Response to reviews #1

Dai et al. investigated the distribution of six key genes and transcripts related to N_2O production and consumption in four main estuaries in China. The authors analyzed the correlation between these genes and N_2O fluxes or concentrations obtained from previous literature and discussed what environmental factors might control the gene distribution pattern. This work implies denitrification might be essential for N_2O emissions in these estuaries. This study provides new insight into microbial divers of N_2O cycling in estuaries in China.

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.

Here are some minor suggestions:

Line 70: cite (Frey et al., 2020 Biogeosciences) for the dominance of nitrate derived N_2O in OMZs.

Response:

Thanks for your suggestion. We have added the citation of the article in the revised manuscript.

Lines 75-79: cite (Ji et al. 2018 Biogeosciences) for N_2O production from denitrification in the Chesapeake Bay.

Response:

Thank you. We have added the citation in the revised manuscript.

"*In addition, the incubation experiments with nitrogen stable isotope tracer reveal active N_2O production by denitrification in the Chesapeake Bay (Ji et al., 2018b). Another research in the Chesapeake Bay reveals that physical processes such as wind events and vertical mixing affected the net balance between N_2O production and consumption, resulting in a variable source and sink for N_2O (Laperriere et al., 2019).*"

Line 82: cite Figure 1 for the location of the four estuaries.

Response:

Thank you. Figure 1 has been cited in the revised manuscript.

Line 126: What were the minor modifications? Please elaborate.

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.*"

Line 145: What kind of alpha diversity? (e.g. Shannon alpha diversity?)

Response:

We have revised it as "*Alpha diversity indices (Shannon, Simpson, and Chao1) of the clade II-type nosZ gene were calculated …*".

Line 148: 'The top 10 most similar sequences of each OTU were used as references.' It is not clear how the taxonomy of the OTU was assigned. Did you use the taxonomy of the top 1 reference as the taxonomy of the OTU or the dominant taxonomy among all 10 references? Please explain this.

Response:

Thank you. These reference sequences were first deduplicated, then the representative sequences of OTUs along with these deduplicated reference sequences were used to build a maximum likelihood phylogenetic tree, and the taxonomy of the OTU was assigned according to the structure of the phylogenetic tree. The relevant statements have been added in the revised manuscript for clarification.

"*The top 10 most similar sequences of each OTU were used as references. The deduplicated reference sequences and the representative sequences of OTUs were aligned using MAFFT (Katoh and Standley, 2013) and automatically trimmed using trimAl (Capella-Gutiérrez et al., 2009). A maximum likelihood (ML) phylogenetic tree*

was constructed using Fasttree (v2.7.1, default parameters) (Price et al., 2010) with 500 bootstrap replicates for node support determination. The taxonomy of the OTU was assigned according to the phylogenetic relationship."

Line 235: accounting for % and % of N_2O production-related gene abundance

Response:

Thank you. We have revised it as suggested.

Line 240: I believe you meant to say 'one to two orders of magnitudes'.

Response:

Thank you. We have corrected it.

Line 291: should be (Figure 4b). Abundance was not reflected in Figure 4a.

Response:

Corrected.

Line 376: need to tune down this sentence here since N_2O emission is controlled by both N_2O production and consumption. You could say 'suggesting that acidification of the ocean may decrease N_2O consumption potential.'

Response:

Thank you. We have revised it as suggested.

Line 345: Since the four estuaries were sampled in different seasons, it would be useful to see some discussion about how different seasons might affect the distribution of genes and transcripts.

Response:

Thank you for the comment. The discussion about the effect of different seasons on the distribution of genes has been added in the revised manuscript.

"*There was a distinct large-scale spatial structure among the detected genes, as shown in Fig. 3. The different sampling seasons between the PRE (January) and the other three estuaries (June to September) may influence the spatial distribution of functional genes across the four estuaries. However, the niche differentiation of functional genes, spatially or temporally, is controlled by environmental factors in essence, such as temperature, salinity, oxygen and nutrient availabilities, and primary productivity.*"

Lines 388-404: *nosZ* clade I was transcribed more even though *nosZ* clade II genes were more abundant (Figure 3 i). The discrepancy between *nosZ* DNA and transcripts is worth discussing.

Response:

Thank you. The discussion about the discrepancy between the *nosZ* DNA and transcripts has been added in the revised manuscript.

"*The nosZ clade I gene was transcribed more actively even though the nosZ clade II gene was more abundant (e.g., the case in the BS shown in Fig. 3e and l). The higher growth yields of clade II-type N_2O-reducing bacteria than those of clade I-type (Yoon et al., 2016) may lead to a preponderance of the nosZ clade II gene. However, a microbial culture of clade I-type N_2O-reducing bacteria has been reported to have the capability of continually synthesizing N_2O reductase enzyme under oxic conditions to allow for a rapid transition into anoxic environments (Lycus et al., 2018). Such a strategy could result in the more abundant nosZ clade I transcripts observed in the estuaries.*"

Lines 415-416: (a) are these datasets measured from the same months or years as the microbial samples? Or they are mean values of some sort? Please provide a little more detail here. (b) why use gene abundance as indicators but not transcripts? The latter shows 'activity' in some sense. Could you present the transcript data in Figure 6 or the supplement?

Response:

Thank you very much for the comments.

(a) The datasets contain almost all available published data on N_2O concentration, N_2O flux, and ΔN_2O in the four estuaries, covering January to November from 2002 to 2015. We suppose that these data could have well covered the annual variations of these parameters in the estuaries for multiple years. The detailed statements have been added in the revised manuscript. A supplementary Table S5 has also been added to the supplementary materials.

"*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."

(b) 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 much 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.

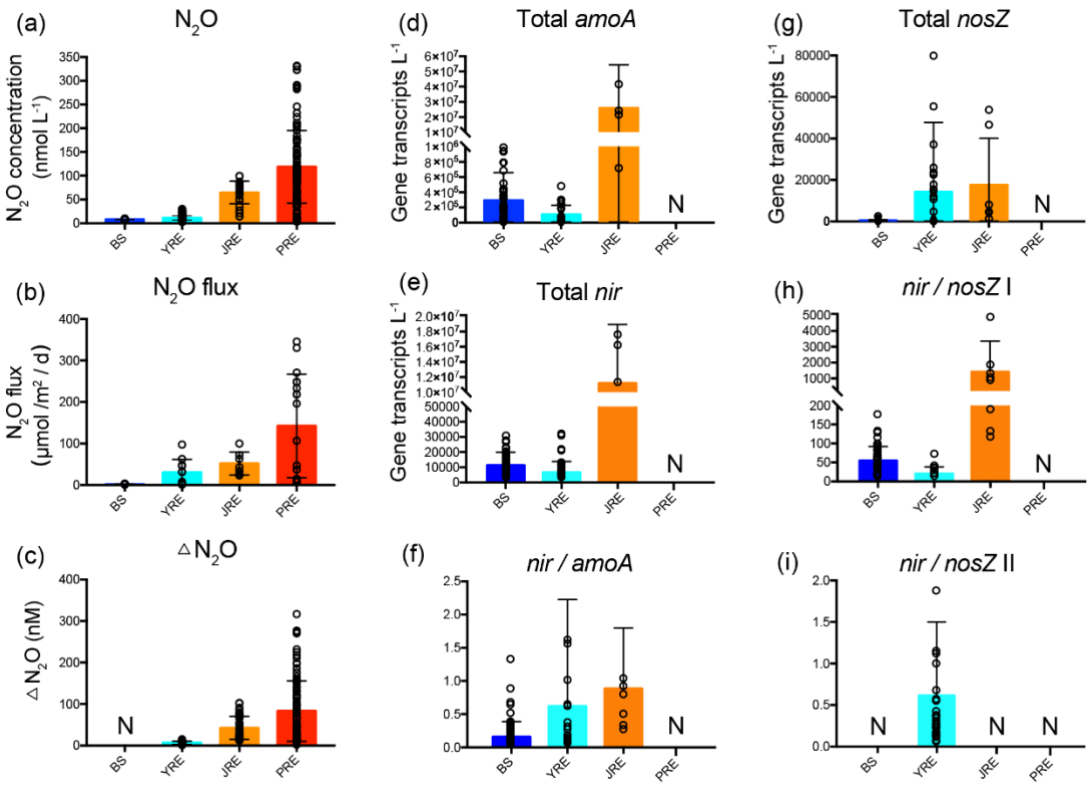

Fig. S4. The ranges of (a) N_2O concentration, (b) N_2O flux, (c) ΔN_2O, (d) total archaeal and bacterial *amoA* gene transcript abundance, (e) total *nirS* and *nirK* gene transcript abundance, (f) the ratio of total *nir* to *amoA* gene transcript abundance, (g) total *nosZ* clade I and II gene transcript 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.

Line 456: additional citations should be included here: (Bertagnolli et al., 2020 Environmental Microbiology reports) and (Sun et al., 2017 Frontiers in Microbiology).

Response:

Thank you. The citations have been added.

"*However, the most abundant nosZ clade II groups found in the OMZs of the eastern tropical South and North Pacific and the Arabian Sea are affiliated with Anaeromyxobacter (Deltaproteobacteria) (Sun et al., 2017; Sun et al., 2021) and those in the coastal OMZ waters of the Golfo Dulce, Costa Rica are affiliated with Gammaproteobacteria, Marinimicrobia, Bacteroidetes, and SAR324 (Bertagnolli et al., 2020).*"

Figure 1: I suggest adding sampling time for each estuary in the figure.

Response:

Thank you. Added.

Figure 2: please label the four estuaries (maybe as row names for all subplots). Latitudes and longitudes for the first few plots were missing. Please add latitudes and longitudes for all subplots. It is hard to tell ammonia, nitrite, and nitrate concentrations in three out of the four estuaries. You could use a different scale bar for PRE, so the other three plots could have a better resolution.

Response:

Thank you very much for the suggestions. Figure 2 has been modified. Latitudes and longitudes for all subplots have been added, and different scale bars have also been added to tell ammonia, nitrite, and nitrate concentrations in the four estuaries clearly.

Figure S2: red and orange in the plots were too similar to each other, please choose another color to distinguish the two better.

Response:

Thank you! We have changed the color and it is better now.

References:

Bertagnolli, A. D., Konstantinidis, K. T. and Stewart, F. J.: Non-denitrifier nitrous oxide reductases dominate marine biomes, Environ. Microbiol. Rep., 12(6), 681–692, doi:10.1111/1758-2229.12879, 2020.

Blackmer AM, Bremner JM.: Inhibitory effect of nitrate on reduction of N_2O to N_2 by soil microorganisms, Soil Biol Biochem., 10(3):187–191, doi:10.1016/0038-0717(78)90095-0, 1978.

Levipan, H. A., V. Molina, and C. Fernandez.: Nitrospina-like bacteria are the main drivers of nitrite oxidation in the seasonal upwelling area of the Eastern South Pacific (Central Chile ~36°S), Environ Microbiol Rep., 6:565–73, doi:10.1111/1758-2229.12158, 2014.

Lycus P, Soriano-Laguna MJ, Kjos M, Richardson DJ, Gates AJ, Milligan DA, et al.: A bet-hedging strategy for denitrifying bacteria curtails their release of N_2O, Proc Natl Acad Sci USA, 2018;115:11820–5, doi:10.1073/pnas.1805000115, 2018.

Molina, V., Belmar, L., and Ulloa, O.: High diversity of ammonia-oxidizing archaea in permanent and seasonal oxygen-deficient waters of the Eastern South Pacific, Environ. Microbiol., 12: 2450–2465, doi:10.1111/1462-2920.14246, 2010.

Sobarzo, M., Bravo, L., Donoso, D., Garcés-Vargas, J., and Schneider, W.: Coastal upwelling and seasonal cycles that influence the water column over the continental shelf off central Chile. Prog Oceanogr 75: 363–382., doi:10.1016/j.pocean. 2007. 08. 022, 2007.

Sun, X., Jayakumar, A. and Ward, B. B.: Community composition of nitrous oxide consuming bacteria in the oxygen minimum zone of the Eastern Tropical South Pacific, Front. Microbiol., 8(JUN), 1–11, doi:10.3389/fmicb.2017.01183, 2017.