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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 Reviewer #1

We greatly thank the reviewer 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.

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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 *amoA* 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 *amoA* 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 *amoA* 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 *amoA* 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 *amoA* 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 *amoA* subgroups were similar to the archaeal *amoA* gene clone library analysis; the inconsistent pattern was present in the hadal zone (below 6000 m). Our clone library analysis of archaeal *amoA* 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 *amoA* 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.”*

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

3. The authors obtained archaeal *amoA* 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 *amoA* genes was identical to a primer set used for the conventional clone analysis. This generally allows complete match between archaeal *amoA* clone sequences and primer sequences in PCR reaction for obtaining standard curves. In contrast, the presence of few mismatch residues is expected between environmental *amoA* gene sequences and primer sequences in qPCR. The gap would be a reason for underestimation of archaeal *amoA* genes in environmental samples. In qPCR of archaeal *amoA*, 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 *amoA* 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 *amoA*, 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 *amoA* 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 *amoA* 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 *amoA* 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 *amoA* 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.”*

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.”

“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).”

“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).”

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.”

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).”

“Notably, all genes were significantly positively correlated to TSM concentrations in PA communities ($P < 0.05$ – 0.01 , Table S5).”

Deleted “This is consistent with the observation that all of the genes were significantly more abundant in the PA communities.”

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.”

“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.”

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.

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

Station	Water Depth (m)	Sampling Depth (m)	Archaeal <i>amoA</i> (copies L ⁻¹) FranAOA				Archaeal <i>amoA</i> (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.)

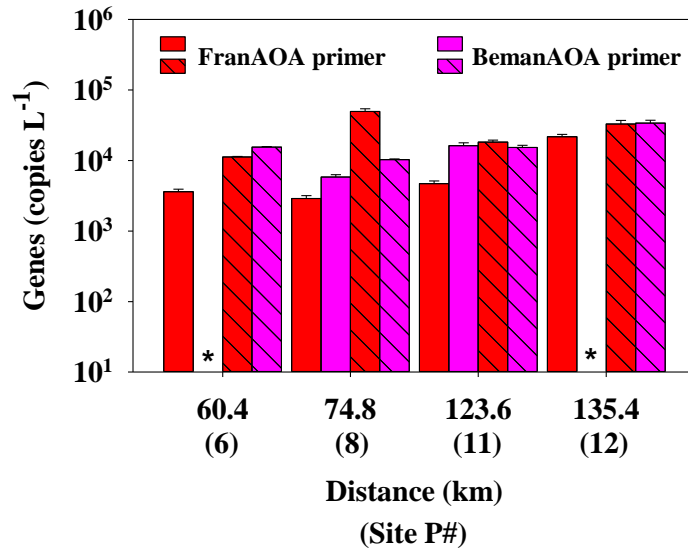


Figure R1. The archaeal *amoA* gene copies from six samples at sites P6 (upper reaches), P8 (middle reaches), P11 and P12 (lower reaches) of PRE using the two primer sets. *, No DNAs.

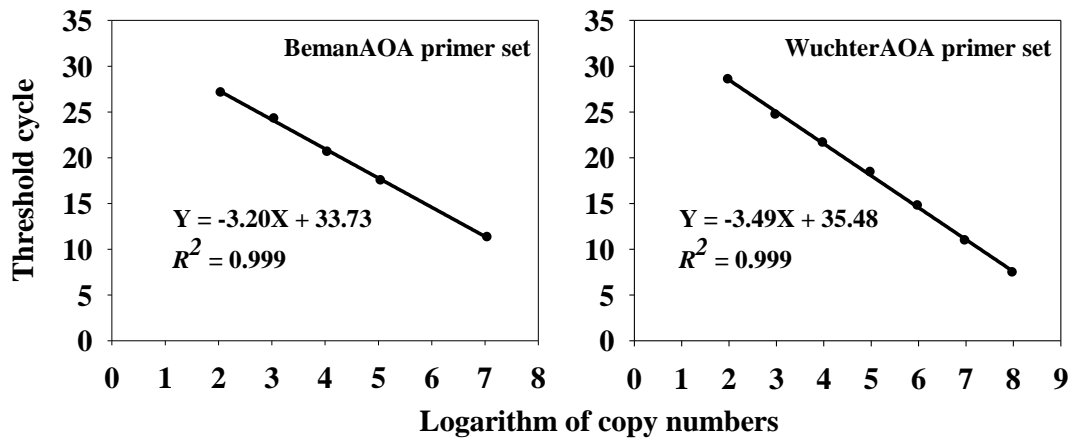


Figure R2. Standard curves for the BemanAOA and WuchterAOA primer sets qPCR measurements.

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.

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

Response:

Revised as suggested.

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.*”

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*.”

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.

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.

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.”

P8, L18-: Did the authors conduct any chimera check programs? It has been reported that more than 10% of the archaeal *amoA* 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.”

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.”*

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

Response:

Genomic DNAs of *Candidatus 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.

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

Response:

Thanks. Revised as suggested.

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.

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.

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.

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.*”

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

Response:

We revised “*ammonia levels*” as “*ammonia concentration/flux*”.

P25, L17: Appropriate references should be provided.

Response:

Added. Please see below.

Sintes, E., Bergauer, K., De Corte, D., Yokokawa, T., and Herndl, G. J.: Archaeal *amoA* gene diversity points to distinct biogeography of ammonia-oxidizing *Crenarchaeota* in the ocean, *Environ. Microbiol.*, 15, 1647–1658, 2013.

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.

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.

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

Response:

We added two citations. Please see below.

Lomas, M. W., and Lipschultz, F.: Forming the primary nitrite maximum: Nitrifiers or phytoplankton?, *Limnol. Oceanogr.*, 51, 2453–2467, 2006.

Merbt, S. N., Stahl, D. A., Casamayor, E. O., Martí, E., Nicol, G. W., and Prosser, J. I.: Differential photoinhibition of bacterial and archaeal ammonia oxidation, *FEMS Microbiol. Lett.*, 327, 41–46, 2012.

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

Response:

Revised throughout the references list.

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 (*nxB*) gene sequences of *Candidatus Nitromaritima* were included. Please see the revised Figure 5 (below).

