Response to reviews #2

I have carefully read the manuscript "Potential contributions of nitrifiers and denitrifiers to nitrous oxide sources and sinks in China's estuarine and coastal areas" by Dai et al. The manuscript describes the spatial distribution and abundance of marker genes and transcripts related to the production and consumption of nitrous oxide in four estuaries in coastal China. Moreover, the diversity of the clade II-type *nosZ* gene was further investigated along the same four estuaries. The manuscript is nicely written and provides valuable information on the potential mechanisms controlling nitrous oxide consumption/production in coastal China. Furthermore, the results are put in context by using water column physicochemical data and by previously published measurements of nitrous oxide fluxes in the same four estuaries. I have, however, the following minor comments on this manuscript:

Response:

Thank you very much for taking the time to review our manuscript. The point-by-point reply to the comments are in blue color as below.

Line 39: add "the" before nitrification.

Response:

Thank you. We have added it.

In the introduction, between lines 52-59 the authors describe the physiology/ecology of microorganism possessing the clade II-type *nosZ*. How is that different from microbes containing the clade I-type?

Response:

Thank you for the comment. We have added the relevant statements on the calde I-type *nosZ* in the revised manuscript.

"*The microorganisms possessing clade I-type nosZ genes are mainly affiliated with alpha-, beta-, and gamma-proteobacteria, and the clade I gene has a higher frequency of co-occurrence with nir and nor genes than the clade II gene. The nosZ clade II genes are present in a much larger range of archaeal and bacterial phyla (Jones et al., 2013), and intergenomic comparisons have revealed that more than half of the microorganisms possessing clade II genes lack nitrite reductase or nitric oxide*

reductase, do not produce N_2O, and thus are expected to drive potential N_2O sinks (Graf et al., 2014; Jones et al., 2008; Marchant et al., 2017; Sanford et al., 2012)."

Check verb tense in the introduction. Usually, present tense is used.

Response:

Thank you! We have carefully checked the verb tense and revised many of them into the present tense in the introduction.

In material and methods please provide the depths from which samples were collected.

Response:

The depth ranges from which samples were collected were provided in the revised manuscript. The depth for each sample can be found in supplementary Table S1.

Lines 125-126. What minor modifications?

Response:

Thank you. The modifications have been elaborated in the revised manuscript.

"*DNA from water samples was extracted using the phenol-chloroform-isoamyl alcohol method (Massana et al., 1997) with minor modifications to maximize the DNA output. Briefly, tubes containing shredded filters, approximately 0.5 g of 0.1 mm glass beads, and 800 µL of STE lysis buffer (0.75 M sucrose, 50 mM Tris-HCl, 40 mM EDTA) were first agitated for 60 s on a FastPrep machine (MP Biomedicals, Solon, OH, USA) at 4.5 m s^{-1}. Then, the mixture was processed with lysozyme (1 mg ml^{-1}), proteinase K (0.5 mg ml^{-1}), and sodium dodecyl sulfate (SDS) (1%) sequentially. At last, the lysate was extracted twice with phenol-chloroform-isoamyl alcohol and once with chloroform-isoamyl alcohol. DNA was precipitated with isopropyl alcohol and washed with 75% ethyl alcohol before dissolved in 50 µL sterile water.*"

Was the same qPCR program (lines 178-180) used for all primer sets?

Response:

We are sorry for the unclear statements. Different qPCR programs were used for each primer set and they were mentioned in supplementary Table S2. We have revised the relevant statement in the revised manuscript. —"*All specific primer sequences, reactions, and programs for qPCR/PCR used in this study are shown in Table S2.*"

Among the six functional genes, only the qPCR program for the primer set designed by this study for the clade II-type *nosZ* gene quantification was shown in the main text (lines 178-180).

Line 199: What characteristic of the community? (i.e., community assembly or structure?)

Response:

Thank you! We have revised "community" as "*community structure*".

Please add units to salinity values (ppt, I guess?)

Response:

Yes, the unit to salinity is ppt or ‰. We have added.

When reporting the qPCR/RT-qPCR copy numbers, it is nice that the authors provided the range for each site. However, the median could also be informative, since the extremes may be outliers.

Response:

Thank you for the suggestion. The medians of the qPCR/RT-qPCR copy numbers for each site have been added in supplementary Table S3. Basically, nearly no outliers were used.

Line 241: Orders of magnitude?

Response:

Thank you. Corrected.

Line 283: It was not clear to me what did the authors mean by "sequencing coverage"?

Response:

Sorry for the unclear statement. We have revised "The sequencing coverage for each clone library …" as "the coverage of each lone library ….". We also have added the calculation equation in the Methods section —"*The coverge (C) of each clone libarary was calculated by C = 100% [1 – (n / N)] (Mullins et al., 1995), where n is the number of unique OTUs and N the total number of clones in a library.*"

Line 311-313. NMDS is only a visualization approach, I think the authors measured the similarity level by performing pairwise comparisons of the Bray Curtis dissimilarity index.

Response:

Thank you. The descriptions have been modified in the revised manuscript.

"*...at a >10% Bray-Curtis similarity level*"

"*...at a >3% Bray-Curtis similarity level*"

In the discussion, as well as in the introduction, when describing previous literature, the present tense is usually preferred.

Response:

Thank you! We have carefully checked the verb tense and revised many of them into the present tense throughout the introduction and discussion sections.

Lines 374-376: Authors mention that the *nosZ* gene/transcript abundance was correlated with pH. Could it also be a confounding effect of DIN concentration, since pH seems to have a similar spatial gradient as DIN, with higher pH and lower DIN in the open ocean (Fig. 2).

Response:

Thank you for the comment. When redundancy analysis (RDA) was performed, the collinearity between environmental parameters had been excluded (variance inflation factors > 10; Palacin-Lizarbe et al., 2019). It means that in theory, the correlation observed between gene/transcript abundance and pH was not caused by collinearity between pH and DIN concentration. However, indeed the *nosZ* gene/transcript abundance also correlated with DIN according to Fig. 5a and b. We have added the relevant discussion in the revised manuscript.

"The nosZ genes and transcripts showed significantly negative correlations with nitrate and/or nitrite (Fig. 5a and b), and similar correlations were also found in mountain lake habitats (Palacin-Lizarbe et al., 2019). It is possible that high abundances of nosZ gene and transcript lead to high consumption of nitrate and nitrite. In addition, it was reported that the presence of nitrate can inhibit the reduction of N_2O *to* N_2 *(Blackmer and Bremner, 1978)."*

As I mentioned above, it is a strength of the manuscript to use N_2O flux, and deltaN_2O data to put in context the results. However, greater background/information on where, when, and how that data was collected would be helpful to the reader.

Response:

Thank you very much for the suggestion! A supplementary Table S5 has been added to the supplementary materials, providing information on where, when, and how the data was collected. The relevant statements have also been added to the main text.

"*To assess how community structure controls the regional N_2O source or sink potential across China's estuaries, we collected the data on N_2O concentration, N_2O flux, and ΔN_2O in the four estuaries from the literature, covering January to November from 2002 to 2015 (Table S5; Chen et al., 2008; Lin et al., 2016, 2020; Ma et al., 2019; Song et al., 2015; Wang et al., 2014, 2016; Wu et al., 2013; Xu et al., 2005; Zhan et al., 2011; Zhang et al., 2008, 2010), and analyzed their relationships with the six functional gene distributions.*"

As reviewer 1 mentioned, I also wonder why the authors decided to use the gene abundance, and not the transcript abundance to correlate it with N_2O flux and deltaN_2O.

Response:

Thank you! According to your suggestion, a supplementary Fig. S4 presenting the transcript data (see below) has been added to the supplementary materials and the citation of Fig. S4 has been added in the revised manuscript. —"*Similarly, the functional gene transcript distribution indicated that the nir/nosZ I and nir/amoA gene transcript abundance ratios also had consistent patterns with the N_2O concentration, N_2O flux, and ΔN_2O across the four estuaries in general (Fig. S4).*"

Given the transcript datasets contain fewer data points compared with the gene datasets due to lacking samples from the PRE and the *nirK* transcript data from the JRE as well as some data below the detection limit, we only present the gene data in Fig. 6 of the main text.

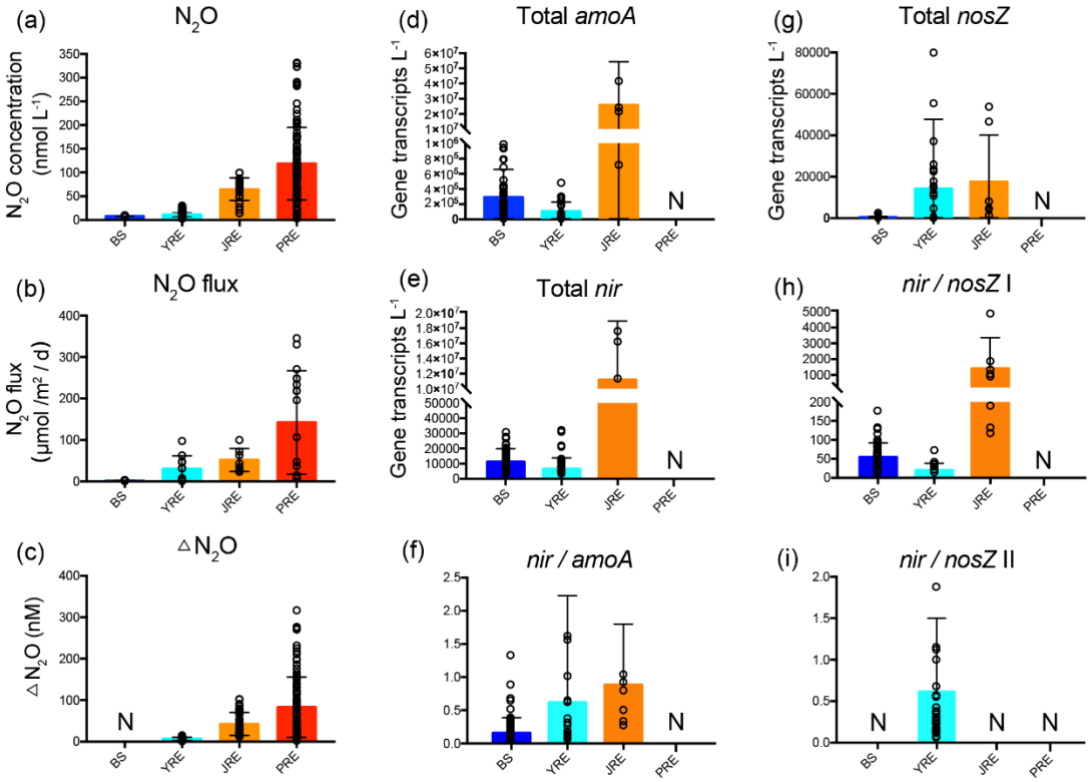

Fig. S4. The ranges of (a) N_2O concentration, (b) N_2O flux, (c) ΔN_2O, (d) total archaeal and bacterial *amoA* gene transcript abundance, (e) total *nirS* and *nirK* gene transcript abundance, (f) the ratio of total *nir* to *amoA* gene transcript abundance, (g) total *nosZ* clade I and II gene transcript abundance, (h) the ratio of total *nir* to *nosZ* clade I gene transcript abundance, and (i) ratio of total *nir* to *nosZ* clade II gene transcript abundance in the Bohai Sea (BS), Yangtze River estuary (YRE), Jiulong River estuary (JRE), and Pearl River estuary (PRE). Black circles represent the value of each sample. Bars represent the mean values. Error bars indicate standard deviation. N, no data or not determined.

Figure 2: What were the depth layers? Adding a label explaining which panel corresponds to which estuary may be helpful for the reader (same for Fig 3).

Response:

Thank you for the suggestion! We have added the labels of estuaries and depth layers in Fig. 2 and Fig. 3. The figure legends have also been modified.

Figure 6: Could it help to log transform the qPCR/RT-qPCR data to plot it in order to avoid breaking the y-axis?

Response:

Thank you! We have tried to log transform the qPCR/RT-qPCR data to plot it (see below). It seems that using raw data can show the differences between different estuaries better and can be compared clearly with the distribution of N_2O concentration and flux across the estuaries. So we still kept the original version of Figure 6.

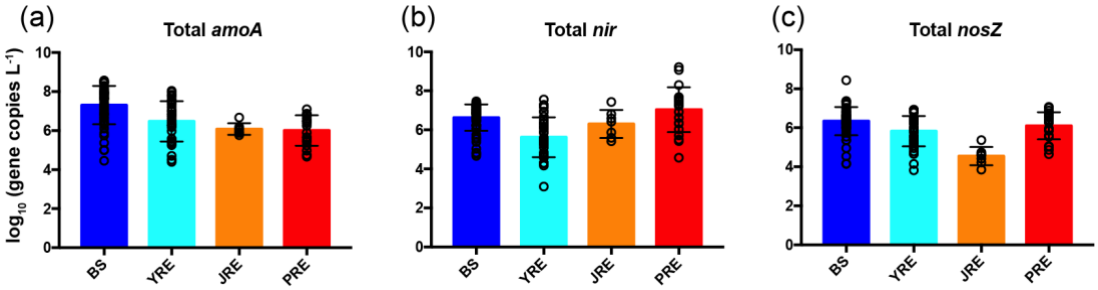

Fig. R2-1. The ranges of log-transformed (a) total archaeal and bacterial *amoA* gene abundance, (b) total *nirS* and *nirK* gene abundance, (c) total *nosZ* clade I and II gene abundance in the Bohai Sea (BS), Yangtze River estuary (YRE), Jiulong River estuary (JRE), and Pearl River estuary (PRE). Black circles represent the value of each sample. Bars represent the mean values. Error bars indicate standard deviation.

References:

Graf, D.R.; Jones, C.M.; Hallin, S.: Intergenomic comparisons highlight modularity of the denitrification pathway and underpin the importance of community structure for N_2O emissions, PLoS ONE, doi:10.1371/journal. pone. 0114118, 2014.

Lin, Hua, Dai, Minhan, Kao, Shuh-Ji, Wang, Lifang, Roberts, Elliott, Yang, Jin-Yu Terence, Huang, Tao, He, Biyan: Spatiotemporal variability of nitrous oxide in a large eutrophic estuarine system: The Pearl River Estuary, China, Marine Chemistry, doi: 10.1016/j.marchem.2016.03.005, 2016.

Zhan, Liyang., Chen, Liqi., Zhang, Jiexia., and Zheng, Airong.: Distribution of N_2O in the Jiulongjiang River Estuary and estimation of its air-sea flux during winter, Journal of Oceanography in Taiwan Strait, 30(MAY), doi:10.3969/J ISSN.2011.02.006, 2011.