287, 2014SepS6800m20
AB592024, kt075, 2000m, Suruga Bay
KF727142, GT2H7AF6, Mesopelagic, Atlantic Ocean
KY388227, 2012AugS6200m55
KF727098, GT2D9AF6, Mesopelagic, Atlantic Ocean
KY388185, 2012AugS6200m13
KF727076, GT2B6AF6, Mesopelagic, Atlantic Ocean
KY388317, 2014SepS6800m51
FJ615389, PU009500.D2+10, 500m, eastern South Pacific
KY388344, 2014SepS6800m78
DQ148769, ETNP26, Eastern Tropical North Pacific
KY388242, 2012AugS6200m70
KY388195, 2012AugS6200m23
KP754347, 1000m17, 1000m, Portuguese continental margin
KY388177, 2012AugS6200m05
LC087900, JTstEAamoA 200m27, 200m, Japan Trench
KF727064, GT2A6AF4, lower bathypelagic, Atlantic Ocean
KY388223, 2012AugS6200m51
KC596360, 2-250m_01, Gulf of California and ETNP
KY388278, 2014SepS6800m11
GU181794, 712200amoA43, 200m, East China Sea
KY388238, 2012AugS6200m66
KF727249, GT3182317, lower bathypelagic, Atlantic Ocean
KY388272, 2014SepS6800m05
KF727248, GT3182313, lower bathypelagic, Atlantic Ocean
KY388209, 2012AugS6200m37
AB592186, kt266, 500m & 2000m, Suruga Bay
KY388381, 2012AugS63000m15
KY388367, 2012AugS63000m01
EU886104, Z2000amoA32, 2000m, South China Sea
EU886176, Y2000amoA5, 2000m, South China Sea
KF727105, GT2E3AF4, upper bathypelagic, Atlantic Ocean
KY388304, 2014SepS6800m38
KF727201, GT31821164, upper bathypelagic, Atlantic Ocean
KY388312, 2014SepS6800m46
DQ148832, MBC130m14, Monterey Bay
KY388180, 2012AugS6200m08
KF970631, GB456421Arch23, deep-sea hydrothermal sediments
KY388428, 2012AugS63000m62
KJ549574, OTU5A46, Hydrothermal Plume
KY388297, 2014SepS6800m31
JQ396287, AOA OTU8, adjacent to the Southwest Indian Ocean Ridge
KY388396, 2012AugS63000m30
KY388274, 2014SepS6800m07
KY388309, 2014SepS6800m43
KF727221, GT31821758, Mesopelagic, Atlantic Ocean
AB591995, kt045, 500 or 2000m, Suruga Bay
KC857541, BW76, central Red Sea
KY388226, 2012AugS6200m54
KY388345, 2014SepS6800m79
GU181757, 712200amoA1, 200m, East China Sea
KY388241, 2012AugS6200m69
KY388239, 2012AugS6200m67
GU181777, 712200amoA21, 200m, East China Sea
KF970697, GB45686Arch47, deep-sea hydrothermal sediments
KY388175, 2012AugS6200m03
KY388224, 2012AugS6200m52
EU885995, S500amoA36, 500m, South China Sea
KY388286, 2014SepS6800m19
EU886096, Z500amoA7, South China Sea
KY388248, 2012AugS6200m76
KY388233, 2012AugS6200m61
KY388295, 2014SepS6800m28
KC349220, D5200mamoA03, 200m, Gulf of Mexico
KC349162, D5100mamoA27, 100m, Gulf of Mexico
KY388335, 2014SepS6800m69
DQ148828, MBC130m10, Monterey Bay
KY388191, 2012AugS6200m19
0.050

Figure S3. Unrooted neighbor-joining (NJ) phylogenetic tree of the archaeal *amo*A gene sequences (expanded view for group Ba and Bb (LAC)). Clone sequences from this study are shown in bold and sequences sharing 95% DNA identity are grouped. Phylogenetic relationships were bootstrapped 1000 times, and bootstrap values greater than 50% are shown. The scale bar indicates 5% estimated sequence divergence.

Comment [YZ7]: Group E belongs to Soil/sediment cluster. We added the cluster information in Figure S3. (RC2)