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Manuscript title: Major role of ammonia-oxidizing bacteria in N$_2$O production in the Pearl River Estuary

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 shown below.

Anonymous Referee #1

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Ma et al. investigated the relationship between N$_2$O production and spatial distribution of AOA and AOB along a salinity gradient in the Pearl River Estuary, China by using qPCR, chemical analysis and in situ incubation experiment. Data are well analyzed and presented. However, the manuscript's structure should be modified because the some results were presented in the discussion section, and some conclusions needs to be rephrased because the main findings in this study were mainly based on the correlation analysis OR statistical analysis (e.g., between N$_2$O production and the abundance of functional genes), which can't provide a solid support for a causal relationship between microbial contributors and N$_2$O production.
Response:

Many thanks for the reviewer's comments. We moved the results pointed out by the reviewer into the Discussion section. We also revised some conclusion sentences with the appropriate tone according to the reviewer's suggestions.

More specific comments and suggestions are given below:

1. As mentioned by authors, both nirK and nirS genes are the key functional genes in the denitrification pathway, so why did not determine the abundance of nirK gene here?
Response:

The *nir*S and *nir*K genes encode cytochrome cd1 and copper-containing nitrite

reductase, respectively. They were functionally and physiologically equivalent, but structurally different and could not be detected in the same strains in the previous research (Coyne et al., 1989), while the recent genomic analyses found a few bacteria contain both *nir*S and *nir*K (Graf et al., 2014). A recent genomic analysis revealed that a great many *nir*K-encoding bacteria have both denitrification and DNRA (Dissimilatory Nitrate Reduction to Ammonium) pathways (Helen et al., 2016). Furthermore, it was reported that *nir*S genes were more widely distributed than the *nir*K genes (Zumft, 1997; Bothe et al., 2000), and *nir*S genes were both more abundant and more diverse than *nir*K in the estuarine water columns (Zhu et al., 2018; Wang et al., 2019) and various estuarine sediments (Nogales et al, 2002; Santoro et al, 2006; Abell et al., 2010; Mosier and Francis, 2010; Beman, 2014; Smith et al., 2015; Lee and Francis, 2017). The previous study on the Pearl River sediment also showed that *nir*K abundance was much lower than *nir*S abundance (Huang et al., 2011). Therefore, *nir*S can be used to identify the distribution of denitrifiers in the PRE, suggesting the denitrification potential and indirectly indicating N_2O production.

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2. Page 7, line 18-19, make subscript for some chemistry formulas (N_2O, NH_3 etc.);

Response:

Revised as suggested.

3. Page 7, line 24, please correct the P value using the Bonferroni correction or other multiple-comparison methods;

Response:

Thanks for the reviewer's suggestion. As normal distribution of the individual data sets was not always met, we could not use Bonferroni correction to correct *P* value. So, we used the False Discovery Rate (FDR)-based procedures to identify truly significant comparisons, which has been considered as the best choice available in many studies of ecology and evolution (Pike, 2011). In the revised manuscript, we corrected the *P* value using the False Discovery Rate (FDR)-based multiple comparison procedures, and added the statement in the "2.5 Statistical analyses": "*False Discovery Rate (FDR)-based multiple comparison procedures were adopted to evaluate the significance of multiple hypotheses and identify truly significant results (FDR-adjusted P value) (Pike, 2011).*" The FDR-adjusted *P* values have been added in the Table 2.

Reference

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4. Page 7, line 25, and Fig. 5. Please check the multicollinearity problems before perform the RDA analysis. Some environmental parameters are highly correlated with each other, some of them should be removed from the RDA analysis;

Response:

Thanks for the reviewer's suggestion. We used the value of Variance Inflation Factor (VIF) to check the multicollinearity. The variables Temperature, Silicate, NH_3, NO_3^-, and pH had a high VIF (> 20) (Ter Braak et al., 1998; Ricart et al., 2010), so we removed these factors from the RDA analysis. We modified this part in the 3.3

subsection of the revised manuscript. —"*The RDA was used to further analyze variations in the relative abundances of ammonia oxidizers under the environmental constraints. The results confirmed that the relatively high AOB abundances in the upper estuary were constrained by low salinity water, high nitrite and TSM concentrations and low DO conditions, as well as high N_2O concentration; whereas the water with high salinity and opposite environmental conditions constrained the high AOA abundances in the Lingdingyang area (Fig. 5). These constraints explained 89.3% of the variation in the ammonia oxidizers distribution along the PRE.*" Please see the revised Figure 5 (below).

References

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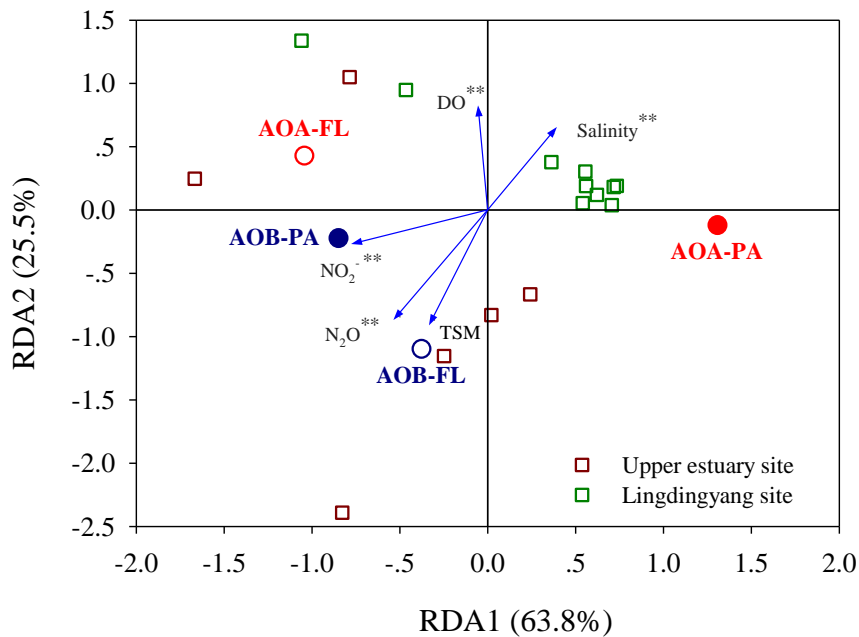
Figure R1 (Figure 5 in the MS): Redundancy analysis of the relative abundance of AOB-*amo*A and AOA-*amo*A under biogeochemical constraints. Each square represents an individual sample. Vectors represent environmental variables. [*]$P <0.05$, [**]$P <0.01$ (Monte Carlo permutation test).

5. Page 8, line 5-8 and Fig. 6. I am not convinced with the usage of Mantel and partial Mantel tests here due to two following reasons: 1) for ammonia oxidizer community, actually there were only four variables based on qPCR analysis (PA AOA, FL AOA, PA AOB and FL AOB) but not community data based on sequencing, so I don't think the results of qPCR reflected the truly community composition of ammonia oxidizers; and 2) the authors divided the environmental into four groups, but the classification seems a bit confusing. For example, why classify silicate into water mass but not substrate parameters? And TSM, DO and pH were classify as water mass parameters by numerous previous studies;

Response:
1) Sequencing-based community structure has higher resolutions than qPCR-based community structure. For community composition based on sequencing, the dissimilarity matrices were calculated with the relative abundance of OTUs (Operational Taxonomic Units). Similarly, for community composition based on

qPCR, the relative abundance of PA AOA, FL AOA, PA AOB, and FL AOB were used to calculate the dissimilarity matrices, just like merging some OTUs into one OTU. Despite lower resolutions of community composition, the dissimilarity matrices can be calculated and the Mantel and partial Mantel tests can be performed. Similarly, Castellano-Hinojosa et al. (2018) and Huang et al. (2011) also used qPCR data in NDMS analysis and CCA analysis of community structure.

2) Silicate has long been recognized as one of the most common indicators to trace river water in the ocean, and the low salinity and high silicate contents were the best indicators for river source (Moore, 1986). We added a three-dimensional scatter plot in the revised MS (Figure S1; see below) to show the relationships between Potential temperature (θ) (^{o}C), salinity, and silicate (SiO_3^{2-}) concentration. The waters from the upper estuary where the salinity of most sites was close to zero, had high potential temperature and silicate concentrations. The mixing behaviors of waters occurred at the Humen outlet (sites P07 and A01), and the waters from the off-shore sites (A10 and A11) had high salinity and low potential temperature and silicate concentrations. Therefore, we chose silicate, temperature, and salinity as the indicators to trace estuarine water masses and mixing.

We defined the substrate parameters as nitrogen substrates (ammonium, nitrite, and nitrate), which are related to the N_2O producing processes of nitrification and denitrification. TSM, DO, and pH are not conservative parameters and thus cannot trace water masses. These factors represent the biogeochemical characteristics of waters and could influence the availability of electron donors (or substrates) during nitrification and denitrification. For example, the suspended particles could be beneficial to microbial activity because of nutrients or substrates supply (Belser, 1979; Crump et al., 1998; Ouverney and Fuhrman, 2000; Teira et al., 2006; Zhang et al., 2014); DO concentration and pH also could influence the availability of ammonia, etc. (Geets et al., 2006; Ward, 2008; Martens-Habbena et al., 2009; Zhu et al., 2013; Huesemann et al., 2002; Hutchins et al., 2009; Fulweiler et al., 2010; Beman et al., 2011).
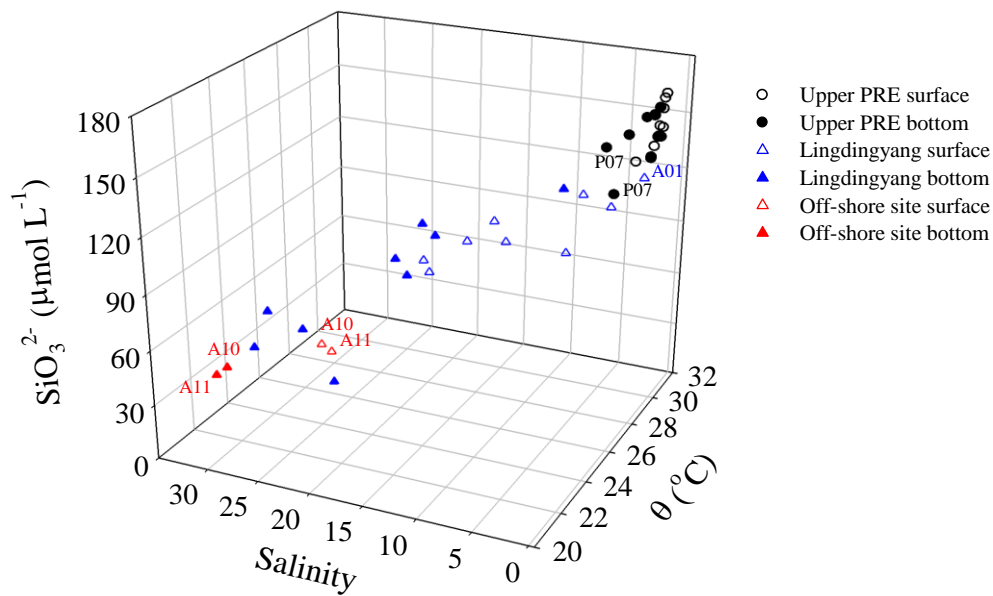
Figure R2 (Figure S1 in the MS): Three-dimensional scatter plot of Potential temperature (θ) (^{o}C), salinity, and silicate (SiO_3^{2-}).

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6. Page 8, line 20, is the 63.0 μmol/L the hypoxic threshold?

Response:

Yes, the DO concentration of 63.0 μmol L^{-1} (equaling to 2 mg L^{-1}) is the hypoxic threshold, which was cited from Rabalais et al. (2010).

Reference

Rabalais, N. N., D´ıaz, R. J., Levin, L. A., Turner, R. E., Gilbert, D., and Zhang, J.: Dynamics and distribution of natural and human-caused hypoxia, Biogeosciences, 7, 585–619, 2010.

7. Page 9, line 11, please re-phrase this subtitle because only the transcripts of *amo*A and *nir*S genes from two freshwater stations were quantified here;

Response:

We re-phrased this subtitle as "*Distributions of amoA and nirS genes along the salinity transect*" in the revised manuscript.

8. Page 12, line 12-13, too much speculation;

Response:

We deleted this sentence.

9. Page 12, line 15-26, please move this part into Results section, and again, I don't think the classification for environmental parameters is on the right way;

Response:

This part was moved into the Results section as suggested by the reviewer. As for the water mass parameters and the parameters influencing substrate availability, please refer to our response above.

10. Page 12, line 23, "positive correlations between AOB *amo*A abundances and all N_2O parameters", should be except for FL AOB;

Response:

This sentence was revised as "*Notably, there were positive correlations between AOB amoA abundances and all N2O parameters as well as ammonia concentration (Table 2; P <0.05−0.01), except for the FL AOB communities, suggesting a significant influence of AOB on N2O production*".

11. Page 12, line 27, the results of RDA analysis also should be presented in Results section;

Response:

We moved this part into the Results section according to the reviewer's suggestion (3.3 subsection).

12. The most part of first paragraph of 4.3 subsection should be moved into Results section;

Response:

The descriptions on Figure 7 in the original 4.3 subsection were moved into the Results section (3.5 subsection).

13. How about the potential role of comammox and *nir*K-type denitrifier for N_2O production in PRE, please discuss it in the 4.3 subsection.

Response:

Thanks for the reviewer's suggestion. We added this discussion in the 4.3 subsection.
—"*The complete nitrification activity in a single organism (comammox) was newly discovered in terrestrial systems (Daims et al., 2015; Santoro, 2016; Kits et al., 2017). Given the similarity of ammonia oxidation pathway to that of classic AOB, it is possible that comammox may be also involved in N_2O production (Hu and He, 2017). It has been further reported that the comammox Nitrospira inopinata has a low N_2O yield due to the lack of NO reductases and N_2O formed by N. inopinata originates from abiotic conversion of hydroxylamine, indicating that comammox microbes may produce less N_2O during nitrification than AOB (Kits et al., 2019). However, comammox has not been observed to be widespread in the estuary waters.*"
"The *previous study in the Pearl River sediment detected both nirK and nirS, however, the nirK gene abundance was much lower than nirS abundance as nirK only prevails in conditionally oxygen-exposed environment (Huang et al., 2011). Recent studies proposed that nirK-type denitrifiers were responsible for N_2O production despite being less abundant than nirS denitrifiers (Maeda et al., 2017). NirS-type denitrifiers are more likely to be capable of complete denitrification, because the nosZ gene had a*

significantly higher frequency of co-occurrence with nirS than with nirK, and thus contribute less to N$_2$O emissions than nirK-type denitrifiers under favorable environmental conditions (Graf et al., 2014). So far, there is no direct evidence that denitrification or nitrifier-denitrification pathways contribute much to N$_2$O production in the PRE water column, but a release of N$_2$O into the overlying waters through denitrification was reported in the PRE sediments (Tan et al., 2019). It was possible that the nirK-type denitrifier contributed to N$_2$O production from the interface between sediment and water. Further study is needed to clarify the potential of nirK-type denitrifier in N$_2$O production in PRE."

References

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14. Fig. 7. It is a little difficult to understand this figure. It seems like the AOA contributed more for N_2O production and yield in site P01, right?

Response:

We attempted to accurately assess the relative contributions of AOA and AOB to N_2O production in the PRE by plotting the N_2O production rates (Fig. 7a) and yields (Fig. 7b) normalized to total AOA and AOB *amo*A gene copies (sum of PA and FL fractions or only PA fractions) or transcripts (only PA fraction) along X-Y axes that represent the relative contributions of AOA and AOB to the total *amo*A gene or transcript pools. For both incubation sites, the more abundant AOB were in the *amo*A gene-based DNA or cDNA pool, the distinctly higher (disproportionately higher relative to enhanced abundance) the average *amo*A gene copy or transcript-specific N_2O production rates (Fig. 7a) and yields (Fig. 7b), suggesting that AOB may have higher cell-specific activity in the upper estuary and thus be more active in producing N_2O than AOA.

15. Table 2, Spearman rank correlation analysis generate a rho () value rather than a R value.

Response:

Sorry for this mistake. We revised "R" as "rho (ρ) ".