Dear Reviewer,

We appreciate your constructive suggestions that have led to an improvement of the manuscript. We have fully addressed these comments during the revision. To assist your assessment of our revised manuscripts, we have provided point-to-point response (**blue in color**) to each of the comments by reviewers below. The location of the change in the revised manuscript is highlighted in our response.

Sincerely yours,

Dr. Hongbin LIU (Corresponding author, Email address: liuhb@ust.hk)

Response to review 2:

I feel that this manuscript contains valuable information regarding ammonia oxidizing archaea in estuarine systems, particularly in that it focuses on processes occurring in the water column rather than the sediment, which, as the authors point out, is understudied. However, there are numerous issues with the manuscript in its current form.

Response: *We thank the reviewer for the comments.*

First and foremost, there are serious issues throughout the manuscript with grammar and syntax. Sometimes these issues are so severe that they obscure the meaning of the text. This made it difficult to grasp the authors' meaning and to review the manuscript effectively.

Response: *We thank the reviewer for the comments. We have improved the manuscript by reducing the grammar and syntax as well as following the important suggestions from the reviewer. We have also added detailed information into the method section. We hope that the current version is much clearer.*

In general, the description of the methods is unclear and lacking in detail.

For example: line 78: "the 10-50m by 10m interval" What does this mean?

Response: *We removed "by 10m interval" for the clarity of the station design. The current version is "In the first leg, 83 stations were designed within the 10-50m isobaths covering from the upper estuary to the continental shelf"* Page 4 Line 76-77

lines 87-89: "Sea water was prefiltered... analysis (Liu et al. 2014)." Which analysis was this performed for?

Response: *This sentence described flow cytometry (for microbial cell abundances) sample preparation. For clarity, the current version is "Seawater for microbial abundance quantification was prefiltered by a 20 μm mesh, fixed with final concentration of 0.5 % seawater-buffed paraformaldehyde in cryotubes, and stored in liquid nitrogen until flow cytometric analysis (Liu et al. 2014)."* Page 4 Line 86-88

line 93: "Community respiration rates were measured" in what? Microcosms? Incubations are

mentioned but no volume is given, whether a headspace was left in the bottle...

line 94: "running seawater" Outside the (unmentioned) bottle?

Response: *We have added the corresponding information of community respiration measurement. The running seawater was used to control incubation temperature. The current version is "Community respiration rates (CR) were measured in triplicate in 60ml BOD bottles without headspace through the dissolved oxygen variance before and after 24 h dark incubation submerged in seawater continuously pumped from sea surface"* Page 5 Line 93-111

line 95: "less 10%" Does this mean "less than 10%"?

Response: *Yes. It was revised to "less than 10%".* Page 5 Line 95

line 96: "The del-15N in NO- x the product of nitrification" I have no idea what this means.

line 97: "denitrifier method" What is that? The authors provide citations but for methods but do not explain what they are or how they are performed. Similarly the measurement of the nitrification rate is not described, only cited in an unpublished manuscript.

Response: *We have added the detailed information of nitrification measurement in the revised manuscript. The current version is "Nitrification were measured by incubating $^{15}NH_4^+$ amended (less than 10 % of ambient concentration) seawater in duplicated 200 ml HDPE bottles in dark for 6-12 h, with temperature controlled by running seawater. After incubation, filtrate (0.2 μm-syringe-filtered) was collected and stored in -20 ℃ for downstream $^{15}NO_x^-$ ($^{15}NO_3^- + {}^{15}NO_2^-$) analysis (Sigman et al. 2001).*

The nitrification rates were calculated using the following equation:

$$AO_b = \frac{(R_t NO_x^- \times [NO_x^-]_t) - (R_{t0} NO_x^- \times [NO_x^-]_{t0})}{T} \times \frac{[{}^{14}NH_4^+] + [{}^{15}NH_4^+]}{[{}^{15}NH_4^+]} \qquad (1)$$

In equation 1, AO_b is the bulk nitrification rate. $R_{t0} NO_x^-$ and $R_t NO_x^-$ are the ratios (%) of ^{15}N in the NO_x^- pool measured at the initial (t_0) and termination time (t) of the incubation. $[NO_x^-]_{t0}$ and $[NO_x^-]_t$ are the concentration of NO_x^- at the initial and termination of the incubation, respectively. T is the incubation time. $[{}^{14}NH_4^+]$ is the ambient NH_4^+ concentration. $[{}^{15}NH_4^+]$ is the final concentration after addition of the stable isotope tracer ($^{15}NH_4^+$). The NO_x^- was

completely converted to N_2O by a single strain of denitrifying bacteria (Pseudomonas aureofaciens, ATCC#13985) which lack N_2O-reductase activity (Sigman et al. 2001). The converted N_2O was further analyzed using IRMS (Isotope Ration Mass Spectrometer, Thermo Scientific Delta V Plus) to calculate the isotopic composition of NO_x^-. (Sigman et al. 2001; Casciotti et al. 2002; Knapp et al. 2005).” Page 5 Line 93-111

lines 110-111: "Fast DNA SPIN Kit for Soil" Why would you use a soil kit for filter samples from seawater?

Response: *Our samples spanned from highly turbid riverine water to oceanic waters. For better purification and consistency of our DNA samples, we used the “Fast DNA SPIN kit for Soil”. We have used this kit in previous studies, and it works well with plankton samples, so the name of the kit is a bit misleading.*

line 117: "transpired" I assume you mean "transferred"

Response: *Yes. We have revised it into “transferred”.* Page 6 Line 123

line 136: "the DNA mixture" I don’t know what is meant by this. DNA and cDNA?

Response: *The DNA mixture consisted of 28 DNA samples from 7 stations along A-transect (A01, A05, A09, A11, A12, A14, A16). The DNA mixture here used as a template for clone construction. We want an amoA clone generated from the local community to reduce the dissimilarity between our standard curve and samples.*

Because the methods were so unclear in general, it is difficult for me to assess whether the claims made in the results and discussion sections are to be believed. For example, AOA and AOB copy numbers are referred frequently as evidence of dominance of one group over the other. Is this a rational claim, particularly without 16S data to support it? How many copy numbers of the amoA gene do AOA have vs AOB? And if archaeal amoA transcripts are more abundant than bacterial amoA transcripts, does that mean the archaea are more abundant or simply more active? Is the difference is gene/transcript number statistically significant?

Response: *The amoA gene copies in AOA is one while it is 2-3 copies in AOB* (Norton, et al.

2002, Hallam et al. 2006). *At DNA level, as the amoA gene abundances of AOA in this study were orders of magnitude higher than AOB, we assumed that AOA should be the dominant ammonia oxidizers (Table S2). On the transcript (RNA use cDNA as template) level, we also performed qPCR. We found that AOA were detectable while AOB were under our detection limit (Table S4). Although we cannot rule out the nitrifying activities of AOB by our method, the current evidences supported that AOA is dominant and active in our study.*

As for the measurement of nitrification rates, so little detail is given regarding how these numbers were reached, as to render the data meaningless. The sections on spatial distribution were in general unclear and difficult to follow.

Response: *We have elaborated the nitrification method.* Page 5 Line 93-111

More specific comments:

line 223: "B-proteobacteria amoA were under detection limit" Not in all your samples though, judging by Figure 5?

Response: *It is not judged by figure 5. The figure 5 only displayed the size fractionated amoA gene abundance along the A-transect on DNA level. The "under detection limit" is specified for cDNA level in the original sentence. We performed qPCR for both AOA and β-proteobacterial amoA gene abundance using cDNA (represent the RNA level) as template. The data were listed in Supplementary Table S4. Using cDNA as template, we found β-proteobacterial amoA gene abundance were under the detection limit (Table S4).*

line 257: "Besides" Besides what? What is meant by this?

Response: *We have removed "Besides" for clarity.* Page 11 Line 266

line 270: "heterotrophic bacteria abundance" How was this determined? It's not described in the methods.

Response: *We had used the term for all non-phototrophic (no-pigmented) microbial cells in flow cytometric analysis. We admit that flow cytometry method cannot distinguish autotrophic non-phototrophic microbial cells. We have changed "heterotrophic bacteria" into "non-*

phototrophic prokaryotic cells" with abbreviation "NPC" in the figure legend in Figure 10. Page 33 Line 663-667; Page11 Line 279; Page 15 Line 401-402

lines 271-272: "Nutrient concentration showed an opposite pattern comparing with salinity" I have no idea what this means.

Response: *We intended to give a general description of the correlation between AOA sublineages and nutrients. Nutrients in PRE were associated with the freshwater discharge. To be clearer, we have revised the sentence as the follow: "In general, WCA sublineages were negatively correlated with nutrient concentration, while SCM1-like sublineages were positively correlated with nutrient concentration."* Page 11 Line 280-281

line 274: "which may be introduced by" Again, no idea.

Response: *We have revised the sentence to "Ammonium showed no significant correlation with AOA sublineages."* Page 11 Line280-282

lines 295-296: "Intensive nitrification... oxygen consumption (Pakulski et al. 1995)." Was that observed in this study or in the study cited?

Response: *It is observed in the cited study. The current version is "Intensive nitrification was observed at intermediate salinities, and it accounted for 20 to over 50 % of oxygen consumption in the Mississippi River plume (Pakulski et al. 1995)"* Page 12 Line 302

lines 300-301: "It is well known... organic matter degradation (respiration)." Be that as it may, you still have to cite it- and it's hardly proof that ammonia is supplied to nitrification by this process.

Response: *We added the citation of paper "Nitrification and ammonification in aquatic systems" (Ward 1996).* Page 12 Line 310

line 305: 229.21% oxygen consumption? How do you consume more than 100% of something in a closed microcosm?

Response: *This may be caused by the methodological difference in the two measurements.*

Nitrification oxygen consumption were estimated via equation 2 ($NH_3 + 1.5O_2 \rightarrow NO_2^- + H_2O + H^+$). Nitrification in this study are measured in HDPE bottle while community respiration rates were measure in BOD bottles without headspace. We only have one data point at station F701 that exceeding 100%. Similar situation was also observed in Nueces estuary (Yoon and Benner, 1992) and Chang Jiang estuary (Hsiao et al. 2014). Although the unreasonably high NOD/CR ratio might be caused by the underestimated community respiration rates under low oxygen condition (Sampou and Kemp 1994), it showed the potential effect of active nitrification on oxygen consumption in the estuarine system suffered by hypoxia. We have discussed the issue in section 4.1. The oxygen limitation was rather strong for community respiration than nitrification activities (in Section 4.1). Thus, we considered that oxygen consumption via nitrification may contribute to hypoxia formation in the bottom waters.

lines 328-329: "Though size-fractionated... were observed." I don't understand what is meant here.

Response: *It was a typo, and we mean "Through". We performed qPCR of the size-fractionated (PA-Particle-attached (>3μm) and FL-Free-living (3-0.2μm)) samples. The amoA gene abundances were listed in table S2. Furthermore, figure 5 displayed the amoA gene abundances of the sized-fractionated samples along the A-transect with an increasing salinity gradient. Our result showed differential distribution of the two group of ammonia oxidizers with AOA more abundant in the free-living fraction while AOB more abundant in particle attached fraction and distributed near the upper estuary. We added the citation of figure 5 and Table S2.* Page 13 Line 336-337

line 330: "higher substrate requirement" of what substrate?

Response: *The substate here means "ammonia". We have revised it. Current version is "..higher substrate (ammonia) concentration requirement…".* Page 13 Line 339

In multiple locations in the document the authors mention previous DNA-based studies of AOA and how such studies may overlook active AOA populations. To begin with, those populations would not be overlooked, but perhaps underrepresented in the data. Additionally, several

culture-independent studies of AOA activity utilizing stable isotope probing (in particular, the use of urea as a substrate, and heterotrophy) have been performed in both salt marsh sediment (Seyler et al., 2014, ISME J) and the open ocean (Seyler et al., 2018, FEMS Microbiol Ecol; Seyler et al., 2019, Frontiers Mar Sci), and none of these studies are cited in the text. AOA activity has also been previously described in an estuarine water column using similar techniques to this manuscript (Horak et al., 2013, ISME J; Happel et al., 2018, Env Microbiol)- these should be cited in the text.

Response: *We thank the reviewer for these suggestions. We have revised the statement of "overlooked" or "neglected" into "underrepresented". We have added the citation of Seyler's and Happel's work in the revised manuscript. We have added the citation of Horak's and Happel's work in the revised Table S1.*

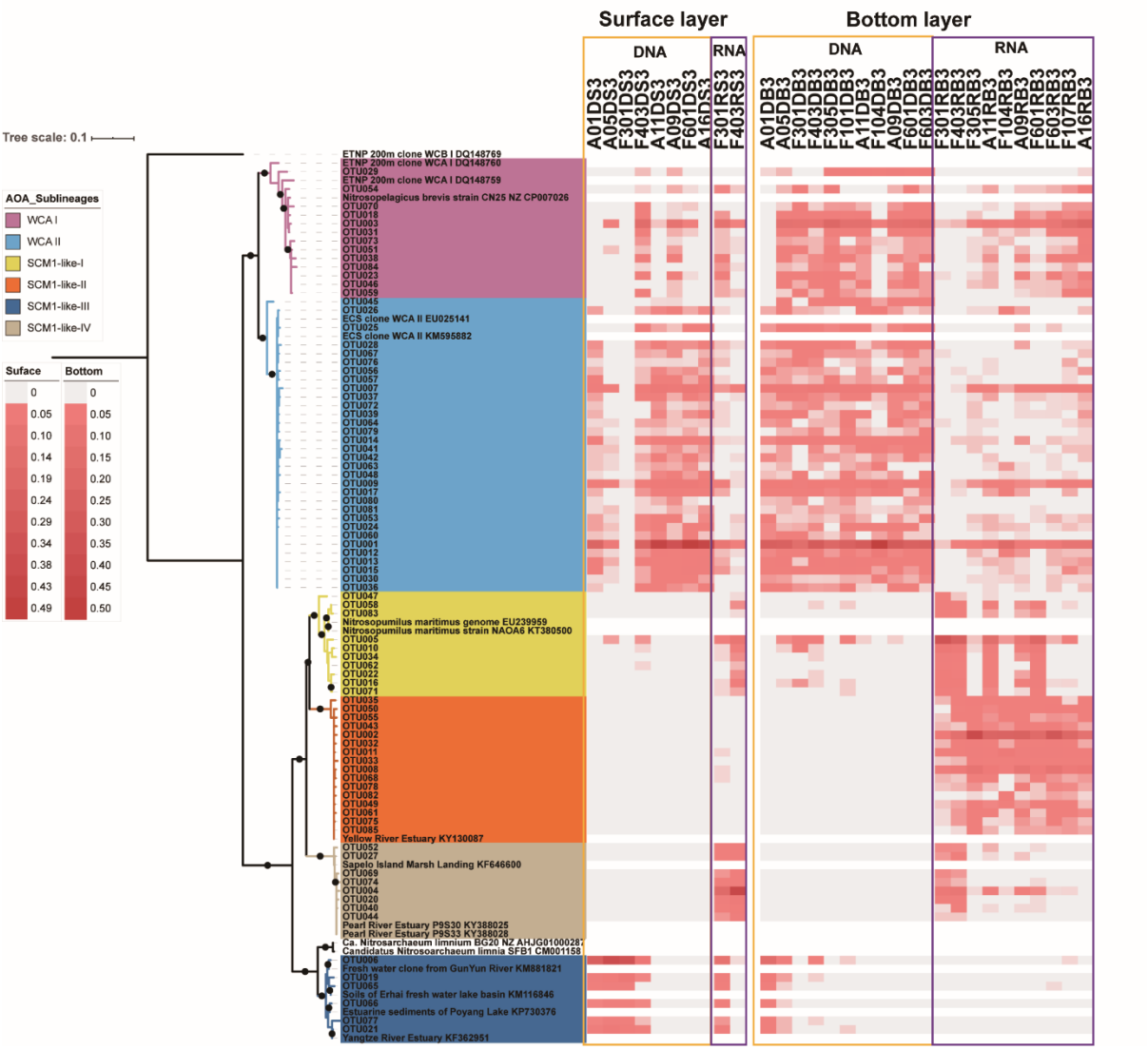
We have cited Seyler's work by adding "Using the stable isotope probing technology, the utilization of organic matter provided evidences of heterotrophy of AOA in the salt marsh sediment and oceanic environment (Seyler, et al. 2014; Seyler et al. 2018; Seyler et al. 2019)." Page 14 Line 395-397.

We have cited Happel's work by adding "In Baltic sea, a distinct AOA community were retrieved from RNA level and a few phylotypes related to Nitrosomarinus showed widespread expression in the coastal region (Happel et al. 2018)." Page 13 Line 350-351.

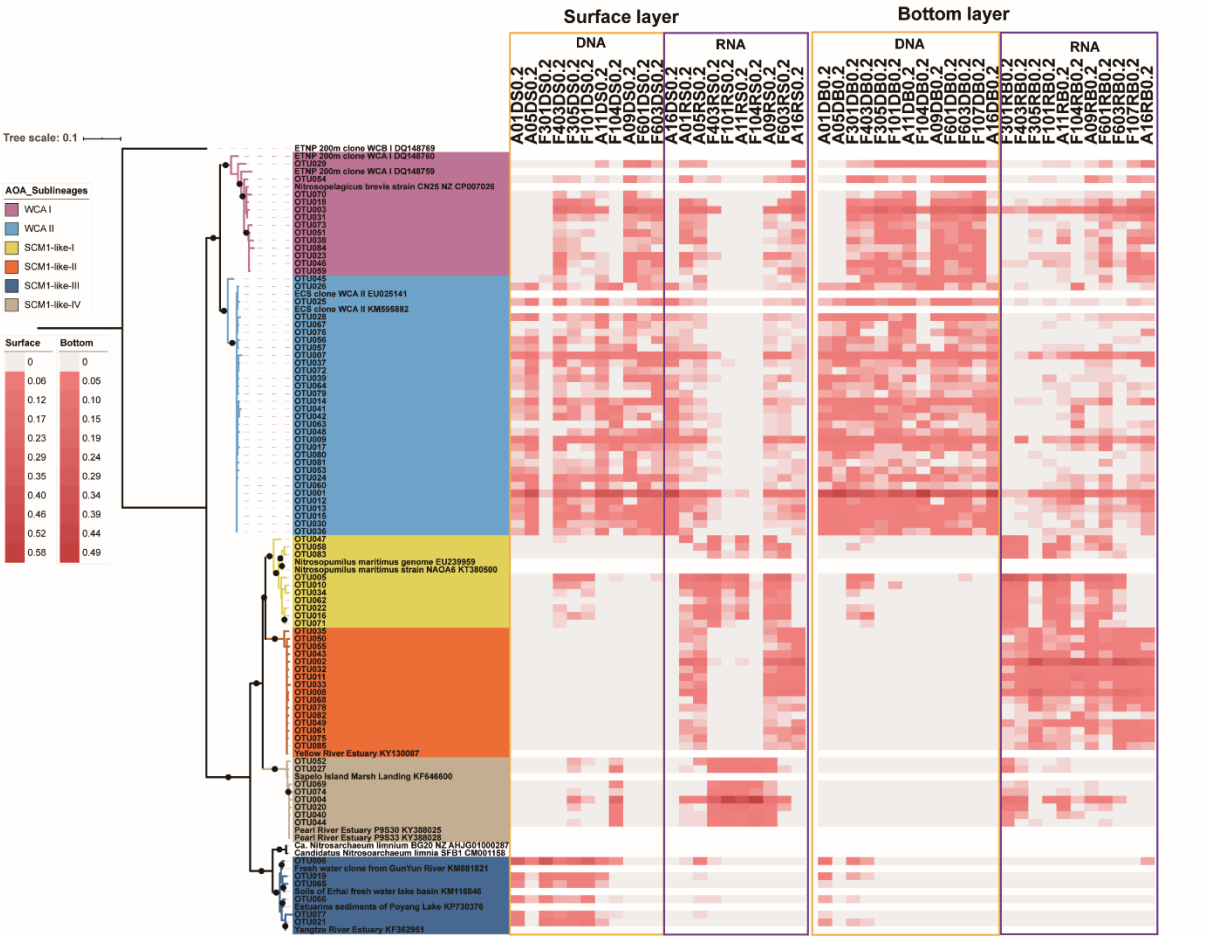
As for the figures:

Figure 6 is impossible to read. Could it be separated into two figures by size fraction? Otherwise there's just too much going on.

Response: *The figure 6 displayed the phylogenetic relationship of top OTUs together with their distinct distribution among samples in the heatmap at both DNA and RNA level. As for the more specific information about the size-fractionated community, we have also displayed in figure 8 by two separated figures. Here, we make a new version for your reference (Figure 6 & 7 below): New Figure 6: Phylogenetic tree and relative abundance (heatmap) of particle attached AOA. New Figure 7: Phylogenetic tree and relative abundance (heatmap) of free-living AOA. Here, we have split original figure 6 into two figures: new figure 6 and new figure 7. The rest of figure legends in the main-text were revised correspondingly.*



(Revised) Figure 6 Maximum likelihood phylogenetic tree of top 85 OTUs based on amoA gene sequences using T92+G+I model with 1000 bootstrap. The associated heat map is generated based on the relative abundance of top OTUs in the particle-attached samples. Samples are listed from left to right along the ascending salinity gradient.



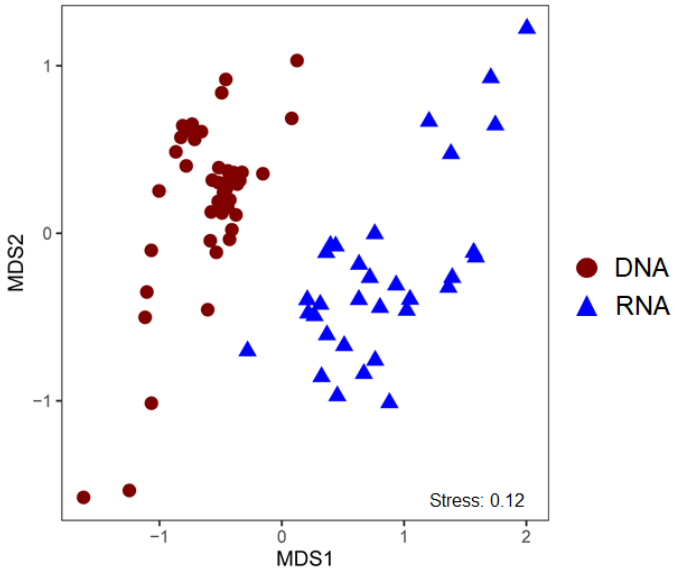
(Newly added) Figure 7. Maximum likelihood phylogenetic tree of top 85 OTUs based on amoA gene sequences using T92+G+I model with 1000 bootstrap. The associated heat map is generated based on the relative abundance of top OTUs in the free-living samples. Samples are listed from left to right along the ascending salinity gradient.

Figure 7 has me completely puzzled. Firstly because the figure has no axes or scale. Secondly because there's no description of how NDMS analysis was performed in the text. But most importantly, how is it possible that there is absolutely no overlap between the DNA and RNA sequences? I find this incredibly difficult to believe. Are the DNA and RNA sequence data even capturing the same community?

Response: *Figure 7 is NMDS plot generate using Primer 5 (Primer-E-Ltd, PML, UK). The input data was the community composition of 76 samples (OTU table, i.e. relative abundance). The community dissimilarities matrix was calculated using "Bray-Curtis dissimilarity". Thus, the dissimilarity between samples were introduced by compositional difference (different relative abundance of each OTU across all samples). As for the sequence data, for example, the heatmap in figures 6 and 7 has showed the relative abundance of WCA sublinseages*

presented in both DNA and RNA samples. So, there are shared OTUs in these samples. The archaeal amoA sequencing samples for DNA and RNA (using cDNA as template) were amplified using same primer pair under same conditions and thermal cycles (Francis et al., 2005). The highly dissimilar community composition retrieved from DNA and RNA as well as the differential distribution AOA sublineages is one of our key findings.

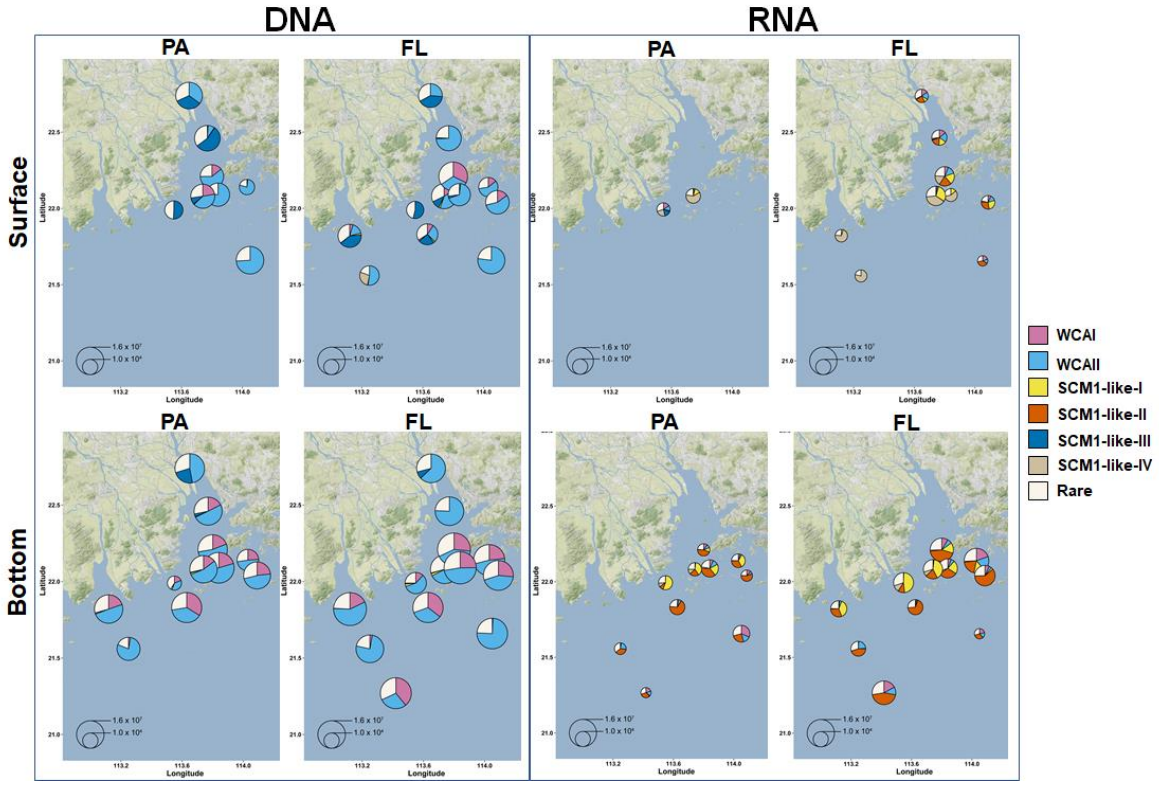
The previous version generated by Primer 5 cannot show axis information. The current version was generated by R via package "vegan" and "ggplot2" (Oksanen, et al. 2019; Wickham, 2016). The method of NMDS plot has been added into Page 7 Line 174-177*. This figure is now figure 8 in the revised main text after splitting figure 6 into new figure 6 and new figure 7 according to your suggestion.*



(Revised) Figure 8. Nonmetric multidimensional scaling (NMDS) plot of AOA community similarity at DNA and RNA level.

Figure 8 I think is very interesting, but some of the pie charts are so small as to be illegible.

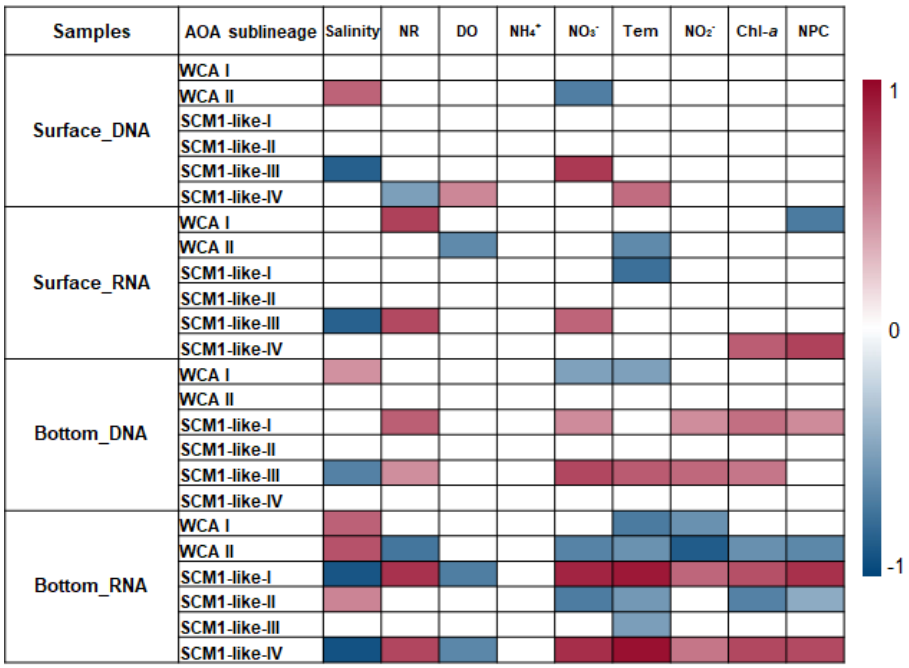
Response: *The revised version is added into the revised manuscript and showed below. The pie charts are enlarged. This figure is now figure 9 after splitting figure 6.*



(Revised) Figure 9. Free-living and particle-attached AOA community composition and distribution in the Pearl River estuary. The size of the pie charts represents the archaeal amoA gene abundance quantified by qPCR. For a clear display of the AOA community composition, the minimum size of the pie charts is set as 500 copies·L^{-1}. The charts were overlaid on Google Maps (© Google Maps) images using "ggmap" with "ggplot" in R (D. Kahle and H. Wickham, 2013)

Figure 9 contains some of the most interesting data in the paper, but the figure needs improvement. I think you could combine this heatmap with your phylogenetic tree, and move Figure 6 to supplemental.

Response: *We have followed the suggestions for figure 6 and the figure 9 were replaced with corrected one. Figure 9 is now figure 10 in the revised main text after splitting figure 6.*



(Revised) Figure 10. Spearman correlation between AOA sublineages (relative abundance at DNA and RNA levels) and environmental factors in the surface and bottom layers of the water column in the Pearl River estuary during summer 2017. Only the significant correlations (P<0.05) are displayed (NR-nitrification rates; DO-dissolved oxygen; Tem-Temperature; NPC-non-phototrophic prokaryotic cells).

Overall I believe the findings presented in this manuscript are likely of interest to the community. The correlations of various AOA lineages to geochemical data and sampling location are very interesting, if difficult to parse in the manuscript's current format. But the issues with the methods in particular and the text in general made it difficult to understand the findings, and some of the claims lack sufficient evidence. I would very much like to see this manuscript again, after significant revisions.

Response: *We thank the reviewer for all insightful and helpful comments. We hope the revised manuscript can meet the standard for publication in Biogeosciences.*

Reference:

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