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Manuscript title: Major role of ammonia-oxidizing bacteria in N₂O production in the Pearl River Estuary

Response to Reviewer #2

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 #2

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Major comments:

More caution is needed on these concentration-based "rate" measurements. Without isotope tracers, very little can be said about actual rates. Evidence for this is in the N₂O yields. The yields reported here are about 100X lower than ever reported from cultures or the field (see Ji et al. 2018 GBC).

Response:

(1) We agree that the ¹⁵N-labeled methods are of high sensitivity, which is more reliable for low nitrification activity in natural environments (Damashek and Francis 2018). However, in the nutrient-rich estuary waters, changes in nutrient concentrations (ammonium, nitrite, and nitrate) during incubations can be used to calculate nitrification rates. Previous studies reported concentration-based nitrification rates ranging from 0–153.6 μM d⁻¹ (Bianchi et al., 1994; Pakulski et al., 1995; Pakulski et al., 2000; De Wilde and De Bie, 2000; Dai et al., 2008; Grundle and Juniper, 2011). In the upper-PRE, where high nitrification activity has been reported in the hypoxic zone (Dai et al., 2008; Hou et al., 2018), the in-situ concentrations of ammonium (33.3-167.2 μM), nitrite (11.6-24.5 μM), and nitrate (82.0-126.1 μM) at the incubation sites were high, so the changing of the nutrients can be sensitively detected during incubations.

(2) We compared our ammonium oxidation rates with the ¹⁵N-labeled-based rates from Hou et al. (2018) (see Table R1 below). Hou et al. reported that during PRE

cruises in July to August 2012 and September 2014, the nitrification rates in the bottom waters of the PRE reached to 40.25 to 40.70 $\mu\text{mol L}^{-1} \text{d}^{-1}$ in the hypoxic sites. Actually, during our cruise, the nitrification rates in the upstream of Humen were also measured using the ^{15}N -labeled method by simulating in-situ condition incubations, which ranged from 51.05–1182.81 $\text{nmol L}^{-1} \text{h}^{-1}$ (1.23–28.32 $\mu\text{mol L}^{-1} \text{d}^{-1}$) (Zhang, 2016, Thesis). Thus, the nitrification rates estimated in our study are comparable to other studies in the upper reach of PRE.

(3) We compared our N_2O yields with reported from cultures (see Table R1 below). The N_2O yield in the estuarine waters in this study is lower than those from the cultured AOB strains (*Nitrosomonas europaea*, N_2O yield of 2.6–26% relative to NO_2^- production), however, the cultures were of high cell densities ($10^9 \text{ cells mL}^{-1}$) (Yoshida and Alexander, 1970) and were incubated with high concentration of ammonium (mM) (~10–100X higher than the natural ammonium concentration in the estuary). Obviously, the N_2O yield is the result of the physiological response of ammonia-oxidizing microorganisms to the environment (Mendum et al., 1999), as shown by the previous study on the AOB strain (*Nitrosomonas marina* C-113a) that N_2O yield increased in higher cell concentration cultures and higher ammonium concentration conditions (Frame and Casciotti, 2010).

(4) We also compared our N_2O yields with reported by Ji et al. (2018, GBC) (see Table R1 below). The N_2O yields were 0.003–0.06% at $>50 \mu\text{M O}_2$ and >2% at $<0.5 \mu\text{M O}_2$ in the Eastern Tropical Pacific (Ji et al., 2018), which are 2–10-fold lower than those from the AOB strain cultures under the 10–100 $\mu\text{M O}_2$ concentration (Goreau et al., 1980). Our N_2O yield ranged from 0.21 to 0.32% during nitrification (the initial in-situ O_2 concentration: 30–61.3 μM ; the terminal O_2 concentration: 0.7–2.5 μM). The estimated range of N_2O yield is 0.16 ± 0.09 to $0.37 \pm 0.23\%$ when fitting our measured O_2 concentrations into the empirical equation of the relationship between N_2O yield (%) from nitrification and O_2 concentration (μM) given by Ji et al. (2018), which was comparable with our measured N_2O yield.

Table R1 Nitrification rates/ammonia oxidation rates and N₂O yield from literatures and our study.

Study area/Microorganisms	Method	Nitrification rates ($\mu\text{M day}^{-1}$)	NH ₃ concentrations (μM)	N ₂ O yield	Reference
Rhône River plume	Nutrients + N-serve	0.23 – 2.20	0 – 10	–	Bianchi et al., 1994
Mississippi River	Nutrients	0 – 13.44	0.3 – 2.4	–	Pakulski et al., 1995
Mississippi & Atchafalaya River plume	Nutrients	0 – 14.16	0.5 – 2.5	–	Pakulski et al., 2000
Scheldt	¹⁴ C+ methylfluoride	Up to 19.2	0 – 400	0.10–0.40%	De Wilde & De Bie, 2000
	Nutrients	Up to 153.6			
Saanich Inlet	Nutrients + allylthiourea	0 – 7.66	0 – 4.9	–	Grundle & Juniper, 2011
Pearl River	Nutrients + allylthiourea	12.47 – 33.10 ^a	1.2 – 341.9	–	Dai et al., 2008
Pearl River	¹⁵ N, denitrifier method	40.25 – 40.70 ^b	–	–	Hou et al., 2018
Pearl River	¹⁵ N, denitrifier method	1.23–28.32	–	–	Zhang, 2016
Eastern Tropical Pacific	¹⁵ N tracer	–	0 – 0.5	0.003–0.06% ^c >2% ^d	Ji et al., 2018
<i>Nitrosomonas europaea</i>	Nutrients, N ₂ O	–	–	2.6–26% ^e	Yoshida & Alexander, 1970

	concentrations	2.38 – 23.8 ^f	7.14–714.3 ^g	2.6–18% ^h	
<i>Nitrosomonas</i> sp. (Marine)	Nutrients, N ₂ O	—	—	0.26–0.99 % ⁱ	Goreau et al., 1980
	concentrations	—	—	2.5–9.9 % ^j	
<i>Nitrosomonas marina</i> <i>C-113a</i>	Nutrients, N ₂ O	—	50 ^g	0.04–2.2%	Frame & Casciotti, 2010
	isotopic analyses	—	—	—	
Pearl River	Nutrients	11.28–26.88 ^a	33.3 – 167.2	0.21–0.32%	This study

^aThe ammonia oxidation rates observed at the upper reach of PRE in summer.

^bThe nitrification rates observed at the upper estuary where the O₂ concentration from 0.67 to 1.41 mg L⁻¹, which were little lower than that ranging from 0.9 to 2.0 mg L⁻¹ in our study.

^cN₂O yield from ammonia oxidation under the O₂>50 μM.

^dN₂O yield from ammonia oxidation under the O₂<0.5 μM.

^eThis experiment was designed to study the influence of different levels of ammonium concentration on N₂O formation by *Nitrosomonas europaea*.

^fThe ammonia oxidation rates were estimated based on the difference of ammonium concentrations between initial- and terminal- incubation time using the data from Yoshida and Alexander, 1970.

^gAmmonium concentrations in the medium.

^hThis experiment was designed to study the influence of cells in different growth stages on N₂O formation by *Nitrosomonas europaea*.

ⁱN₂O yield from ammonia oxidation under the O₂ ranging from 5–20% (56.3 –218.8 μM).

^jN₂O yield from ammonia oxidation under the O₂ ranging from 0.5–1% (5.6 –10.9 μM).

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The strength of the correlation between genes and rates absolutely cannot be used to apportion a relative importance of one group of ammonia oxidizers or the other to the total rates. Nothing can be concluded from the data presented about who the important nitrifiers are. One possibility would be to obtain a range of cell-specific ammonia oxidation rates from the literature and then use those in combination with the qPCR data to calculate the relative contribution of each group to the observed "rates."

Response:

The cell-specific ammonia oxidation rates, nitrite production rates, and N₂O production rates from the literature on AOA and AOB strains varied in a very large range, due to the different species cultures, cell densities, cell stages, and incubation conditions such as O₂ or substrates concentrations (see Table R2 below). It is fairly uncertain to use these greatly varying cell-specific rates from cultures to estimate the contribution of AOA and AOB to the N₂O production in natural environments. Notably, although the cell-specific N₂O production rates from AOB and AOA strains varied greatly, the N₂O yields from the AOB strains, ranging from 0.09 to 26 % (Table R2), were generally higher than the N₂O yield from the AOA strains (0.002–0.09%; Table R2). In addition, the higher N₂O yield from AOB has been observed in soils although the abundance of AOB was lower than AOA (Hink et al., 2017, 2018).

We admit that the conclusions of this study mainly based on the correlation analysis and statistical analysis between multi-parameters. But there are two analyses providing more strong evidence supporting these statistical analyses:

- (1) We attempted to accurately assess the relative contributions of AOA and AOB to N₂O production in the PRE by plotting the N₂O production rates (Fig. 7a in the MS) and yields (Fig. 7b in the MS) normalized to total AOA and AOB *amoA* gene copies (sum of PA and FL fractions or only PA fractions) or transcripts (only PA fractions) along X-Y axes that represent the relative contributions of AOA and AOB to the total *amoA* gene or transcript pools. For both incubation sites, the more abundant AOB were in the *amoA* gene-based DNA or cDNA pool, the distinctly higher (disproportionately higher relative to enhanced abundance) the average *amoA* gene copy or transcript-specific N₂O 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 N₂O than AOA.
- (2) The values of N stable isotopes in N₂O ($\delta^{15}\text{N}$) were analyzed. The much lower $^{15}\text{N-N}_2\text{O}$ (-27.9 to $-12.6\text{\textperthousand}$) upstream of the Humen outlet is consistent with AOB nitrification or denitrification processes, whereas enriched $^{15}\text{N}_2\text{O}$ (5.2 – $7.1\text{\textperthousand}$) in the lower reaches approaches AOA nitrification and air $^{15}\text{N-N}_2\text{O}$ (Santoro et al., 2011). Taken together, the isotopic compositions of N₂O (Fig. 2h in the MS) and N₂O concentration distribution (Fig. 2e–g) suggest that the high concentrations of N₂O (oversaturation) were produced from strong nitrification by AOB and probably concurrent minor denitrification in the upper estuary, however in the lower reaches, low concentrations of N₂O could be explained by AOA nitrification or water atmospheric exchange of N₂O.

Table R2 Cell-specific ammonia oxidation rates, cell-specific N₂O production rates, and N₂O yield from archaeal and bacterial strains.

Microorganisms	Species (source of isolate)	Ammonia oxidation rates (fmol cell ⁻¹ h ⁻¹) ^a	N ₂ O production rates (fmol cell ⁻¹ h ⁻¹) ^b	N ₂ O yield ^c	Reference
AOA		19.0	–	–	Martens-Habbena et al., 2009
	<i>Nitrosopumilus maritimus</i> (Marine)	–	0.02–1.01	0.002–0.026%	Löscher et al., 2012
		–	–	0.03–0.05%	Stieglmeier et al., 2014
	<i>Nitrososphaera viennensis</i> (Soil)	2.6–2.8	0.004–0.005	0.03–0.09%	Stieglmeier et al., 2014
AOB	<i>Nitrosomonas</i> sp. (Marine)	2.0–15.4	0.04–0.21	0.26–9.9%	Goreau et al., 1980
	<i>Nitrosomonas marina</i> (Marine)	0.9–4.9	–	–	Glover, 1985
		13.7–31.3	–	–	Glover, 1985
	<i>Nitrosococcus oceanus</i> (Ocean)	83.3	–	–	Waston, 1965
		–	–	0.26±0.1%	Goreau et al., 1980
		12.4–18.3	–	2.6–26%	Yoshida & Alexander, 1970
	<i>Nitrosomonas europaea</i> (Soil)			0.47±0.1%	Goreau et al., 1980
	<i>Nitrosospira tenuis</i> NV12 (Soil)	–	0.002	–	Shaw et al., 2006
	<i>Nitrosomonas europaea</i> ATCC 19718	–	0.06	–	Shaw et al., 2006
	<i>Nitrosospira multiformis</i> (Soil)	–	–	0.09–0.27%	Stieglmeier et al., 2014

<i>Nitrosolobus multiformis</i> (Soil)	–	–	0.09 ±0.02%	Goreau et al., 1980
<i>Nitrosospira briensis</i> (Soil)	–	–	0.11 ±0.04%	Goreau et al., 1980

^aThe units for cell-specific ammonia oxidation rates in the cited references were unified as fmol cell⁻¹ h⁻¹.

^bThe units for cell-specific N₂O production rates in the cited references were unified as fmol cell⁻¹ h⁻¹.

^cThe range of N₂O yield of different cell densities under different O₂ conditions.

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The literature review, both in the Introduction and Discussion, is severely lacking.

There is a substantial literature about nitrification and N₂O production in estuaries, almost none of which are referenced here. Normally I would provide some specific suggestions, but the omissions are too vast to list. One place to start would be a review by Damashek and Francis 2018 Estuaries and Coasts, or a nice earlier paper with a summary of nitrification rates in estuaries, Damashek et al. 2016 Estuaries and Coasts.

Response:

Very sorry for this problem. We added the estuarine studies literature review on nitrification and N₂O production in the Introduction and Discussion of the revised version.

“Estuaries, being highly impacted by coastal nutrient pollution and eutrophication due to anthropogenic activity, play a significant role in nitrogen cycling at the land-sea interface (Bricker et al., 2008; Damashek et al., 2016). Estuarine and coastal regimes have long been recognized major zones of N₂O production in the marine system (Seitzinger and Kroeze, 1998; Mortazavi et al., 2000; Usui et al., 2001; Kroeze et al., 2010; Allen et al., 2011). In particular, the eutrophic estuaries with significant nitrification and extensive oxygen-deficient zones (ODZs) has been considered as the hot spot regions for N₂O production (Abril et al., 2000; DeWilde and De Bie, 2000; Garnier et al., 2006; Lin et al., 2016), and nitrification is often credited as the dominant N₂O production pathway in estuaries (deBie et al. 2002; Barnes and Upstill-Goddard 2011; Kim et al. 2013; Lin et al. 2016; Huertas et al., 2018; Laperriere et al., 2019). The estuaries have been reported with high of N₂O saturation and large N₂O flux range, and N₂O concentrations are highly variable (Hashimoto et al., 1999; deWilde and de Bie 2000; deBie et al. 2002; Xu et al., 2005; Chen et al., 2008; Rajkumar et al., 2008; Zhang et al., 2010; Barnes and Upstill-Goddard 2011; Stocker et al. 2013; Wu et al., 2013; Murray et al., 2015; Lin et al., 2016; Wells et al., 2018). The dynamics of N₂O emissions in these ecosystems are regulated by complex physical and biological processes, e.g. mixing between freshwater and oceanic waters influenced biogeochemistry of estuarine waters and microbial activity in the water column (Huertas et al., 2018; Laperriere et al., 2019), yet studies on estuarine N₂O production and emission in the water column based on integrated biogeochemical parameters, function genes, and in-situ incubations remain sparse.”

“Previous studies also proposed that nitrification may be the major source of N₂O production in the water column in estuarine systems, such as the Guadalquivir estuary (Huertas et al., 2018), the Schelde estuary (De Wilde and De Bie, 2000), and the Chesapeake Bay estuary (Laperriere et al., 2019). However, in the estuarine sediments, N₂O production was attributed to both nitrification and denitrification, such as the Tama estuary of Japan (Usui et al., 2001) and the Yangtze Estuary of China (Liu et al., 2019; Wang et al., 2019), where denitrification is the major nitrogen removal pathway with the N₂O production and consumption”

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The nirS data are not very useful to this manuscript in that there is essentially no relationship between nirS abundance and N₂O production from denitrification. nirS presence could just as easily be a marker for N₂O consumption.

Response:

NO_2^- is reduced by a copper-containing (NirK) or cytochrome cd1-containing nitrite reductase (NirS) to nitric oxide (NO), then by a heme-copper NO reductase (NOR) to N_2O (Coyne et al., 1989; Treusch et al., 2005; Abell et al., 2010; Bartossek et al., 2010; Canfield et al., 2010; Lund et al., 2012; Graf et al., 2014), and finally by nosZ gene to N_2 (Sanford et al., 2002; Simon et al., 2004; Sanford et al., 2012; Graf et al., 2014). We measured the *nirS* gene in this study to identify the distribution of denitrifiers in the PRE, but there were no any significant correlations between *nirS* abundance and N_2O parameters. Furthermore, our incubation experiments indicated that nitrification occurred during the entire incubations, but very minor complete denitrification (N loss) may be present in the ending phase of the incubation at the O_2 -minimum site P01, suggesting that denitrification was not the main process contributing to N_2O production in the water column of the Pearl River Estuary.

References

- Abell, G. C. J., Revill, A. T., Smith, C., Bissett, A. P., Volkman, J. K., and Robert, S. S.: Archaeal ammonia oxidizers and *nirS*-type denitrifiers dominate sediment nitrifying and denitrifying populations in a subtropical macrotidal estuary, *ISME J.*, 4, 286–300, 2010.
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- Lund, M. B., Smith, J. M., and Francis, C. A.: Diversity, abundance and expression of

nitrite reductase (*nirK*)-like genes in marine thaumarchaea, ISME J., 6, 1966–1977, 2012.

Sanford, R. A., Cole, J. R., and Tiedje, J. M.: Characterization and Description of Anaeromyxobacter dehalogenans gen. nov., sp. nov., an Aryl-HaloRespiring Facultative Anaerobic Myxobacterium, Appl Environ Microbiol., 68, 893–900, 2002.

Sanford, R. A., Wagner, D. D., Wu, Q., Chee-Sanford, J. C., Thomas, S. H., Cruz-Garc á, C., Rodr íquez, G., Massol-Dey á, A., Krishnani, K. K., Ritalahti, K. M., Nissen, S., Konstantinidis, K. T., and Löffler, F. E.: Unexpected nondenitrifier nitrous oxide reductase gene diversity and abundance in soils. Proc Natl Acad Sci U S A 109, 19709–19714, 2012.

Simon, J., Einsle, O., Kroneck, P. M. H., Zumft W. G.: The unprecedented nos gene cluster of Wolinella succinogenes encodes a novel respiratory electron transfer pathway to cytochrome c nitrous oxide reductase, FEBS Lett., 569, 7–12, 2004.

Treusch, A. H., Leininger, S., Kletzin, A., Schuster, S. C., Klenk, H., and Schleper, C.: Novel genes for nitrite reductase and Amo-related proteins indicate a role of uncultivated mesophilic crenarchaeota in nitrogen cycling, Environ. Microbiol., 7, 1985–1995, 2005.

All the physical dynamics in the system have been reduced to a very naive "water mass" identification. Basic concepts in estuarine biogeochemistry are absent—for example, using salinity as a conservative tracer in a two-end member mixing model to determine production and loss of the various biogeochemical parameters.

Response:

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). Therefore, we used temperature, salinity, and silicate to trace water masses and mixing in the estuary transect. We believe that fresh and saline water masses mixing might directly mix nitrifiers and denitrifiers as well as N₂O from fresh and saline waters. Thus, in order to peel off the directly mixing effects, we used Partial Mantel tests to eliminate the co-varying of water mixing, substrate concentrations, and N₂O production along the transect and to identify the intrinsic/direct relationship between ammonia oxidizers and N₂O

production. We revised the relevant statements on “water mass” and emphasized “water mixing” throughout the MS for a clearer expression.

According to the reviewer’s suggestion, we also performed the end-member mixing analysis in the supplementary materials of the revised MS.

(1) Figure R1 (see below) is a three-dimensional scatter plot showing the relationships between Potential temperature (θ) ($^{\circ}$ 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. Figure R2 (below) shows the linear relationships between Potential temperature (θ) or silicate and salinity as well as between observed and conservative silicate. These analyses indicate a two end-member mixing in this estuary and silicate, temperature, and salinity can be used as the indicators to trace estuarine water masses and mixing.

(2) Figure R3 (below) shows the scatter plot of RN_2O (the two end-member mixing model prediction minus field observation) versus salinity as well as the relationship between RN_2O and $\Delta\text{NH}_3/\text{NH}_4^+$ in the Lingdingyang. RN_2O indicates biogeochemical produced and then outgassing N_2O through the water-air exchange (see Lin et al., 2016). RN_2O decreased with salinity indicating N_2O removal through the estuarine mixing behavior and/or water-air exchange. Meanwhile, the positive correlation between RN_2O and $\Delta\text{NH}_3/\text{NH}_4^+$ (ammonium consumption) suggested that N_2O may be mostly related to ammonia oxidation in Lingdingyang.

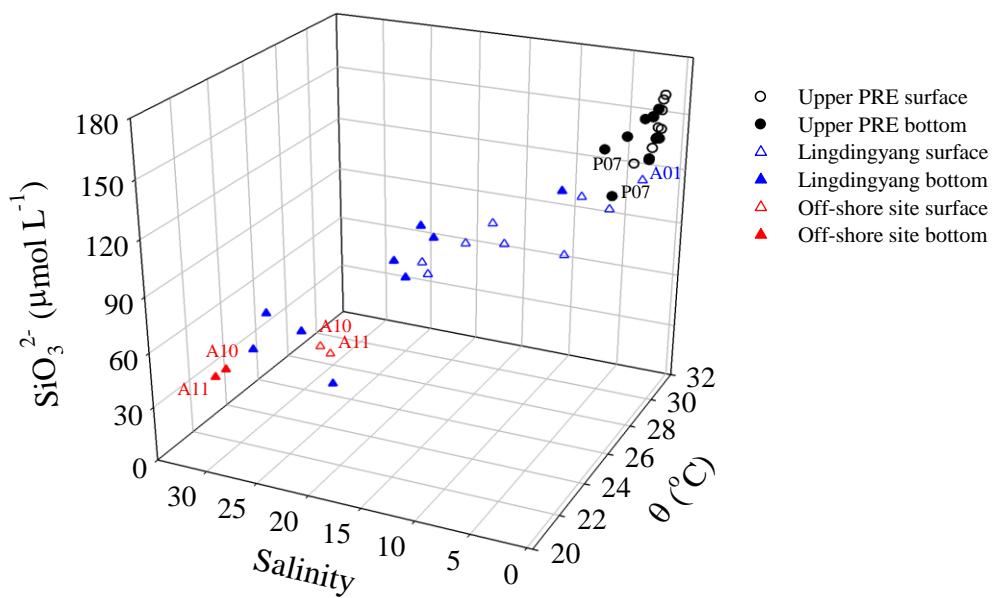


Figure R1: Three-dimensional scatter plot of Potential temperature (θ) ($^{\circ}\text{C}$), salinity, and silicate (SiO_3^{2-}).

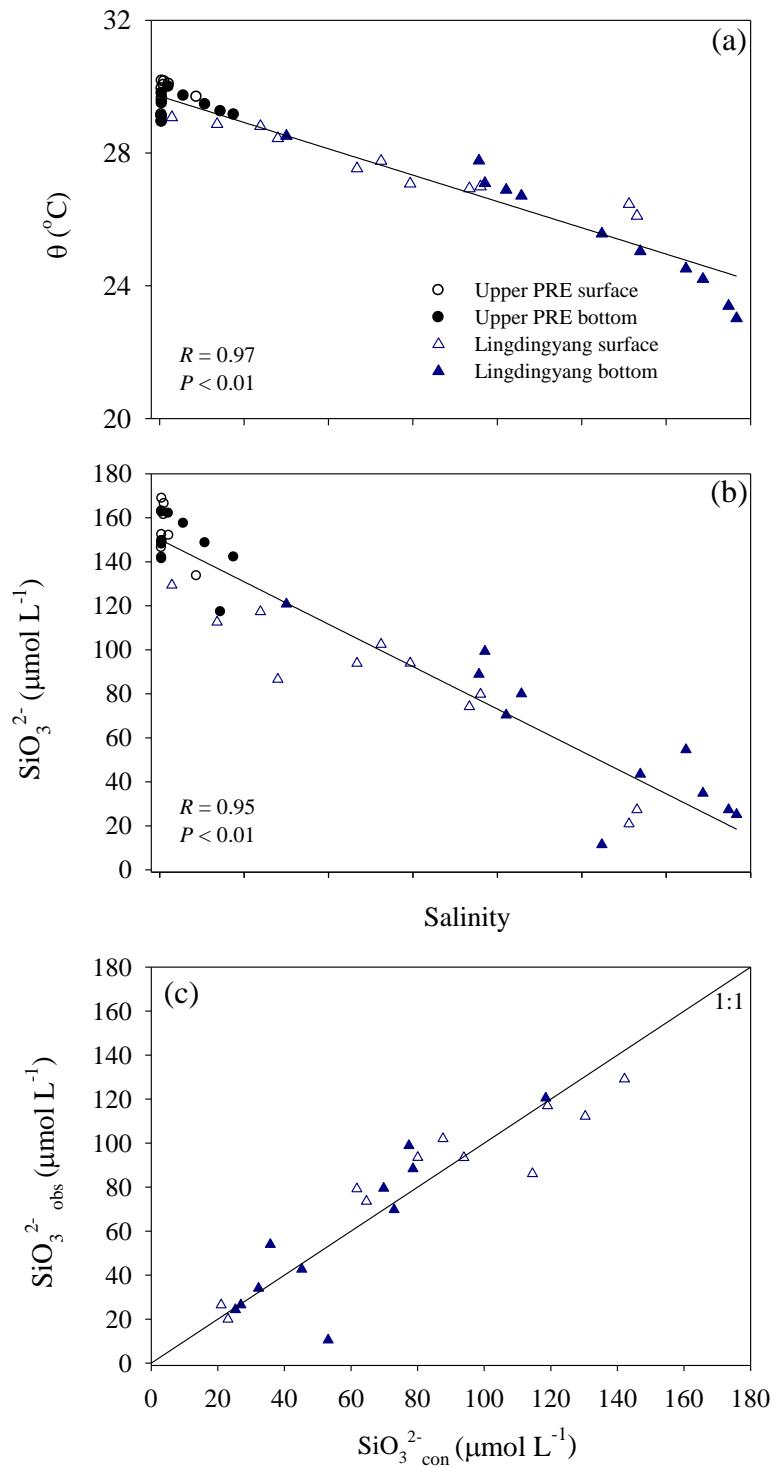


Figure R2: Relationships between (a) potential temperature (θ) ($^{\circ}\text{C}$) or (b) silicate and salinity in the PRE estuary. The fitted curves represent the conservative distribution controlled by physical mixing processes. (c) Relationship between observed and conservative silicate concentrations. The straight line represents a 1:1 reference line.

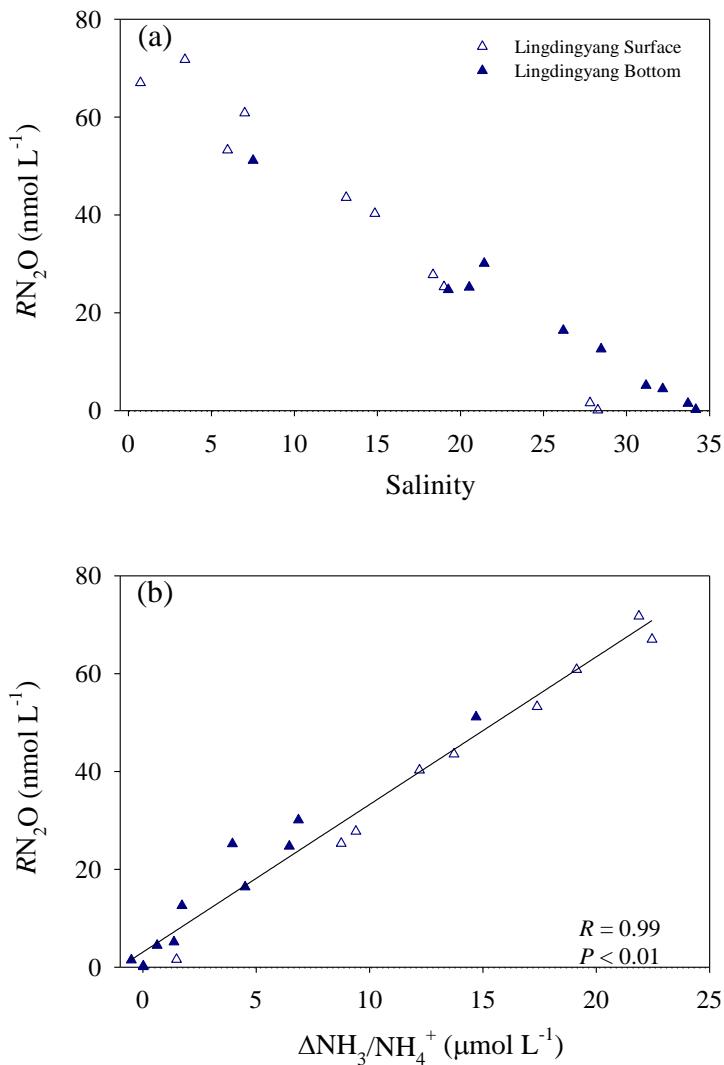


Figure R3: (a) RN_2O versus salinity in Lingdingyang; (b) the relationship between RN_2O and $\Delta NH_3/NH_4^+$.

References

- Lin, H., Dai, M., Kao, S. J., Wang, L., Roberts, E., Yang, J., Huang, T., and He, B.: Spatiotemporal variability of nitrous oxide in a large eutrophic estuarine system: The Pearl River Estuary, China, Mar. Chem., 182, 14–24, 2016.
- Moore, W. S., Sarmiento, J. L., and Key, R. M.: Tracing the Amazon component of surface Atlantic water using ^{228}Ra , salinity and silica, J. Geophys. Res., 91, 2574–2580, 1986.

Specific comments:

p. 3 lines 15-16 Unclear to me what is meant by “runoff ranked 17th”.

Response:

Sorry for the unclear sentence. We modified this sentence as “*The Pearl River Estuary (PRE) is one of the world's most complex estuarine systems, with the total discharge of $285.2 \times 10^9 \text{ m}^3 \text{ yr}^{-1}$, which makes the Pearl River system the 17th largest river in the world.*”

p. 5 lines 2-4 What N₂O standards were used? How was the GC calibrated?

Response:

N₂O standard gases of 1.02 and 2.94 ppmv N₂O/N₂ (National Center of Reference Material, China, Beijing) were used. N₂O concentration was analyzed using a purge and trap system coupled with gas chromatography described by Lin et al. (2016). The repeatability based on the relative standard deviation (RSD) of the slope of the standard working curve was investigated. The results showed that RSD of curve slopes was 1.77% (n=8). The detection limit of the method was calculated to be about 0.1 nmol L⁻¹. The precision of this method was estimated to be better than $\pm 5\%$. When water samples were analyzed, every 5–10 samples were inserted with N₂O standards to calibrate GC.

Reference

Lin, H., Dai, M., Kao, S. J., Wang, L., Roberts, E., Yang, J., Huang, T., and He, B.: Spatiotemporal variability of nitrous oxide in a large eutrophic estuarine system: The Pearl River Estuary, China, Mar. Chem., 182, 14–24, 2016.

p. 5 line 6 How was N₂O_{aquatic} calculated?

Response:

We defined the measured concentration of dissolved N₂O as N₂O_{aquatic}, which were measured with the analytical method described by Lin et al. (2016). The dissolved N₂O concentration was calculated using the measured peak area of samples and the standard working curve of standard gases of 1.02 and 2.94 ppmv N₂O/N₂ (National Center of Reference Material, China, Beijing). Calibration of N₂O concentrations was calculated from the peak areas with standard gases. Certain volumes of standard gas

were transferred into the glass purge vessel and subsequently analyzed by the same procedure used for water samples.

p.7 lines 3 How much did DO concentration change over the course of the 24 h incubations? What effect would this have on the measured N₂O production?

Response:

During 24 hour incubation at site P01 with in-situ DO below 1.0 mg kg⁻¹, DO in the incubation system was fast consumed and below the detection limit of the Winkler method. In the late phase (18–24 hours) of the incubation, obvious reduction of both NH₃ and NO₃⁻ and NO₂⁻ accumulation was observed (Fig. 5 a-c), suggesting that nitrification and denitrification might be coupled under suboxic/anaerobic conditions. The reduction of N₂O accumulation along with the incubation time may be caused by N₂O consumption during denitrification in the late phase. When N₂O production and consumption co-occurred, the N₂O yield during nitrification would be underestimated. Thus we only calculated the N₂O production rate and yield during the early-middle phase of the incubation where DIN was in balance.

At site P05, ~55% of DO was consumed during the 12 hours incubation in the bottom water, decreasing from 54.7 to 24.6 μmol L⁻¹; ~34% of DO was consumed in the surface water during 12 hours, decreasing from 61.3 to 40.3 μmol L⁻¹. But there was no N loss and DIN was in balance during the incubations, so there was no effect on the measurement of N₂O production.

p. 7 lines 18-19 Were both N₂O yield equations used? Compared? Were they equal?

Response:

Sorry for the confusion. We only used Eq. (8) to estimate N₂O yield (the ratio of N₂O production rate to ammonia oxidation rate). Eq. (9) was deleted in the revised MS.

In addition, we compared the N₂O yield estimated by Eq (8) and Eq (9) for site P05, where the only nitrification occurred during 12 hour-incubation. The N₂O yield estimated by Eq (8) and Eq (9) was 0.21% and 0.19%, respectively in the surface water and 0.32% and 0.33%, in the bottom water.

More details are needed about how you arrived at the Schmidt number for N₂O. Is this the Raymond and Cole reference?

Response:

In the Eq. (5) for N₂O flux estimation, k (cm h⁻¹) is the gas transfer velocity depending on wind and water temperatures. In this study, k_{600} , the gas transfer velocity at a Schmidt number of 600, was used for the estuarine system (Raymond and Cole, 2001). The Schmidt number (Sc) is defined as the kinematic viscosity of water divided by the diffusion coefficient of the gas, and is usually expressed as a function of temperature and salinity (Wanninkhof, 1992). For steady winds with the average climatological wind speed at 10 m above the water surface, the relationship between gas transfer and wind speed is estimated using Eq. (6) according to Wanninkhof (1992):

$$k_{600} = 0.31 \times u^2_{10} \times (Sc/600)^{-0.5} \quad (6)$$

For N₂O in waters of salinity <35 and temperature ranging from 0–30 °C, Sc_{N₂O} is estimated using the following Eq. (7) according to Wanninkhof (1992):

$$Sc_{N_2O} = 2055.6 - 137.11 t + 4.3173 t^2 - 0.05435 t^3 \quad (7)$$

We added more details in the revised 2.2 section as suggested by the reviewer.

Need additional details of the calibration of the isotopic values.

Response:

We added more details of the calibration of the isotopic values in the revised version (2.2 subsection).

“The δ¹⁵N values in N₂O were analyzed by quantifying the molecular ions (N₂O⁺, m/z 44, 45 and 46) of N₂O by isotope ratio mass spectrometry (IRMS) at the State Key Laboratory of Soil and Sustainable Agriculture, Institute of Soil Science, Chinese Academy of Sciences, Nanjing. The values for δ¹⁵N-N₂O in the sample were calculated using the raw peak area ratios of 45/44 for a reference gas, which was previously calibrated using stable isotope N₂O standard gas produced by SHOKO, Co., Ltd., Japan (δ¹⁵N_{Air} = -0.320‰), and the sample peak (Frame and Casciotti, 2010; Mohn et al., 2014). In this study, the precision of the isotope method for δ¹⁵N-N₂O was estimated to be 0.3‰.”

References:

Frame, C. H., and Casciotti, K. L.: Biogeochemical controls and isotopic signatures of nitrous oxide production by a marine ammonia-oxidizing bacterium, *Biogeosciences*, 7, 2695–2709, 2010.

Mohn, J., Wolf, B., Toyoda, S., Lin, C. T., Liang, M. C., Brüggemann, N., Wissel, H., Dyckmans, A. E. S. J., Szwec, L., Ostrom, N. E., Casciotti, K. L., Forbes, M., Giesemann, A., R., Doucett, R. R., Well, Yarnes, C. T., Ridley, A. R., Kaiser, J., and Yoshida, N.: Interlaboratory assessment of nitrous oxide isotopomer analysis by isotope ratio mass spectrometry and laser spectroscopy: current status and perspectives, *Rapid Commun Mass Spectrom.* 28, 1995–2007, 2014.

p.7 Why is N₂O yield in units of permil? (line 18-19, and also in the Discussion). Also would be more conventional to list this as N₂O-N not N-N₂O

Response:

We deleted the unit and replaced N-N₂O with N₂O-N as suggested.

No discussion of particle attached versus free living amoA copies. Data is presented in multiple figures. Previous literature show no association. Did the filters clog?

Response:

(1) We mentioned in section 2.1 that “*total of 1000 mL of water for gene analysis was serially filtered through 0.8 µm and then 0.22 µm pore size polycarbonate membrane filters (47 mm diameter, Millipore) within 30 min at a pressure <0.03 MPa, whereas for the upper estuary waters that high of TSM, we filtered each 250/500 mL waters through 4/2 pieces of 0.8 µm and 0.2 µm membrane filters, to avoid filter clog.*”

(2) We described in section 3.2 that “*these three genes were predominantly distributed in the PA communities compared to the FL communities.*”

(3) We discussed in section 4.2 that “*The more abundant AOA amoA genes than AOB as well as the more abundant genes in the PA communities than the FL communities are consistent with our previous study in the PRE (Hou et al., 2018)*”.

(4) Moreover, based on RDA analysis and correlations between AOB and TSM (Table 2), we discussed that “*AOB-amoA abundance was significantly correlated (P <0.05–0.01) to TSM concentration (positively), which is consistent with our previous PRE study that found high TSM concentrations influenced substrate availability and thus AOB distribution (Hou et al., 2018)*”.

(5) In addition, we added more discussion. —“*We speculated that AOA and AOB could be better adapted to particle-rich estuarine environments with highly active in ammonia oxidation (Zhang et al., 2014; Hou et al., 2018). Additionally, qPCR quantification results revealed that significantly higher proteobacterial and archaeal amoA and nirS gene abundances in the PA than in the FL community, suggesting that higher potentials for both nitrification and denitrification occurring in particle-associated rather than free-living communities (Zhang et al., 2014). The lower oxygen availability in the micro-niche of particles has been reported to be favorable for both nitrification and denitrification potentials in oxygenated water (Kester et al., 1997). The statistical analysis in this study also revealed that low DO concentrations and high TSM conditions favored AOB-amoA (Table 2), suggesting that AOB might be more active in the hypoxic upper reach of PRE with high TSM.*”

References:

- Hou, L., Xie, X., Wan, X., Kao, S. J., Jiao, N., and Zhang, Y.: Niche differentiation of ammonia and nitrite oxidizers along a salinity gradient from the Pearl River estuary to the South China Sea, *Biogeosciences*, 15, 5169–5187, 2018.
- Kester, R. A., de Boer, W., and Laanbroek, H. J.: Production of NO and N₂O by pure cultures of nitrifying and denitrifying bacteria during changes in aeration, *Appl. Environ. Microbiol.*, 63, 3872–3877, 1997.
- Zhang, Y., Xie, X., Jiao, N., Hsiao, S. S. Y., and Kao, S. J.: Diversity and distribution of amoA-type nitrifying and nirS-type denitrifying microbial communities in the Yangtze River estuary, *Biogeosciences*, 11, 2131–2145, 2014.

P. 9 lines 4-6 “the entire PRE acts as a N₂O source” but negative air-sea fluxes are reported in the previous sentence?

Response:

The estimated water-air N₂O fluxes were 100.4 to 344.0 μmol m⁻² d⁻¹ upstream and decreased in Lingdingyang (42.4 to -2.6 μmol m⁻² d⁻¹). Taken together, the PRE was a strong source. We revised this sentence as “*Together, the PRE acts as a N₂O source*”.

p.11 lines 19-26: This paragraph confuses some important concepts. Some of these numbers are the isotopic composition of N₂O produced by ammonia oxidizers, but

some of these numbers are the isotope effect (epsilon). Also, the isotopic composition of the N₂O being produced by nitrification is dependent on the isotopic composition of the NH₃ being oxidized, for which no measurements or even estimates are provided.

Response:

We only used the isotopic composition of N₂O in this paragraph and supplementary Table S2. But sorry for the wrong supplementary Table S2 title. We revised it as “Isotopic composition of ¹⁵N-N₂O during bacterial and archaeal ammonia oxidation, bacterial nitrifier-denitrification, and bacterial denitrification.” These data all are from literature.

p.12 lines 15-17: Doesn’t make sense to refer to ’water masses’ in estuaries. There is a tremendous amount of mixing that leads to variation in these parameters. Just because something is a different salinity doesn’t mean it’s a different ’water mass.’ These parameters are just ’hydrography.’

Response:

We revised “water masses parameters” as “hydrographic parameters”.

p. 12 lines 15-28 and p. 13 lines 1-18 A lot of results presented that should be moved to the results section.

Response:

Thanks for the reviewer’s suggestion. We moved this part into the Results section (3.3 subsection).

p. 12 line 27 “ammonia oxidizer community” The use of the word “community” throughout the paper is confusing. More accurate to state the abundances of AOA and AOB?

Response:

We revised “ammonia oxidizer community” as “AOA and AOB distribution”, and moved this part into the Results section (3.3 subsection) according to the reviewer’s suggestion.

p. 24 Fig 1 i,j It looks like two different slopes in the data upstream and Lingdingyang. This could be quantified using a break point analysis.

Response:

Thanks for the suggestion. We re-quantified using a break point analysis. See below.

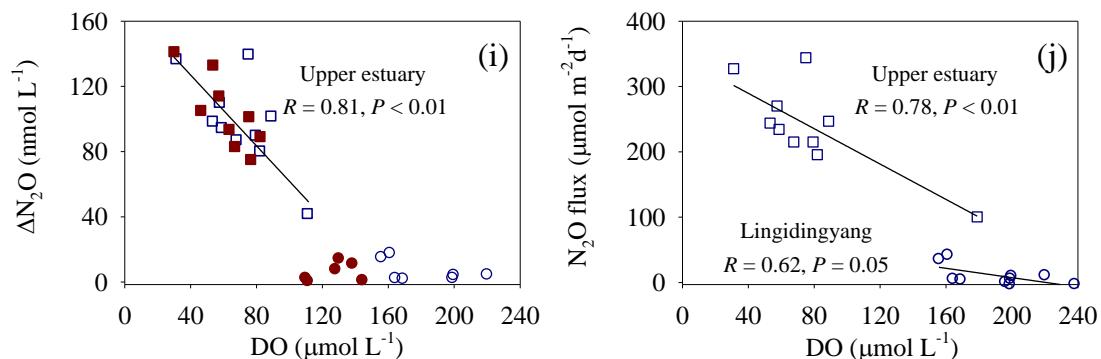


Figure R4: (i) $\Delta\text{N}_2\text{O}$ vs. DO and (j) N_2O flux vs. DO.

p. 32 I found this figure confusing. Perhaps it would be useful to have a table with the data presented in the figure? It is unclear using AOB and AOA% if the normalized N_2O production values are a result of the N_2O yield or low/high amoA abundance.

Response:

We added Table S3 (below) with the data that presented in the figure. 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 amoA gene copies (sum of PA and FL fractions or only PA fraction) or transcripts (only PA fraction) along X-Y axes that represent the relative contributions of AOA and AOB to the total amoA gene or transcript pools. For both incubation sites, the more abundant AOB were in the amoA gene-based DNA or cDNA pool, the distinctly higher (disproportionately higher relative to enhanced abundance) the average amoA gene copy or transcript-specific N_2O production rates (Fig. 7a) and yields (Fig. 7b), suggesting that AOB may be more active in producing N_2O than AOA. AOB may contribute the major part in N_2O production with their high cell-specific activity in the upper estuary.

Table R3 (Table S3 in the MS) The abundances of DNA/cDNA-based *amoA* gene and the N₂O production rates and yields normalized to total *amoA* gene copy or transcript numbers of AOA and AOB in a given sample at the incubation experiment sites.

Site_Layer	DNA-based AOB (All) (copies L ⁻¹)	DNA-based AOA (All) (copies L ⁻¹)	N ₂ O production rates (All) (f mol cell ⁻¹ h ⁻¹)	N ₂ O yields (All) (10 ⁻⁶)	DNA-based AOB (PA) (copies L ⁻¹)	DNA-based AOA (PA) (copies L ⁻¹)	N ₂ O production rates (PA) (f mol cell ⁻¹ h ⁻¹)	N ₂ O yields (PA) (10 ⁻⁶)	cDNA-based AOB (PA) (copies L ⁻¹)	cDNA-based AOA (PA) (copies L ⁻¹)	N ₂ O production rates (PA) (f mol cell ⁻¹ h ⁻¹)	N ₂ O yields (PA) (10 ⁻⁶)
P05_S	14030	34427	23.70	21.30	12125	29082	27.90	25.00	382928	138646	2.20	1.97
P05_B	87915	397740	2.90	3.25	77820	357308	3.24	3.63	89559	12559	13.80	15.50
P01_S	19623	642905	0.91	1.93	9343	578974	1.02	2.18	500	461578	1.30	2.77
P01_B	21334	251163	5.91	5.47	16458	221184	6.77	6.27	362	7436	206.00	191.00

S, surface; B, bottom; All, sum of particle-attached and free-living fractions; PA, particle-attached fraction.

Technical corrections

p. 2 lines 18-22 Needs citation “Denitrification by heterotrophic denitrifiers is another major pathway of N₂O production in marine environments. NO₂⁻ is reduced by a copper-containing (NirK) or cytochrome cd1-containing nitrite reductase (NirS) to nitric oxide (NO), and then by a heme-copper NO reductase (NOR) to N₂O.”

Response:

We added citations.

“Denitrification is another pathway of N₂O production in marine environments, occurring under anoxic conditions or at the suboxic-anoxic interface (Naqvi et al., 2000; Yamagishi et al., 2007; Ji et al., 2018). NO₂⁻ is reduced by a copper-containing (NirK) or cytochrome cd1-containing nitrite reductase (NirS) to nitric oxide (NO), and then by a heme-copper NO reductase (NOR) to N₂O (Coyne et al., 1989; Treusch et al., 2005; Abell et al., 2010; Bartossek et al., 2010; Lund et al., 2012; Graf et al., 2014). As an intermediary product during denitrification, production and further reduction of N₂O are sensitive to different O₂ conditions (Babbin et al., 2015; Ji et al., 2015).”

References:

- Abell, G. C. J., Revill, A. T., Smith, C., Bissett, A. P., Volkman, J. K., and Robert, S. S.: Archaeal ammonia oxidizers and *nirS*-type denitrifiers dominate sediment nitrifying and denitrifying populations in a subtropical macrotidal estuary, ISME J., 4, 286–300, 2010.
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- Bartossek, R., Nicol, G.W., Lanzen, A., Klenk, H. P., and Schleper, C.: Homologues of nitrite reductases in ammonia-oxidizing archaea: diversity and genomic context, Environ. Microbiol., 12, 1075–1088, 2010.
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- Graf, D. R. H., Jones, C. M., and Hallin, S.: Intergenomic comparisons highlight

- modularity of the denitrification pathway and underpin the importance of community structure for N₂O emissions. PloS One 9: e114118. <https://doi.org/10.1371/journal.pone.0114118.s008>. 2014.
- Ji, Q., Babbin, A. R., Jayakumar, A., Oleynik, S., and Ward, B. B.: Nitrous oxide production by nitrication and denitrification in the Eastern Tropical South Pacific oxygen minimum zone, Geophy. Res. Lett., 42, 10, 755–10, 764, 2015.
- Ji, Q., Buitenhuis, E., Suntharalingam, P., Sarmiento, J. L., and Ward, B. B.: Global Nitrous Oxide Production Determined by Oxygen Sensitivity of Nitrification and Denitrification, Global Biogeochem. Cy., 32, 1790–1802, 2018.
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- Naqvi, S. W. A., Bange, H. W., Farás, L., Monteiro, P. M. S., Scranton, M. I., and Zhang, J.: Marine hypoxia/anoxia as a source of CH₄ and N₂O, Biogeosciences, 7, 2159–2190, 2010.
- Yamagishi, H., Westley, M. B., Popp, B. N., Toyoda, S., Yoshida, N., Watanabe, S., Koba, K., and Yamanak, Y.: Role of nitrification and denitrification on the nitrous oxide cycle in the eastern tropical North Pacific and Gulf of California, J Geophy. Res., 112, G02015, doi:10.1029/2006JG000227, 2007
- Treusch, A. H., Leininger, S., Kletzin, A., Schuster, S. C., Klenk, H., and Schleper, C.: Novel genes for nitrite reductase and Amo-related proteins indicate a role of uncultivated mesophilic crenarchaeota in nitrogen cycling, Environ. Microbiol., 7, 1985–1995, 2005.

p.3 lines 3 citation should be after “soil” “and arable (Clark et al., 2012; Jones et al., 2014) soils”

Response:

Sorry for this. Revised.

p. 3 lines 10-11 Needs citation

“Moreover, there is a potential niche overlap between nitrifiers and denitrifiers in low oxygen conditions.”

Response:

We added three citations as follows:

Frame, C. H., and Casciotti, K. L.: Biogeochemical controls and isotopic signatures of nitrous oxide production by a marine ammonia-oxidizing bacterium, *Biogeosciences*, 7, 2695–2709, 2010.

Penn, J., Weber, T., and Deutsch, C.: Microbial functional diversity alters the structure and sensitivity of oxygen deficient zones, *J. Geophys. Res. Lett.*, 43, 9773–9780, 2016.

Zhang, Y., Xie, X., Jiao, N., Hsiao, S. S. Y., and Kao, S. J.: Diversity and distribution of *amoA*-type nitrifying and *nirS*-type denitrifying microbial communities in the Yangtze River estuary, *Biogeosciences*, 11, 2131–2145, 2014.

p. 4 lines 15-16 Should be moved to results section 2.2 discussing ammonia analysis
“Ammonia/ammonium concentrations were analyzed onboard.”

Response:

We moved this sentence to section 2.2.

“Ammonia was measured using the indophenol blue spectrophotometric method (Pai et al., 2001) on board”

p. 4 line 25-26 What salinity, temperature and DO probes were used?

Response:

We revised this sentence.

“Temperature and salinity were determined with a SBE 25 conductivity–temperature–depth/pressure unit (Sea-Bird Co.). DO were determined with a SBE 43 Dissolved Oxygen Sensor (Sea-Bird Co.). All DO concentrations used in this study was measured using the Winkler method.”

p. 5 lines 5-23 Not all variables in the equations are defined.

Response:

We added more details for the equations and defined the variables in the revised section 2.2 (see below highlighted).

“The excess N_2O (ΔN_2O) and N_2O saturation were calculated with Eq. (1) and (2):

$$\Delta N_2O = N_2O_{\text{observed}} - N_2O_{\text{equilibrium}} \quad (1)$$

$$S(\%) = N_2O_{\text{observed}} / N_2O_{\text{equilibrium}} \times 100\% \quad (2)$$

where N_2O_{observed} represents the measured concentrations of N_2O in the water, and the equilibrium values of N_2O ($N_2O_{\text{equilibrium}}$) are calculated by Eq. (3) and (4) (Weiss and Price, 1980):

$$N_2O_{\text{equilibrium}} = xF \quad (3)$$

$$\ln F = A_1 + A_2(100/T) + A_3 \ln(T/100) + A_4(T/100)^2 + S[B_1+B_2(T/100) + B_3(T/100)^2] \quad (4)$$

where x is the mole fraction of N_2O in the atmosphere and T is the absolute temperature. In this study, we used the global mean atmospheric N_2O (327 ppb) from 2015 (<http://www.esrl.noaa.gov/gmd>). The fitted function F with constants $A_1, A_2, A_3, A_4, B_1, B_2$, and B_3 was proposed by Weiss and Price (1980).

The N_2O flux (F_{N_2O} , $\mu\text{mol m}^{-2} \text{ d}^{-1}$) through the air-sea interface was estimated based on Eq. (5):

$$F_{N_2O} = k_{N_2O} \times \rho \times K_H^{N_2O} \times \Delta p_{N_2O} = k_{N_2O} \times 24 \times 10^2 \times (N_2O_{\text{observed}} - N_2O_{\text{equilibrium}}) \quad (5)$$

where k_{N_2O} (cm h^{-1}) is the N_2O gas transfer velocity depending on wind and water temperatures, $K_H^{N_2O}$ is the solubility of N_2O , and Δp_{N_2O} is the average sea-gas N_2O partial pressure difference. In this study, k_{600} , the gas transfer velocity at a Schmidt number of 600, is used for the estuarine system (Raymond and Cole, 2001). The Schmidt number (Sc) is defined as the kinematic viscosity of water divided by the diffusion coefficient of the gas, and is usually expressed as a function of temperature and salinity (Wanninkhof, 1992). For steady winds with the average climatological wind speed at 10 m above the water surface, the relationship between gas transfer and wind speed is estimated using Eq. (6) according to Wanninkhof (1992):

$$k_{600} = 0.31 \times u_{10}^2 \times (S_c/600)^{-0.5} \quad (6)$$

For N_2O in waters of salinity <35 and temperature ranging from 0–30 °C, Sc_{N_2O} is estimated by the following Eq. (7) according to Wanninkhof (1992):

$$S_{cN_2O} = 2055.6 - 137.11 t + 4.3173 t^2 - 0.05435 t^3 \quad (7)$$

where t is in situ temperature of the sampling site.

p. 31 Fig 6 Should axes be swapped?

Response:

We swapped X and Y axes in Fig 6 of the revised version according to the reviewer's suggestion.