

Dear Dr. Pantoja,

Thank you very much for the valuable comments and careful revisions, based on which we have revised the manuscript again. Our responses to all comments are listed below. Thank you again for your time and kind efforts.

Best wishes,

Yao Zhang

**Manuscript Number: bg-2019-132**

**Manuscript title: Major role of ammonia-oxidizing bacteria in N<sub>2</sub>O production in the Pearl River Estuary**

**Response to Editor**

Comments to the Author:

Review of bg-2019-132

October 25, 2019

Dear authors,

Thank you for providing a revised version of the manuscript. From my point of view some of the aspects brought up by the reviewers could be addressed more carefully:

1. Abstract, L.10 “However, biological sources of N<sub>2</sub>O in estuarine ecosystems remain controversial, but are of great importance for understanding global N<sub>2</sub>O emission patterns.”

“However, knowledge on or discrimination of biological sources of N<sub>2</sub>O in estuarine ecosystems remains controversial, but are of great importance for understanding global N<sub>2</sub>O emission patterns. ”

Knowing “biological sources of N<sub>2</sub>O is of great importance for understanding global N<sub>2</sub>O emission patterns”, not sources by themselves. Please rephrase accordingly

**Response:**

We thank the editor for this correction. We have revised this sentence as “*However, knowledge on biological sources of N<sub>2</sub>O in estuarine ecosystems remains controversial, but are of great importance for understanding global N<sub>2</sub>O emission*

*patterns.*” (Page 1, Lines 9–10)

2. Abstract, L. 14. “Our results indicated that nitrification predominantly occurred, with significant N<sub>2</sub>O production during ammonia oxidation, in the hypoxic waters of the upper estuary where the maximum N<sub>2</sub>O and DN<sub>2</sub>O excess concentrations were observed, although minor denitrification might be concurrent at the site with the lowest dissolved oxygen.” This sentence is difficult to understand as it is. Do you mean this?:

“Our results indicated that nitrification predominantly occurred, with significant N<sub>2</sub>O production during ammonia oxidation. In the hypoxic waters of the upper estuary where the maximum N<sub>2</sub>O and DN<sub>2</sub>O excess concentrations were observed, although minor denitrification might be concurrent at the site with the lowest dissolved oxygen.”

**Response:**

We thank the editor for this correction. We have revised this sentence as “*Our results indicated that nitrification predominantly occurred, with significant N<sub>2</sub>O production during ammonia oxidation. In the hypoxic waters of the upper estuary, strong nitrification resulted in the observed maximum N<sub>2</sub>O and  $\Delta N_2O_{excess}$  concentrations, although minor denitrification might be concurrent at the site with the lowest dissolved oxygen.*” (Page 1, Line 14–18)

3. Page 4, L. 5. “The Pearl River Estuary (PRE) is one of the world’s most complex estuarine systems with a total discharge of 285.2×10<sup>9</sup> m<sup>3</sup> yr<sup>-1</sup> (Dai et al., 2014). The PRE is surrounded by complex regions with a rich nitrogen supply that produces eutrophic waters (Dai et al., 2008).”

complex is repeated in both continuous sentences.

**Response:**

We have revised this sentence as “*The Pearl River Estuary, surrounded by several big cities, is one of the world’s most complex estuarine systems with a total discharge of 285.2×10<sup>9</sup> m<sup>3</sup> yr<sup>-1</sup> (Dai et al., 2014). A rich nitrogen supply with the river discharge produces eutrophic waters in the estuary (Dai et al., 2008).*” (Page 4, Line 5–7).

4. Discussion, How is that “The in situ incubation experiments clearly indicated that nitrification predominantly occurred ...” from Figure 5?

**Response:**

This sentence was based on the results presented in Figure 2 and Figure 5. Figure 2 showed the distribution of biogeochemical factors along the estuary transect, including the N<sub>2</sub>O related parameters, dissolved oxygen concentration, etc. **Sites P01 and P05 were located in the hypoxic zone (DO < 63.0 μmol L<sup>-1</sup>; Rabalais et al., 2010)** and the maximum N<sub>2</sub>O and ΔN<sub>2</sub>O<sub>excess</sub> concentrations were observed at P01 and P05. Figure 5 showed the incubations experiments results of P01 and P05, indicating that nitrification predominantly occurred at the both sites (i.e. in the hypoxic waters). The detailed explanation on Figure 5 results have been stated in the Result 3.4 subsection.

For a clearer statement, we have revised this sentence as “*The in situ incubation experiments clearly indicated that nitrification predominantly occurred in the hypoxic waters (e.g. both the P01 and P05 sites) of the upper estuary along with significant N<sub>2</sub>O production, and suggested that denitrification could be concurrent at the lowest DO site (P01) where the maximum N<sub>2</sub>O and ΔN<sub>2</sub>O<sub>excess</sub> concentrations were observed (Figs. 2 and 5).*” (Page 13, Lines 20–24)

**Reference:**

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

5. Show Lingdingyang in Fig. 1.

**Response:**

We have revised Figure 1 as suggested.

6. Caption Fig. 1. Biogeochemical analyses are in red and green, but gene analyses are green; same color? This is confusing for readers. Please modify

**Response:**

We have revised the Fig. 1 legend as “... *Biochemical parameters analyses were performed on samples from all sites (green and red circles). The green circles indicate sites where genes were additionally analyzed. ...*” (Page 29, Lines 2–4)

7. Figure 2. Is Upper estuary in graphs i and j same as upstream of the Humen outlet? Please normalize names and explain.

**Response:**

Yes, they are the same. We have revised “upper estuary” as “upstream of the Humen outlet” in Figure 2i and j and the corresponding text (Page 10, Line 18) as suggested.

We also have explained “upper estuary = upstream of the Humen outlet” in the Methods 2.1 subsection of the manuscript. —“*A total of 22 sites along the salinity gradient of the Pearl River Estuary were sampled during a research cruise in July 2015, including 11 sites in the upper reaches (upstream of the Humen outlet) and 11 sites in the lower reaches (Lingdingyang) (Fig. 1).*” (Page 4, Lines 21–23). So we kept the use of “upper estuary” in some other places of the manuscript.

8. Page 10, lines 20-22. “Overall, upstream of the Humen outlet was characterized by hypoxic waters rich in nitrogen-based nutrients, where ammonium concentrations decreased and the sum of nitrite and nitrate concentrations increased seaward, corresponding to distinctly higher N<sub>2</sub>O fluxes released to the atmosphere. ”

a) In Fig. 2, only one data point (P01) is almost hypoxic (ca. 25 micromol O<sub>2</sub> /L.

**Response:**

According to Rabalais et al. (2010, Biogeoscience) indicating that “...a commonly used definition of hypoxia is dissolved oxygen < 2 mg L<sup>-1</sup>, the equivalent of 1.4 ml L<sup>-1</sup> or 63 μM”, we used 63 μM as a definition of hypoxia in this manuscript. We have stated this definition in subsection 3.1 —“*The DO concentrations were distinctly lower upstream of the Humen outlet with nearly one-half of the samples below the hypoxic threshold (63.0 μmol L<sup>-1</sup>; Rabalais et al., 2010)*”. (Page 10, 5–7)

In addition, a commonly used definition of “suboxic” is DO < 10 μM and “anoxic” is DO ≈ 0 μM according to Stramma et al. (2008, Science) —“Regions with oxygen concentrations below about 10 μmol kg<sup>-1</sup> are termed suboxic.” “Anoxic regions have

no dissolved oxygen.”

Babbin et al. (2015, Science) also stated a similar definition of suboxic waters —“The most concentrated oceanic sources of N<sub>2</sub>O to the atmosphere are the suboxic (0 to 20  $\mu\text{mol L}^{-1}$ ) waters.”

#### References:

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

Stramma, L., Johnson, G. C., Sprintall, J., and Mohrholz, V.: Expanding oxygen-minimum zones in the tropical oceans, *Science*, 320, 655–658, 2008.

Babbin, A. R., Bianchi, D., Jayakumar, A., and Ward, B. B.: Rapid nitrous oxide cycling in the suboxic ocean, *Science*, 348, 1127–1129, 2015.

b) “...increased seaward, corresponding to distinctly higher N<sub>2</sub>O fluxes released to the atmosphere”. Where are you seeing “higher N<sub>2</sub>O fluxes released” in Fig. 2d?

#### Response:

Figure 2g showed N<sub>2</sub>O flux. The plus values of this parameter represent the excess N<sub>2</sub>O in water release to the atmosphere through the air-sea interface. We have stated the detailed Fig. 2g results directly above this sentence (Page 10, Lines 14–17).

c) In the discussion, the hypoxia issue continues: “The in situ incubation experiments clearly indicated that nitrification predominantly occurred in the hypoxic waters of the upper estuary along with significant N<sub>2</sub>O production, and suggested that denitrification could be concurrent at the lowest DO site (P01) where the maximum N<sub>2</sub>O and DN<sub>2</sub>O excess concentrations were observed.”

This aspect needs to be fixed throughout the whole ms.

#### Response:

Please refer to our above response. According to Rabalsais et al. (2010, Biogeoscience) indicating that “...a commonly used definition of hypoxia is dissolved oxygen < 2 mg L<sup>-1</sup>, the equivalent of 1.4 ml L<sup>-1</sup> or 63  $\mu\text{M}$ ”, we used 63  $\mu\text{M}$  as a definition of hypoxia in this manuscript. We have stated this definition in subsection 3.1 —“*The DO*

*concentrations were distinctly lower upstream of the Humen outlet with nearly one-half of the samples below the hypoxic threshold ( $63.0 \mu\text{mol L}^{-1}$ ; Rabalais et al., 2010)”. (Page 10, 5–7)*

For a clearer statement, we also have revised this sentence as “*The in situ incubation experiments clearly indicated that nitrification predominantly occurred in the hypoxic waters (e.g. both the P01 and P05 sites) of the upper estuary along with significant  $\text{N}_2\text{O}$  production, and suggested that denitrification could be concurrent at the lowest DO site (P01) where the maximum  $\text{N}_2\text{O}$  and  $\Delta\text{N}_2\text{O}_{\text{excess}}$  concentrations were observed (Figs. 2 and 5).*” (Page 13, Lines 20–24)

9. Could you avoid non-standard abbreviations such as PRE, PA, FA, FDR, RDA, etc.

It is very difficult to follow for readers

**Response:**

Thanks for this comment. We have removed these abbreviations throughout the manuscript.

# 1 Major role of ammonia-oxidizing bacteria in N<sub>2</sub>O production in the Pearl River Estuary

2 Li Ma<sup>1,2</sup>, Hua Lin<sup>1,2,3</sup>, Xiabing Xie<sup>1</sup>, Minhan Dai<sup>1,2</sup>, Yao Zhang<sup>1,2</sup>

3 <sup>1</sup>State Key Laboratory of Marine Environmental Science, Xiamen University, Xiamen 361101, China

4 <sup>2</sup>College of Ocean and Earth Sciences, Xiamen University, Xiamen 361101, China

5 <sup>3</sup>Key Laboratory of Marine Ecosystem and Biogeochemistry, State Oceanic Administration, Second  
6 Institute of Oceanography, Ministry of Natural Resources, Hangzhou 310012, China

7 Correspondence to: Yao Zhang (yaozhang@xmu.edu.cn)

8 **Abstract.** Nitrous oxide (N<sub>2</sub>O) has significant global warming potential as a greenhouse gas. Estuarine  
9 and coastal regimes are the major zones of N<sub>2</sub>O production in the marine system. However, **knowledge**  
10 **on** biological sources of N<sub>2</sub>O in estuarine ecosystems remain controversial, but are of great importance  
11 for understanding global N<sub>2</sub>O emission patterns. Here, we measured concentrations and isotopic  
12 compositions of N<sub>2</sub>O as well as distributions of ammonia-oxidizing bacterial and archaeal *amoA* and  
13 denitrifier *nirS* genes by quantitative polymerase chain reaction along a salinity gradient in the Pearl  
14 River Estuary, and performed in situ incubation experiments to estimate N<sub>2</sub>O yields. Our results  
15 indicated that nitrification predominantly occurred, with significant N<sub>2</sub>O production during ammonia  
16 oxidation. **In the hypoxic waters of the upper estuary, strong nitrification resulted in the observed**  
17 **maximum N<sub>2</sub>O and ΔN<sub>2</sub>O<sub>excess</sub> concentrations, although minor denitrification might be concurrent at the**  
18 **site with the lowest dissolved oxygen. Ammonia-oxidizing β-proteobacteria (AOB) were significantly**  
19 **positively correlated with all N<sub>2</sub>O-related parameters, although their *amoA* gene abundances were**  
20 **distinctly lower than ammonia-oxidizing Archaea (AOA) throughout the estuary. Furthermore, the N<sub>2</sub>O**  
21 **production rate and the N<sub>2</sub>O yield normalized to *amoA* gene copies or transcripts estimated a higher**  
22 **relative contribution of AOB to the N<sub>2</sub>O production in the upper estuary. Taken together, the in situ**  
23 **incubation experiments, N<sub>2</sub>O isotopic composition and concentrations, and gene datasets suggested that**  
24 **the high concentration of N<sub>2</sub>O (oversaturated) is mainly produced from strong nitrification by the**  
25 **relatively high abundance of AOB in the upper reaches and is the major source of N<sub>2</sub>O emitted to the**  
26 **atmosphere in the Pearl River Estuary.**

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## 1 **1 Introduction**

2 Nitrous oxide ( $\text{N}_2\text{O}$ ) is a potent greenhouse gas with global warming potential 298 times that of carbon  
3 dioxide ( $\text{CO}_2$ ) on a 100 yr timescale, and contributes to stratospheric ozone depletion as a major  
4 precursor of free radicals (Ravishankara et al., 2009).  $\text{N}_2\text{O}$  emissions from soils and marine systems are  
5 estimated to account for 56%–70% (6–7 Tg  $\text{N}_2\text{O}$ -N  $\text{yr}^{-1}$ ) (Syakila and Kroeze, 2011; Butterbach-Bahl et  
6 al., 2013; Hink et al., 2017) and 30% (4 Tg  $\text{N}_2\text{O}$ -N  $\text{yr}^{-1}$ ) (Nevison et al., 2004; Naqvi et al., 2010; Voss  
7 et al., 2013) of the total global  $\text{N}_2\text{O}$  emissions, respectively. The main processes responsible for  $\text{N}_2\text{O}$   
8 emissions are microbial transformation of ammonia, nitrite, and nitrate through nitrification and  
9 denitrification (Butterbach-Bahl et al., 2013). It has been estimated that oceanic  $\text{N}_2\text{O}$  production is  
10 dominated by nitrification, whereas only 7% is contributed by denitrification (Freing et al., 2012).

11  $\text{N}_2\text{O}$  is released as a byproduct during nitrification via incomplete oxidation of hydroxylamine  
12 ( $\text{NH}_2\text{OH}$ ) to nitrite ( $\text{NO}_2^-$ ) by ammonia-oxidizing bacteria (AOB) (Stein, 2011). This process may be  
13 enhanced under suboxic conditions (Naqvi et al., 2010). While no equivalent of the hydroxylamine-  
14 oxidoreductase that catalyzes  $\text{N}_2\text{O}$  formation through  $\text{NH}_2\text{OH}$  oxidation has been found in ammonia-  
15 oxidizing archaea (AOA) (Hatzenpichler, 2012), recent studies indicated that AOA possibly produces  
16 hybrid  $\text{N}_2\text{O}$  via a combination of an ammonia oxidation intermediate ( $\text{NH}_2\text{OH}$ ,  $\text{HNO}$ , or  $\text{NO}$ ) and  $\text{NO}_2^-$   
17 (Stieglmeier et al., 2014; Frame et al., 2017). In addition, AOB have been shown to produce  $\text{N}_2\text{O}$  from  
18  $\text{NO}_2^-$  during nitrifier denitrification (Shaw et al., 2006). This process is also promoted under micro-oxic  
19 and anoxic conditions (Yu et al., 2010). Denitrification by heterotrophic denitrifiers is another major  
20 pathway of  $\text{N}_2\text{O}$  production in marine environments, occurring under anoxic conditions or at the  
21 suboxic–anoxic interface (Naqvi et al., 2010; Yamagishi et al., 2007; Ji et al., 2018).  $\text{NO}_2^-$  is reduced by  
22 a copper-containing (NirK) or cytochrome cd1-containing nitrite reductase (NirS) to nitric oxide ( $\text{NO}$ ),  
23 and then by a heme-copper  $\text{NO}$  reductase (NOR) to  $\text{N}_2\text{O}$  (Coyne et al., 1989; Treusch et al., 2005;  
24 Abell et al., 2010; Bartossek et al., 2010; Lund et al., 2012; Graf et al., 2014). As an intermediary  
25 product during denitrification, production and further reduction of  $\text{N}_2\text{O}$  are sensitive to different  $\text{O}_2$   
26 conditions (Babbin et al., 2015; Ji et al., 2015).

27 Biological nitrogen transformations are catalyzed by various microbial enzymes, of which  
28 ammonium monooxygenase (AMO) and nitrite reductases (NIRs) are key enzymes responsible for



1 nitrification and denitrification, respectively (Canfield et al., 2010). The genes encoding for AMO  
2 subunit A (*amoA*) and NIRs (*nirS* and *nirK*) have been widely applied as functional marker genes to  
3 identify the distribution of ammonia oxidizers and denitrifiers. Previous studies have shown significant  
4 correlations of *amoA* with spatial variations of N<sub>2</sub>O emissions or N<sub>2</sub>O production rates in soils and  
5 oceans (Avrahami and Bohanann, 2009; Santoro et al., 2011; Löscher et al., 2012). In addition,  
6 significant relationships between *nirK* or *nirS* abundances and N<sub>2</sub>O emissions were observed in  
7 grasslands (Čuhel et al., 2010), arable soils (Clark et al., 2012; Jones et al., 2014), and the ocean  
8 (Arévalo-Martínez et al., 2015).

9 Estuaries are highly impacted by coastal nutrient pollution and eutrophication because of  
10 anthropogenic activity; they play a significant role in nitrogen cycling at the land–sea interface (Bricker  
11 et al., 2008; Damashek et al., 2016; Damashek and Francis, 2018). Estuarine and coastal regimes have  
12 long been recognized as major zones of N<sub>2</sub>O production in the marine system (Seitzinger and Kroeze,  
13 1998; Mortazavi et al., 2000; Usui et al., 2001; Kroeze et al., 2010; Allen et al., 2011). In particular,  
14 eutrophic estuaries with extensive oxygen-deficient zones have been considered hotspot regions for  
15 N<sub>2</sub>O production (Abril et al., 2000; De Wilde and De Bie, 2000; Garnier et al., 2006; Lin et al., 2016),  
16 with oversaturated N<sub>2</sub>O and high N<sub>2</sub>O concentrations and flux (De Wilde and De Bie, 2000; De Bie et  
17 al., 2002; Garnier et al., 2006; Rajkumar et al., 2008; Barnes and Upstill-Goddard, 2011; Lin et al.,  
18 2016). The dynamics of N<sub>2</sub>O emissions in these ecosystems are regulated by complex physical and  
19 biogeochemical processes; for example, mixing between freshwater and oceanic waters influences the  
20 biogeochemistry of estuarine waters as well as microbial activity (Huertas et al., 2018; Laperriere et al.,  
21 2019).

22 Nitrification is often credited as the dominant N<sub>2</sub>O production pathway in estuaries (De Bie et al.,  
23 2002; Barnes and Upstill-Goddard, 2011; Kim et al., 2013; Lin et al., 2016; Huertas et al., 2018;  
24 Laperriere et al., 2019). Although AOA frequently outnumber AOB and dominate microbial  
25 communities, their contribution to nitrification remains controversial in estuarine and coastal waters  
26 (Bernhard et al., 2010; Zhang et al., 2014; Hou et al., 2018). Furthermore, the relative contributions of  
27 AOB and AOA to N<sub>2</sub>O production is inconclusive (Monteiro et al., 2014) and there is a potential niche  
28 overlap between nitrifiers and denitrifiers in low oxygen conditions (Frame and Casciotti, 2010; Zhang

1 et al., 2014; Penn et al., 2016). AOB are reported to thrive in hypoxic environments and denitrification  
2 in the oxic ocean is suggested to occur within anoxic particle interiors (Frame and Casciotti, 2010; Ni et  
3 al., 2014). It is therefore of great importance to elucidate the biological sources of N<sub>2</sub>O production in  
4 estuarine ecosystems to better understanding global N<sub>2</sub>O emission patterns.

5 The Pearl River Estuary, surrounded by several big cities, is one of the world's most complex  
6 estuarine systems with a total discharge of  $285.2 \times 10^9 \text{ m}^3 \text{ yr}^{-1}$  (Dai et al., 2014). A rich nitrogen supply  
7 with the river discharge, produces eutrophic waters in the estuary (Dai et al., 2008). Moreover, increased  
8 oxygen consumption by organic matter degradation leads to the formation of hypoxic zones in the upper  
9 reaches of the estuary (Dai et al., 2006; He et al., 2014), which may support strong nitrification,  
10 denitrification, and N<sub>2</sub>O production (Lin et al., 2016). In this study, N<sub>2</sub>O-related biogeochemical  
11 parameters were measured, and distributions of AOB and AOA *amoA* and denitrifier *nirS* genes were  
12 quantified by quantitative polymerase chain reaction (qPCR) to investigate the relationship between  
13 N<sub>2</sub>O production and spatial distribution of nitrifiers and denitrifiers along a salinity gradient in the Pearl  
14 River Estuary (Fig. 1). Moreover, in situ incubation experiments were performed in the hypoxic upper  
15 estuary to estimate (1) nitrification and N<sub>2</sub>O production rates, (2) whether denitrification occurred  
16 during nitrification, and (3) N<sub>2</sub>O yield (mol N<sub>2</sub>O-N produced per mol ammonia oxidized). By  
17 combining the genetic datasets and incubation estimates, this study thus identified the relative  
18 contributions of AOB and AOA in producing N<sub>2</sub>O in the Pearl River Estuary.

## 19 2 Materials and methods

### 20 2.1 Study area and sampling

21 A total of 22 sites along the salinity gradient of the Pearl River Estuary were sampled during a research  
22 cruise in July 2015, including 11 sites in the upper reaches (upstream of the Humen outlet) and 11 sites  
23 in the lower reaches (Lingdingyang) (Fig. 1). Water samples were taken from the surface (2 m) and  
24 bottom (4–15 m) of each site by using a conductivity, temperature, and depth (CTD) rosette sampling  
25 system (SBE 25; Sea-Bird Scientific, USA) fitted with 12 L Niskin bottles (General Oceanics). A total  
26 of 16 samples (from two depths at eight sites) were subjected to gene analysis (Fig. 1). A total of 1 L of

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1 water for gene analysis was serially filtered through 0.8  $\mu\text{m}$  and then 0.22  $\mu\text{m}$  pore size polycarbonate  
2 membrane filters (47 mm diameter, Millipore) within 30 min at a pressure  $<0.03$  MPa to retain the  
3 particle-associated communities ( $>0.8$   $\mu\text{m}$ ) and free-living communities (0.22–0.8  $\mu\text{m}$ ). For the upper  
4 estuary samples, more membrane filters were used to avoid the filters clogging. RNAlater solution  
5 (Ambion, Austin, Texas, USA) was quickly added to the samples to prevent RNA degradation. All of  
6 the filters were immediately flash frozen in liquid nitrogen and then stored at  $-80$   $^{\circ}\text{C}$  until further  
7 analysis. Water samples for nutrient determination were filtered through 0.45  $\mu\text{m}$  pore size cellulose  
8 acetate membranes and then immediately frozen at  $-20$   $^{\circ}\text{C}$  until further analysis. Water samples for  
9 dissolved  $\text{N}_2\text{O}$  were collected into 125 mL headspace glass bottles to which 100  $\mu\text{L}$  of saturated  $\text{HgCl}_2$   
10 was added; the bottles were immediately closed with rubber stoppers and aluminum crimp-caps and  
11 stored in the dark at 4  $^{\circ}\text{C}$  until analysis in the laboratory. All  $\text{N}_2\text{O}$  samples were collected during the July  
12 2015 cruise except for samples from sites P03, P05, A01, A06, and A10 intended for  $\text{N}_2\text{O}$  isotopic  
13 composition analyses, which were sampled during a cruise in March 2010. Total suspended material  
14 (TSM) was collected by filtering 1–4 L of water onto pre-combusted and pre-weighed glass fiber filters  
15 (GF/Fs) (Whatman), and then stored at  $-20$   $^{\circ}\text{C}$  until weighing in the laboratory.

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## 16 **2.2 Biogeochemical parameters, $\text{N}_2\text{O}$ emissions and isotopic analysis of environmental samples**

17 Temperature and salinity were measured with the SBE 25 CTD system. Dissolved oxygen (DO)  
18 concentrations were measured using the Winkler method (Dai et al., 2006). Ammonia was measured  
19 using the indophenol blue spectrophotometric method (Pai et al., 2001) on board; nitrate, nitrite, and  
20 silicate were analyzed using routine spectrophotometric methods with a Technicon AA3 Auto-Analyzer  
21 (Bran-Lube, GmbH) (Han et al., 2012).  $\text{N}_2\text{O}$  concentrations were analyzed by gas chromatography (GC,  
22 Agilent 6890  $\mu\text{ECD}$ ) coupled with a purge-trap system (Tekmar Velocity XPT) at 25  $^{\circ}\text{C}$  (Lin et al.,  
23 2016).  $\text{N}_2\text{O}$  standard gases of 1.02 and 2.94 ppmv  $\text{N}_2\text{O}/\text{N}_2$  (National Center of Reference Material,  
24 China, Beijing) were used. The relative standard deviation of the slope of the standard working curve  
25 was 1.77% ( $n=8$ ). The detection limit was calculated to be  $\sim 0.1$   $\text{nmol L}^{-1}$  and the precision was better  
26 than  $\pm 5\%$ . When water samples were analyzed, every 5–10 samples were spiked with  $\text{N}_2\text{O}$  standards to  
27 calibrate the GC.

1 The excess N<sub>2</sub>O ( $\Delta N_{2}O_{\text{excess}}$ ) and N<sub>2</sub>O saturation (S%) were calculated with Eq. (1) and (2):

$$2 \Delta N_{2}O_{\text{excess}} = N_{2}O_{\text{observed}} - N_{2}O_{\text{equilibrium}} \quad (1)$$

$$3 S\% = N_{2}O_{\text{observed}} / N_{2}O_{\text{equilibrium}} \times 100\% \quad (2)$$

4 where  $N_{2}O_{\text{observed}}$  represents the measured concentrations of N<sub>2</sub>O in the water, and the equilibrium  
5 values of N<sub>2</sub>O ( $N_{2}O_{\text{equilibrium}}$ ) were calculated by Eq. (3) and (4) (Weiss and Price, 1980):

$$6 N_{2}O_{\text{equilibrium}} = xF \quad (3)$$

$$7 \ln F = A_1 + A_2(100/T) + A_3 \ln(T/100) + A_4(T/100)^2 + S[B_1 + B_2(T/100) + B_3(T/100)^2] \quad (4)$$

8 where  $x$  is the mole fraction of N<sub>2</sub>O in the atmosphere and  $T$  is the absolute temperature. In this study,  
9 we used the global mean atmospheric N<sub>2</sub>O (327 ppb) from 2015 (<http://www.esrl.noaa.gov/gmd>). The  
10 fitted function  $F$  and constants  $A_1$ ,  $A_2$ ,  $A_3$ ,  $A_4$ ,  $B_1$ ,  $B_2$  and  $B_3$  were proposed by Weiss and Price  
11 (1980).

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

$$13 F_{N_2O} = k_{N_2O} \times \rho \times K_H^{N_2O} \times \Delta p N_2O = k_{N_2O} \times 24 \times 10^{-2} \times (N_{2}O_{\text{observed}} - N_{2}O_{\text{equilibrium}}) \quad (5)$$

14 where  $k_{N_2O}$  ( $\text{cm h}^{-1}$ ) is the N<sub>2</sub>O gas transfer velocity depending on wind and water temperature,  $K_H^{N_2O}$  is  
15 the solubility of N<sub>2</sub>O, and  $\Delta p N_2O$  is the average sea–gas N<sub>2</sub>O partial pressure difference.  $k_{N_2O}$  was  
16 estimated using Eq. (6) according to Wanninkhof (1992):

$$17 k_{N_2O} = 0.31 \times u_{\text{av}}^2 \times (\text{Sc}_{N_2O}/600)^{-0.5} \quad (6)$$

18 where  $u_{\text{av}}$  is the average wind speed 10 m above the water surface. In this study, a CO<sub>2</sub> Schmidt number  
19 ( $\text{Sc}$ ) of 600 at 20 °C in fresh water (Wanninkhof, 1992) was used for estuarine systems (Raymond and  
20 Cole, 2001). The  $\text{Sc}$  is defined as the kinematic viscosity of water divided by the diffusion coefficient of  
21 the gas and calculated from temperature (Wanninkhof, 1992). For N<sub>2</sub>O in waters with salinities <35 and  
22 temperatures ranging from 0–30 °C,  $\text{Sc}_{N_2O}$  was estimated using Eq. (7) according to Wanninkhof (1992):

$$23 \text{Sc}_{N_2O} = 2055.6 - 137.11 t + 4.3173 t^2 - 0.05435 t^3 \quad (7)$$

24 where  $t$  is the in situ temperature of the sampling site.

25 To determine the isotopic composition of N<sub>2</sub>O, the gas samples were introduced into a trace gas  
26 cryogenic pre-concentration device (PreCon, Thermo Finnigan), as described in Cao et al. (2008) and  
27 Zhu et al. (2008), and then  $\delta^{15}\text{N-N}_2\text{O}$  was analyzed using an isotope ratio mass spectrometer (IRMS,

1 Thermo Finnigan MAT-253, Bremen, Germany). The molecular ions of N<sub>2</sub>O (N<sub>2</sub>O<sup>+</sup>, m/z 44, 45 and 46)  
2 were quantified by IRMS to calculate isotope ratios for the entire molecule (<sup>15</sup>N/<sup>14</sup>N and <sup>18</sup>O/<sup>16</sup>O). The  
3 δ<sup>15</sup>N values of N<sub>2</sub>O in samples were calculated using the <sup>15</sup>N/<sup>14</sup>N of the pure N<sub>2</sub>O reference gas and  
4 samples (Frame and Casciotti, 2010; Mohn et al., 2014). The reference gas was previously calibrated  
5 against N<sub>2</sub>O isotopic standard gas (δ<sup>15</sup>N (vs Air-N<sub>2</sub>) = -0.320‰) produced by Shoko Co. Ltd. (Tokyo,  
6 Japan) and the δ<sup>15</sup>N value (vs Air-N<sub>2</sub>) of the N<sub>2</sub>O reference gas is 6.579±0.030‰. The precision of the  
7 method for δ<sup>15</sup>N-N<sub>2</sub>O was estimated as 0.3‰.

### 8 **2.3 Nucleic acid extraction and qPCR**

9 DNA was extracted using the FastDNA<sup>TM</sup> SPIN Kit for Soil (MP, USA) according to the  
10 manufacturers' protocol with minor modifications. RNA was extracted using TRIzol reagent (Ambion,  
11 Austin, Texas, USA), and then eluted with 50 µL of RNase-free water. The extracted RNA was treated  
12 with DNase I (Invitrogen, Carlsbad, CA) to remove any residual DNA. DNA contamination was  
13 checked by amplifying the bacterial 16S rRNA genes before reverse transcription. Total RNA without  
14 DNA contamination was reverse transcribed to synthesize single-strand complementary DNA (cDNA)  
15 using the First-Strand cDNA Synthesis Kit (Invitrogen, Austin, Texas, USA).

16 The transcript and copy abundances of bacterial and archaeal *amoA* genes and bacterial *nirS* genes  
17 were examined using qPCR and a CFX96 Real Time PCR system (BIO-RAD, Singapore). The β-  
18 proteobacterial and archaeal *amoA* were amplified using primer sets *amoA*-1F and *amoA*-2R (Kim et  
19 al., 2008) and Arch-*amoA*F and Arch-*amoA*R (Francis et al., 2005), respectively; *nirS* was amplified  
20 using primers *nirS*-1F and *nirS*-3R (Braker et al., 1998; Huang et al., 2011). Quantitative PCR  
21 amplification for the β-proteobacterial and archaeal *amoA* was carried out as described previously  
22 (Mincer et al., 2007; Hu et al., 2011). For the amplification of *nirS*, the qPCR reaction mixture was  
23 prepared in accordance with Zhang et al. (2014) and thermal cycling conditions were as described in  
24 Huang et al. (2011). Standards for the qPCR reactions consisted of serial 10-fold dilutions (10<sup>7</sup> to 10<sup>0</sup>)  
25 copies per µL) of plasmid DNA containing amplified fragments of the targeted genes (accession  
26 numbers MH458281 for β-proteobacterial *amoA*, KY387998 for archaeal *amoA* and KF363351 for  
27 *nirS*). The amplification efficiencies of qPCR were always between 85%–95% with R<sup>2</sup> >0.99. The

1 specificity of the qPCR reactions was confirmed by melting curve analysis, agarose gel electrophoresis  
2 and sequencing analysis. Inhibition tests were performed by 2-fold and 5-fold dilutions of all samples  
3 and indicated that our samples were not inhibited.

#### 4 **2.4 Incubation experiments**

5 Incubation experiments were performed in the surface and bottom waters at sites P01 (2 and 5 m water  
6 depth) and P05 (2 and 12 m) upstream of the Humen outlet (Fig. 1). Water samples were collected from  
7 Niskin bottles through a clean polytetrafluoroethylene (Teflon) silicone hose, and carefully filled into  
8 125 mL clean headspace glass bottles without gas bubbles. The bottles were immediately closed with an  
9 air-tight butyl rubber stopper and aluminum crimp-cap. A total of 43 bottles were set up for surface and  
10 bottom at sites P01 and 34 bottles at P05. Samples from four parallel bottles were taken to determine the  
11 initial ( $t_0$ ) dissolved  $N_2O$  concentration, and triplicate samples were taken to measure the initial  
12 dissolved inorganic nitrogen (DIN) concentration, which included ammonium, nitrite, and nitrate. The  
13 remaining 36 (P01) and 27 (P05) bottles were incubated in the dark at in situ temperatures ( $\pm 1$  °C). At  
14 site P01, samples from six parallel bottles were taken at 3, 6, 18, and 24 h during the incubation  
15 experiment for  $N_2O$  determination after injecting saturated mercuric chloride ( $HgCl_2$ , 1:100 v:v) into the  
16 bottles; triplicate samples were also taken at the same time for DIN measurements by filtering through  
17 0.7  $\mu m$  pore size GF/Fs under pressure  $<0.03$  MPa. Concentrations of  $N_2O$ , ammonium, nitrite, and  
18 nitrate were measured as described in Sect. 2.2. At site P05, samples were taken after 3, 6, and 12 h  
19 incubation and the other procedures were the same as described for site P01.

20 The effect of DIN assimilation is negligible during incubation in the dark (Ward, 2008). Therefore,  
21 the potential processes of nitrogen transformation and  $N_2O$  production can be determined according to  
22 “mass balance” in a closed incubation system. The main processes were analyzed based on the dynamic  
23 variations of DIN ( $\Delta DIN$ ), ammonia ( $\Delta NH_3 + NH_4^+$ ), nitrite ( $\Delta NO_2^-$ ), nitrate ( $\Delta NO_3^-$ ), and  $N_2O$  ( $\Delta N_2O$ )  
24 concentrations during incubation. The average rates of nitrification and  $N_2O$  production were estimated  
25 using the slopes of the linear regression between concentrations versus incubation time when DIN was  
26 in balance (i.e. no denitrification). All of the concentration-based rates described from the incubations  
27 represent net rates. The  $N_2O$  yield during nitrification was calculated with Eq. (8):

$$1 \quad \text{N}_2\text{O}_{\text{yield}} (\%) = \Delta\text{N}_2\text{O-N} / \Delta(\text{NH}_3 + \text{NH}_4^+)\text{-N} \quad (8)$$

## 2 **2.5 Statistical analyses**

3 Since a normal distribution of the individual data sets was not always met, we used the non-parametric  
4 Wilcoxon rank-sum tests for comparing two variables. The bivariate correlations between  
5 environmental factors and functional genes were described by Spearman correlation coefficients ( $\rho$   
6 value). False discovery rate-based multiple comparison procedures were applied to evaluate the  
7 significance of multiple hypotheses and identify truly significant comparisons (~~False discovery rate-~~  
8 adjusted  $P$  value) (Pike, 2011). The maximum gradient length of detrended correspondence analysis  
9 was shorter than 3.0, thus redundancy analysis based on the qPCR data was used to analyze variations  
10 in the AOA and AOB distributions under environmental constraints in the software R (version 3.4.4)  
11 Vegan 2.5–3 package. The qPCR-based relative abundances and environmental factors were normalized  
12 via Z transformation (Magalhães et al., 2008). The null hypothesis, that the community was independent  
13 of environmental parameters, was tested using constrained ordination with a Monte Carlo permutation  
14 test (999 permutations). Significant environmental parameters ( $P < 0.05$ ) without multicollinearity  
15 (variance inflation factor  $< 20$ ) (Ter Braak, 1986) were obtained. Standard and partial Mantel tests were  
16 run in R (version 3.4.4, Vegan 2.5–3 package) to determine the correlations between environmental  
17 factors and the AOA and AOB distributions. Dissimilarity matrices of communities and environmental  
18 factors were based on Bray-Curtis and Euclidean distances between samples, respectively. Based on  
19 Spearman correlation, the significance of the Mantel statistics was obtained after 999 permutations.  
20 Statistical tests were assumed to be significant at a  $P$  value of  $< 0.05$ .

## 21 **3 Results**

### 22 **3.1 Distribution of nutrients, DO, and N<sub>2</sub>O along a salinity transect of the Pearl River Estuary,**

23 The studied transect was divided into a northern region upstream of the Humen outlet and southern area  
24 (Lingdingyang) (Fig. 1); these regions have distinct biogeochemical characteristics. Salinity exhibited  
25 low values (0.1 to 4.4) upstream of the Humen outlet, and sharply increased from 0.7 to 34.2  
26 downstream in Lingdingyang (Fig. 2a). The ammonium/ammonia concentrations decreased from 167.2

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1  $\mu\text{mol L}^{-1}$  (site P01 surface water) to  $20.9 \mu\text{mol L}^{-1}$  (site P07 bottom water) upstream of the Humen  
2 outlet and consistently decreased downstream in Lingdingyang ( $5.7 \mu\text{mol L}^{-1}$  to below detection limit)  
3 (Fig. 2b). Correspondingly, the sum of nitrate and nitrite concentrations increased from  $93.6 \mu\text{mol L}^{-1}$   
4 (site P01 bottom water) to  $172.3 \mu\text{mol L}^{-1}$  (site P03 surface water) upstream, but it sharply decreased  
5 seaward to Lingdingyang (Fig. 2c). The DO concentrations were distinctly lower upstream of the  
6 Humen outlet with nearly one-half of the samples below the hypoxic threshold ( $63.0 \mu\text{mol L}^{-1}$ ; Rabalais  
7 et al., 2010). Generally, the DO concentrations increased seaward from  $155.7$  to  $238.0 \mu\text{mol L}^{-1}$  in the  
8 surface waters of the Lingdingyang area, whereas they varied from  $74.0$  to  $183.3 \mu\text{mol L}^{-1}$  in the  
9 bottom waters (Fig. 2d).

10 In contrast to the DO concentrations, the  $\text{N}_2\text{O}$  concentrations were distinctly higher upstream of the  
11 Humen outlet ( $48.9$ – $148.2 \text{ nmol L}^{-1}$ ) than in Lingdingyang, where they decreased seaward from  $24.6$  to  
12  $5.4 \text{ nmol L}^{-1}$  (Fig. 2e). Similarly, higher  $\Delta\text{N}_2\text{O}_{\text{excess}}$  ( $42.0$ – $141.3 \text{ nmol L}^{-1}$ ) with saturations from  
13  $701.1\%$  to  $2175.1\%$  was observed upstream; lower  $\Delta\text{N}_2\text{O}_{\text{excess}}$  ( $-1.4$ – $17.8 \text{ nmol L}^{-1}$ ) was present in the  
14 Lingdingyang area with the saturations ranging from  $86\%$  to  $363\%$  (Fig. 2f). The estimated water–air  
15  $\text{N}_2\text{O}$  fluxes were  $100.4$  to  $344.0 \mu\text{mol m}^{-2} \text{ d}^{-1}$  upstream and decreased in Lingdingyang ( $42.4$  to  $-2.6$   
16  $\mu\text{mol m}^{-2} \text{ d}^{-1}$ ) (Fig. 2g). Together, the Pearl River Estuary acts as a  $\text{N}_2\text{O}$  source that releases to the  
17 atmosphere and notably, a significant negative relationship was observed between  $\Delta\text{N}_2\text{O}_{\text{excess}}$  or  $\text{N}_2\text{O}$   
18 flux and DO ( $P < 0.01$  for each) in the upstream of the Humen outlet (Fig. 2i and j). The isotopic  
19 compositions of  $\text{N}_2\text{O}$  ( $\delta^{15}\text{N}$ - $\text{N}_2\text{O}$ ) showed an enrichment of  $^{15}\text{N}_2\text{O}$  seaward, varying from  $-27.9$  to  $7.1\%$   
20 (Fig. 2h). Overall, upstream of the Humen outlet was characterized by hypoxic waters rich in nitrogen-  
21 based nutrients, where ammonium concentrations decreased and the sum of nitrite and nitrate  
22 concentrations increased seaward, corresponding to distinctly higher  $\text{N}_2\text{O}$  fluxes released to the  
23 atmosphere.

### 24 3.2 Distributions of *amoA* and *nirS* genes along the salinity transect

25 The total abundance of AOA *amoA* (sum of free-living and particle-associated communities) varied  
26 from  $3.10 \times 10^3$  to  $6.87 \times 10^5$  copies  $\text{L}^{-1}$  in the surface waters (Fig. 3a) and  $6.40 \times 10^4$  to  $4.21 \times 10^7$  copies

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1 L<sup>-1</sup> in the bottom waters; an increase along the salinity transect was observed in the bottom (Fig. 3b). In  
2 contrast, the total abundance of AOB *amoA* generally decreased seaward along the salinity transect for  
3 the surface (4.23×10<sup>2</sup> to 2.13×10<sup>4</sup> copies L<sup>-1</sup>) and bottom waters (4.49×10<sup>3</sup> to 8.79×10<sup>4</sup> copies L<sup>-1</sup>) (Fig.  
4 3c and d). Overall, the abundance of AOA *amoA* was significantly higher than AOB (*P* < 0.01). The  
5 total abundance of *nirS* varied from 9.12×10<sup>4</sup> to 2.00×10<sup>7</sup> copies L<sup>-1</sup> and was higher than both AOA (*P*  
6 < 0.05) and AOB *amoA* (*P* < 0.01) in the surface waters and AOB *amoA* in the bottom water (*P* < 0.01)  
7 (Fig. 3e and f). Notably, these three genes were predominantly distributed in the particle-associated,  
8 communities compared to the free-living communities in the estuary transect (Fig. 3). The transcripts of  
9 the three genes were analyzed in the particle-associated communities of the two incubation sites  
10 upstream of the Humen outlet. The transcript abundances of AOA *amoA* (7.44×10<sup>3</sup> to 4.62×10<sup>5</sup>  
11 transcripts L<sup>-1</sup>) were one to three orders of magnitude higher than AOB *amoA* (3.62×10<sup>2</sup> to 5.00×10<sup>2</sup>  
12 transcripts L<sup>-1</sup>) at P01 (Fig. 3a–d), whereas the transcript abundances of AOB *amoA* were relatively  
13 higher at P05 (AOB = 8.96×10<sup>4</sup> to 3.83×10<sup>5</sup> transcripts L<sup>-1</sup>; AOA = 1.26×10<sup>4</sup> to 1.39×10<sup>5</sup> transcripts  
14 L<sup>-1</sup>). The *nirS* gene showed a similar transcript level with AOA *amoA* at P01 (2.20×10<sup>4</sup> to 6.69×10<sup>4</sup>  
15 transcripts L<sup>-1</sup>), but one order of magnitude lower transcript level than both AOA and AOB *amoA* at  
16 P05 (8.59×10<sup>3</sup> to 1.12×10<sup>4</sup> transcripts L<sup>-1</sup>) (Fig. 3e and f).

### 17 3.3 Correlations between genes abundances and biogeochemical parameters

18 We analyzed the correlations between the genes abundances of AOA, AOB, or denitrifiers and  
19 biogeochemical parameters. The results indicate that AOA *amoA* abundance was significantly  
20 correlated (*P* < 0.05–0.01) to the hydrographic parameters temperature (negative) and salinity (positive),  
21 as well as silicate concentration (negative) (Table 1). However, AOB *amoA* abundance was  
22 significantly correlated (*P* < 0.05–0.01) to TSM concentration (positive), pH (negative), and DO  
23 (negative). Notably, there were positive correlations between AOB *amoA* abundances and all N<sub>2</sub>O  
24 parameters as well as ammonia concentrations (Table 1; *P* < 0.05–0.01) except for the extremely low-  
25 abundance of free-living AOB. No significant Spearman correlations were found between bacterial  
26 nitrite reductase *nirS* abundance and the measured biogeochemical parameters.

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1 The redundancy analysis was used to further analyze variations in the AOA and AOB distributions  
2 under environmental constraints. The results confirmed that the relatively high AOB abundances in the  
3 upper estuary were constrained by low salinity water, high nitrite and TSM concentrations, low DO  
4 conditions, and high N<sub>2</sub>O concentrations whereas high salinity water and opposite environmental  
5 conditions constrained the relatively high AOA abundances in the Lingdingyang area (Fig. 4). These  
6 constraints explained 89.3% of the variation in the ammonia oxidizer distribution along the estuary.  
7 Apparently, the communities with relatively high AOB abundances in the upper estuary positively  
8 influenced the concentration of N<sub>2</sub>O in the water.

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### 9 **3.4 Nitrogen transformation and N<sub>2</sub>O production in the incubation experiments**

10 The in situ biogeochemical conditions of the incubation experiments are shown in Fig. 2 and listed in  
11 Table S1. Site P01 exhibited the lowest in situ DO concentrations (30.0 μmol L<sup>-1</sup> in the bottom water  
12 and 30.9 μmol L<sup>-1</sup> in the surface water). The concentration of DIN was generally unchanged in the  
13 early-to-middle (0–18 h) phase for the P01 surface water and early (0–6 h) phase for the P01 bottom  
14 water, but showed a distinct decrease in the end phase (Fig. 5a). The ammonia and nitrite concentrations  
15 consistently decreased and increased, respectively, during the incubation experiments; the nitrate  
16 concentrations decreased in the end phase after a slight increase (Fig. 5b). These results clearly indicate  
17 that nitrification occurred during the entire P01 incubations, and suggest that denitrification may be  
18 present in the end phase (Fig. 5g). The rates of ammonia oxidation during the entire incubations and  
19 nitrite oxidation during the early or early-to-middle phases were estimated by linear regressions of  
20 ammonia and nitrate concentrations, respectively (Fig. 5a and b; Table 2). Correspondingly, the  
21 estimated average N<sub>2</sub>O production rate (24 h) was 0.62 nmol L<sup>-1</sup> h<sup>-1</sup> in P01 surface water and 0.70 nmol  
22 L<sup>-1</sup> h<sup>-1</sup> in P01 bottom water; the estimated N<sub>2</sub>O production rates from nitrification were 0.60 nmol L<sup>-1</sup>  
23 h<sup>-1</sup> in the surface water (18 h) and 1.61 nmol L<sup>-1</sup> h<sup>-1</sup> in the bottom water (6 h; Fig. 5c). Thus, the  
24 estimated N<sub>2</sub>O yield in the surface and bottom waters based on nitrification was 0.26% and 0.30%  
25 (Table 2).

26 In the incubation experiments at site P05, the DIN concentrations remained unchanged (Fig. 5d)  
27 and the ammonia concentrations consistently decreased and the nitrite and nitrate concentrations

1 increased (Fig. 5e). The rates of ammonia and nitrite oxidation were also estimated by linear regressions  
2 of ammonia and nitrate concentrations, respectively (Fig. 5d and e; Table 2). The ammonia oxidation  
3 rates were approximately equal to the sum of the increased nitrite and nitrate concentration rates. Thus,  
4 nitrification occurred during the incubation experiments without denitrification. The estimated N<sub>2</sub>O  
5 production rates from nitrification were 1.15 nmol L<sup>-1</sup> h<sup>-1</sup> in the P05 surface water and 1.41 nmol L<sup>-1</sup>  
6 h<sup>-1</sup> in the P05 bottom water (Fig. 5f); the estimated N<sub>2</sub>O yields based on nitrification were 0.21%  
7 (surface) and 0.32% (bottom) (Table 2).

8 The N<sub>2</sub>O production rates and yields normalized to total AOA and AOB *amoA* gene copies (sum  
9 of particle-associated and free-living fractions or only particle-associated fractions) or transcripts (only  
10 particle-associated fraction) were calculated (Table S3). The highest average *amoA* gene copy-specific  
11 N<sub>2</sub>O production rates and yields were in the surface water of site P05, where the highest nitrification  
12 rate was observed (Table 2). The highest average *amoA* gene transcript-specific N<sub>2</sub>O production rates  
13 and yields were in the bottom water of site P01, where the highest N<sub>2</sub>O production rate was observed  
14 (Table 2).

## 15 4 Discussion

### 16 4.1 Contribution of nitrification versus denitrification to N<sub>2</sub>O production in the hypoxic upper 17 estuary

18 The spatial variations of N<sub>2</sub>O concentration, its saturation, and water–air N<sub>2</sub>O flux along the Pearl River  
19 Estuary are consistent with our previous study (Lin et al., 2016), indicating that higher N<sub>2</sub>O in the upper  
20 estuary ensures the Pearl River Estuary acts as a source of atmospheric N<sub>2</sub>O. The in situ incubation  
21 experiments clearly indicated that nitrification predominantly occurred in the hypoxic waters (e.g. both  
22 the P01 and P05 sites) of the upper estuary along with significant N<sub>2</sub>O production, and suggested that  
23 denitrification could be concurrent at the lowest DO site (P01) where the maximum N<sub>2</sub>O and ΔN<sub>2</sub>O<sub>excess</sub>  
24 concentrations were observed (Figs. 2 and 5). These results confirm previous speculation that extreme  
25 enrichment of ammonia in the water column due to high loads of anthropogenic-sourced nutrients and  
26 organic matter in an upper estuary (Dai et al., 2008; He et al., 2014) could result in strong nitrification  
27 under low O<sub>2</sub> solubility conditions (Dai et al., 2008); thus, N<sub>2</sub>O is produced as a byproduct through

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1 | nitrification and is oversaturated in the Pearl River Estuary (Lin et al., 2016). The estuary sediments  
2 also act as a source of N<sub>2</sub>O, which is released into the overlying waters through denitrification (Tan et  
3 al., 2019); however, in estuarine waters, nitrification apparently is the main source of N<sub>2</sub>O production.  
4 Previous studies also proposed that nitrification may be the major source of N<sub>2</sub>O production in the water  
5 column in estuarine systems, such as the Guadalquivir (Huertas et al., 2018), Schelde (De Wilde and De  
6 Bie, 2000), and Chesapeake Bay (Laperriere et al., 2019). However, in the estuarine sediments, N<sub>2</sub>O  
7 production was attributed to both nitrification and denitrification, such as in the Tama (Japan) (Usui et  
8 al., 2001) and Yangtze (China) estuaries (Liu et al., 2019; Wang et al., 2019), where denitrification is  
9 the major nitrogen removal pathway with N<sub>2</sub>O production and consumption.

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10 The isotopic composition of N<sub>2</sub>O ( $\delta^{15}\text{N-N}_2\text{O}$ ) was consistent with the above interpretation.  
11 According to previous studies (Table S2), the  $\delta^{15}\text{N}$  of N<sub>2</sub>O produced during ammonia oxidation by  
12 AOB strains ranged from -68‰ to -6.7‰ (Yoshida, 1988; Sutka et al., 2006; Mandernack et al., 2009;  
13 Frame and Casciotti, 2010; Jung et al., 2014; Toyoda et al., 2017) and 6.3–10.2‰ in a marine AOA  
14 strain (Santoro et al., 2011). The  $\delta^{15}\text{N}$  of N<sub>2</sub>O produced during denitrification ranged from -37.2‰ to  
15 -7.9‰ (Toyoda et al., 2005); during nitrifier-denitrification by AOB strains it ranged from -57.6 ±  
16 4.1‰ to -21.5‰ (Sutka et al., 2003; Sutka et al., 2006; Frame and Casciotti, 2010). Therefore, the  
17 much lower  $\delta^{15}\text{N-N}_2\text{O}$  (-27.9‰ to -12.6‰) upstream of the Humen outlet is consistent with AOB  
18 nitrification or denitrification processes, whereas enriched  $^{15}\text{N-N}_2\text{O}$  (5.2–7.1‰) in the lower reaches  
19 approaches AOA nitrification and air  $^{15}\text{N-N}_2\text{O}$  (Santoro et al., 2011). Taken together, the isotopic  
20 compositions of N<sub>2</sub>O (Fig. 2h) and N<sub>2</sub>O concentration distribution (Fig. 2e–g) suggest that the high  
21 concentrations of N<sub>2</sub>O (oversaturation) were produced from strong nitrification by AOB and probably  
22 concurrent minor denitrification in the upper estuary, however in the lower reaches, low concentrations  
23 of N<sub>2</sub>O could be explained by AOA nitrification or water atmospheric exchange of N<sub>2</sub>O.

24 **4.2 Correlations of AOB versus AOA with N<sub>2</sub>O-related biogeochemical parameters along the**  
25 **Pearl River Estuary,**

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26 The more abundant AOA *amoA* genes, relative to AOB, and the more abundant genes in the particle-  
27 associated communities than free-living communities are consistent with our previous study in the Pearl

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1 | River Estuary (Hou et al., 2018), which also reported significant positive correlations between the AOB  
2 | *amoA* gene abundance and the oxidation rate of ammonia to nitrate. This suggests that AOB might be  
3 | active in the ammonium and particle-enriched estuary, despite their low abundance (Füssel, 2014; Hou  
4 | et al., 2018). Lower oxygen availability in particle micro-niches has been reported to be favorable for  
5 | both nitrification and denitrification potential in oxygenated water (Kester et al., 1997). The Spearman  
6 | correlations and redundancy analysis in this study indicate that high nutrient and TSM concentrations  
7 | and low DO and pH conditions were favourable for relatively high abundance of AOB in the upper  
8 | estuary, which is also consistent with our previous Pearl River Estuary study that found high TSM  
9 | concentrations and low DO and pH influenced substrate availability and thus AOB distribution (Hou et  
10 | al., 2018). Moreover, AOB *amoA* abundances positively correlated to all N<sub>2</sub>O-related parameters as  
11 | revealed by the Spearman correlations and redundancy analysis, suggesting a significant influence of  
12 | AOB (mainly the particle-associated fraction) on N<sub>2</sub>O production/emission in the upper estuary.  
13 | However, compared to AOB, AOA *amoA* distribution along the estuary transect appears to be regulated  
14 | more by water mixing since AOA was significantly correlated to the hydrographic parameters and  
15 | silicate concentration.

16 | To further eliminate the co-varying effects of water mixing, substrate availability, and N<sub>2</sub>O-related  
17 | parameters along the salinity transect, and to identify the intrinsic/direct relationship between ammonia  
18 | oxidizers and N<sub>2</sub>O production, we performed standard and partial Mantel tests. We defined four types of  
19 | environmental constraints: water mixing parameters (temperature, salinity, and silicate), substrate  
20 | parameters (ammonia/ammonium, nitrite, and nitrate), parameters influencing substrate availability  
21 | (DO, TSM, and pH), and N<sub>2</sub>O-related parameters (N<sub>2</sub>O and  $\Delta N_2O_{\text{excess}}$ ). For the water mixing  
22 | parameters, we analyzed the relationships between potential temperature ( $\theta$ ), salinity, and silicate  
23 | concentration with a three-dimensional scatter plot (Fig. S1) that indicates low salinity and high silicate  
24 | contents were the best indicators for river input in the ocean (Moore, 1986). Thus, we chose temperature,  
25 | salinity, and silicate as proxies to trace estuarine water masses and mixing. Water mixing parameters  
26 | (standard and partial Mantel tests,  $P < 0.01$ ) and those influencing substrate availability (standard and  
27 | partial Mantel tests,  $P < 0.05$ ) significantly controlled variations in the distribution of AOA and AOB  
28 | along the estuary transect (Fig. 6a and c), supporting the Spearman and redundancy analyses

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1 | conclusions. Notably, variations in the distribution of AOA and AOB were significantly correlated with  
2 | N<sub>2</sub>O production (standard and partial Mantel test,  $P < 0.01$ ) after eliminating the co-varying effects of  
3 | other parameters (Fig. 6d), demonstrating the significant contribution of ammonia oxidizers to N<sub>2</sub>O  
4 | production.

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### 5 | **4.3 Contribution of AOB versus AOA to N<sub>2</sub>O production**

6 | We attempted to accurately assess the relative contributions of AOA and AOB to N<sub>2</sub>O production in the  
7 | Pearl River Estuary by plotting the N<sub>2</sub>O production rates (Fig. 7a) and yields (Fig. 7b) normalized to  
8 | total (sum of AOA and AOB) *amoA* gene copies or transcripts at sites P01 and P05 along the x-y axes  
9 | that represent the relative contributions of AOA and AOB to the total *amoA* gene or transcript pools.  
10 | Notably, compared to AOA, higher AOB abundance in the *amoA* gene-based DNA or cDNA pool  
11 | resulted in distinctly higher (disproportionately higher relative to enhanced abundance) average *amoA*  
12 | gene copy or transcript-specific N<sub>2</sub>O production rates (Fig. 7a) and yields (Fig. 7b), suggesting that  
13 | AOB may have higher cell-specific activities in the upper estuary and thus be more active in producing  
14 | N<sub>2</sub>O than AOA. Previous studies based on pure cultures of AOB and AOA strains provided evidence  
15 | that AOB have higher N<sub>2</sub>O yields (0.09 to 26%) (Yoshida and Alexander, 1970; Goreau et al., 1980)  
16 | than AOA (0.002 to 0.09%) during ammonia oxidation (Löscher et al., 2012; Stieglmeier et al., 2014).  
17 | The higher N<sub>2</sub>O yield from AOB has also been observed in soils despite a lower abundance of AOB  
18 | (Hink et al., 2017; Hink et al., 2018). Based on results indicated by Fig. 7, we conclude that AOB may  
19 | have higher relative contributions to the high N<sub>2</sub>O production in the upper estuary where low DO, high  
20 | concentrations of N<sub>2</sub>O and  $\Delta N_2O$ , and high N<sub>2</sub>O flux were observed.

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21 | Ammonia oxidizers are sensitive to oxygen during N<sub>2</sub>O production (Santoro et al., 2011; Löscher  
22 | et al., 2012; Stieglmeier et al., 2014). Studies based on pure cultures of AOB strains *Nitrosomonas*  
23 | *marina* NM22 and *Nitrosococcus oceani* NC10, and AOA strain *Nitrosopumilus maritimus* showed  
24 | higher N<sub>2</sub>O yields and production during nitrification by both AOA and AOB when O<sub>2</sub> concentrations  
25 | varied from aerobic to hypoxic conditions (Löscher et al., 2012). However, when O<sub>2</sub> concentrations  
26 | varied from hypoxic to anaerobic conditions (i.e. in a lower O<sub>2</sub> concentration range), the AOB strain  
27 | *Nitrospira multiformis* and AOA strains *Nitrososphaera viennensis* and *Nitrosopumilus maritimus*

1 showed that AOB had distinctly higher N<sub>2</sub>O yields at lower oxygen conditions and, in contrast, AOA  
2 had lower N<sub>2</sub>O yields at lower oxygen concentrations (Stieglmeier et al., 2014). In addition, results from  
3 the cultured AOB strain *Nitrosomonas marina* C-113a indicated increasing N<sub>2</sub>O yields with higher cell  
4 concentrations (Frame and Casciotti, 2010). This evidence supports our conclusions that the high  
5 concentration of N<sub>2</sub>O (oversaturated) may be mainly produced from strong nitrification by the high  
6 abundance of AOB in the low DO conditions in the upper estuary.

7 In addition, it is possible that comammox (COMplete AMMonia OXidiser) species, newly  
8 discovered in terrestrial systems (Daims et al., 2015; Santoro, 2016; Kits et al., 2017), are also involved  
9 in N<sub>2</sub>O production (Hu and He, 2017) given the similar ammonia oxidation pathway to AOB. It has  
10 been further reported that the comammox *Nitrospira inopinata* has a lower N<sub>2</sub>O yield than AOB due to  
11 a lack of NO reductases and the formation of N<sub>2</sub>O from the abiotic conversion of hydroxylamine (Kits  
12 et al., 2019). However, comammox has not been widely observed in estuarine waters. Also, *nirK*-type  
13 denitrifiers may contribute to N<sub>2</sub>O production despite being much less abundant than *nirS*-  
14 type denitrifiers (Huang et al., 2011; Maeda et al., 2017). Furthermore, *nirS*-type denitrifiers are more  
15 likely to be capable of complete denitrification because of a higher co-occurrence of the N<sub>2</sub>O reductase  
16 gene (*nosZ*) with *nirS* than *nirK* (Graf et al., 2014). However, there is currently no direct evidence that  
17 denitrification or nitrifier-denitrification is responsible for N<sub>2</sub>O production in the [Pearl River Estuary](#)  
18 [water column](#). A release of N<sub>2</sub>O into the overlying waters through denitrification was reported for [the](#)  
19 [estuary](#) sediments (Tan et al., 2019). Further study is needed to clarify the potential of both *nirK* and  
20 *nirS*-type denitrifiers in N<sub>2</sub>O production from the interface between sediment and water in the [Pearl](#)  
21 [River Estuary](#).

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## 22 5 Conclusions

23 This study explored the relative contributions of AOB and AOA in producing N<sub>2</sub>O in the [Pearl River](#)  
24 [Estuary](#) by combining isotopic compositions and concentrations of N<sub>2</sub>O, distributions and transcript  
25 levels of AOB and AOA *amoA* and denitrifier *nirS* genes, and incubation estimates of nitrification and  
26 N<sub>2</sub>O production rates. Our findings indicate that the high concentrations of N<sub>2</sub>O and  $\Delta N_2O_{\text{excess}}$  and the  
27 much lower  $\delta^{15}N\text{-}N_2O$  are primarily attributed to strong nitrification by AOB. There is also probably

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1 concurrent minor denitrification in the upper estuary where AOB abundances are higher before  
2 decreasing seaward along the salinity transect. Low concentrations of  $N_2O$  and  $\Delta N_2O_{\text{excess}}$  and enriched  
3  $^{15}N_2O$  could be explained by AOA nitrification in the lower reaches of the estuary. Collectively, AOB  
4 contributed the major part of  $N_2O$  production in the upper estuary, which is the major source of  $N_2O$   
5 emitted to the atmosphere in the [Pearl River Estuary](#).

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#### 6 **Data availability**

7 All data can be accessed in the form of Excel spreadsheets via the corresponding author.

8 **The Supplement related to this article is available online.**

#### 9 **Author contribution**

10 M.D. and Y.Z. conceived and designed the experiments. L.M., H.L., and X.X. performed the  
11 experiments. L.M., Y.Z., H.L., and X.X. analyzed the data. L.M. and Y.Z. wrote the paper. All authors  
12 contributed to the interpretation of results and critical revision.

#### 13 **Competing interests**

14 The authors declare no conflicts of interest.

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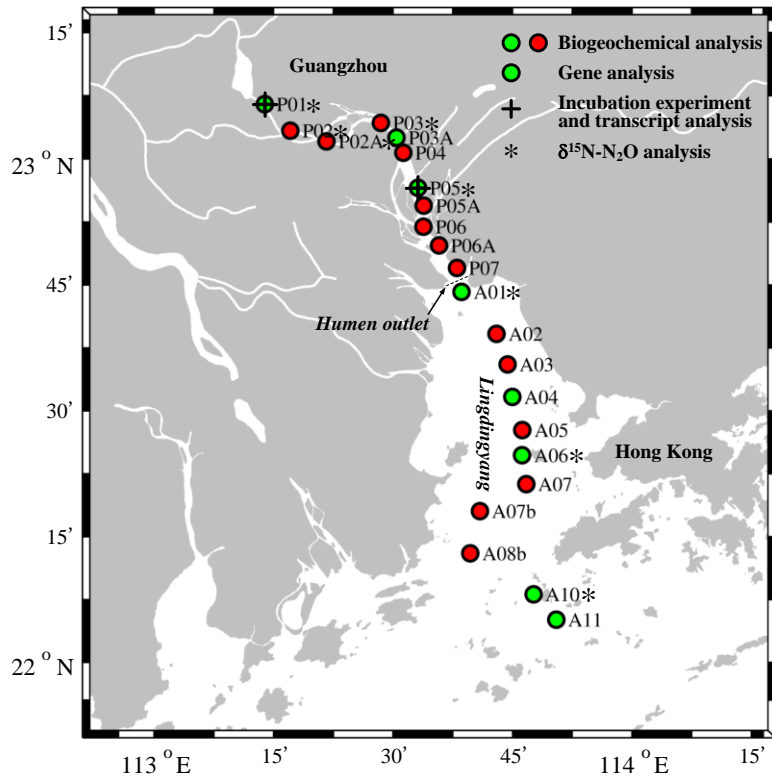
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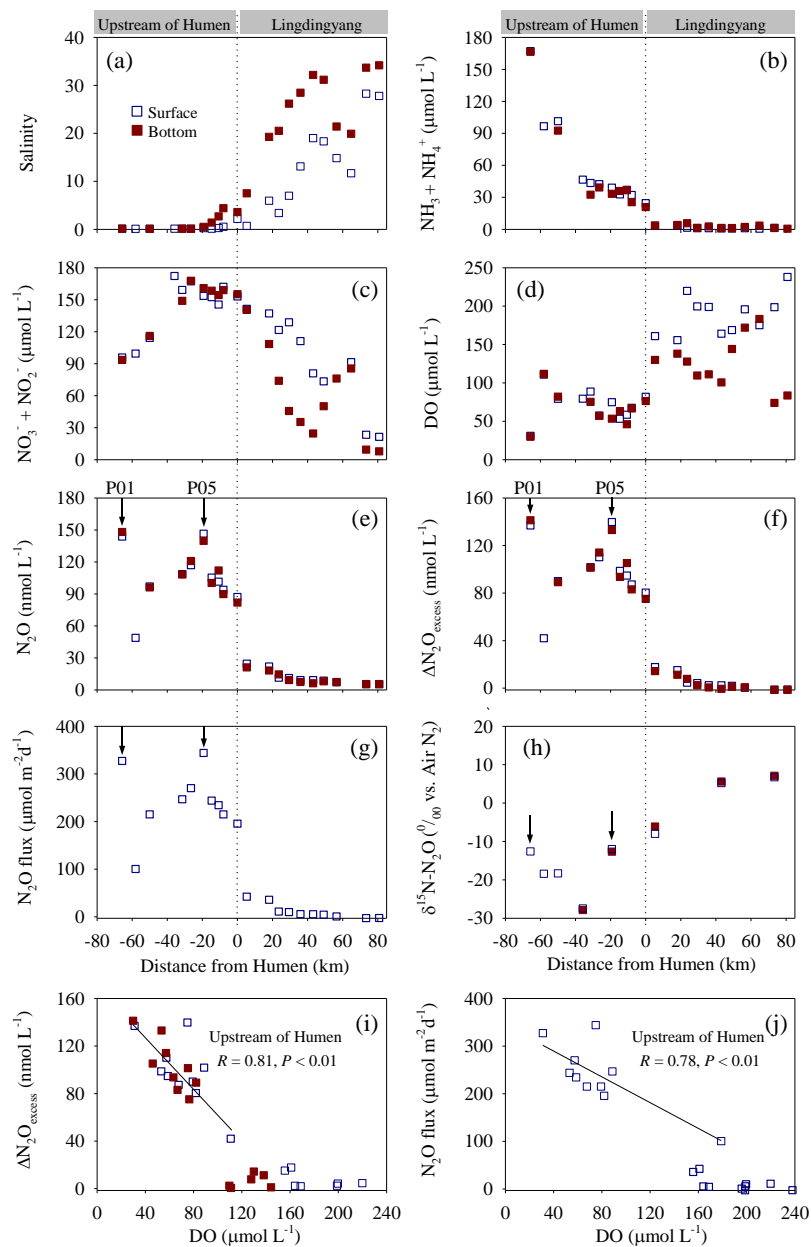
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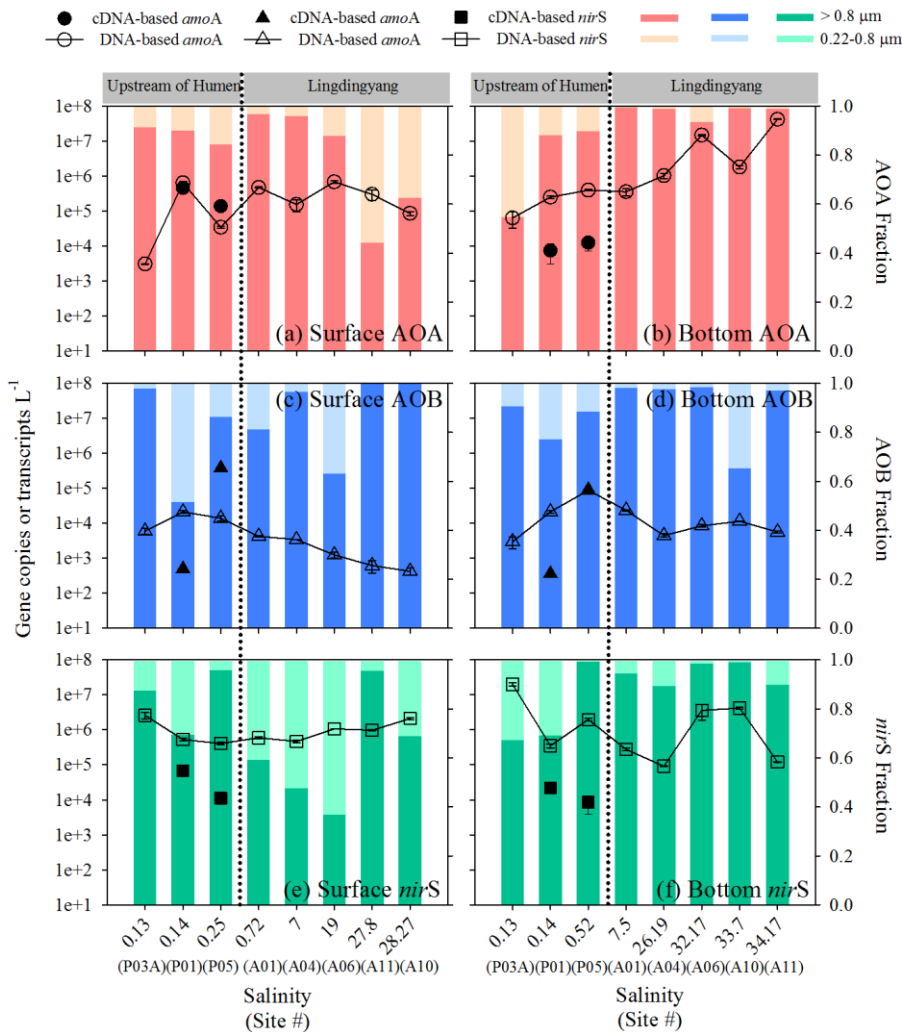
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 2 | **Figure 1:** Map of the Pearl River Estuary showing the sampling sites. Biogeochemical analyses were  
 3 performed on samples from all sites (green and red circles). The green circles indicate sites where genes  
 4 were additionally analyzed. The black crosses indicate in situ incubation experiment sites (P01 and  
 5 P05). The black asterisks indicate sites where the isotopic composition of  $\text{N}_2\text{O}$  was analyzed.

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1 | **Figure 2:** Distribution of biogeochemical factors along the Pearl River Estuary transect. (a) Salinity, (b)  
2  $\text{NH}_3+\text{NH}_4^+$ , (c)  $\text{NO}_2^- + \text{NO}_3^-$ , (d) DO, (e)  $\text{N}_2\text{O}$ , and (f)  $\Delta\text{N}_2\text{O}_{\text{excess}}$  concentrations, (g)  $\text{N}_2\text{O}$  flux, (h)  $\delta^{15}\text{N}$ -  
3  $\text{N}_2\text{O}$ , (i)  $\Delta\text{N}_2\text{O}_{\text{excess}}$  vs. DO, and (j)  $\text{N}_2\text{O}$  flux vs. DO. The dashed lines show the division of the transect  
4 into the northern (upstream of the Humen outlet) and southern (Lingdingyang) areas. The arrows  
5 indicate the sites where in situ incubation experiments were performed.

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1  
 2 **Figure 3:** Abundance distribution of AOA and AOB *amoA* and bacterial *nirS* along the salinity  
 3 gradient in the Pearl River Estuary. Abundances of AOA *amoA* genes (open circles) and particle-  
 4 associated transcripts (closed circles) and the relative abundances of particle-associated and free-living  
 5 AOA *amoA* genes in (a) surface and (b) bottom waters. Abundances of AOB *amoA* genes (open  
 6 triangles) and particle-associated transcripts (closed triangles) and the relative abundances of particle-

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1 ~~associated~~ and ~~free-living~~ AOB *amoA* genes in (c) surface and (d) bottom waters. Abundances of  
2 bacterial *nirS* genes (open squares) and ~~particle-associated~~ transcripts (closed squares) and the relative  
3 abundances of ~~particle-associated~~ and ~~free-living~~ *nirS* genes in (e) surface and (f) bottom waters. The  
4 dashed lines indicate the division into the northern (upstream of the Humen outlet) and southern  
5 (Lingdingyang) areas.

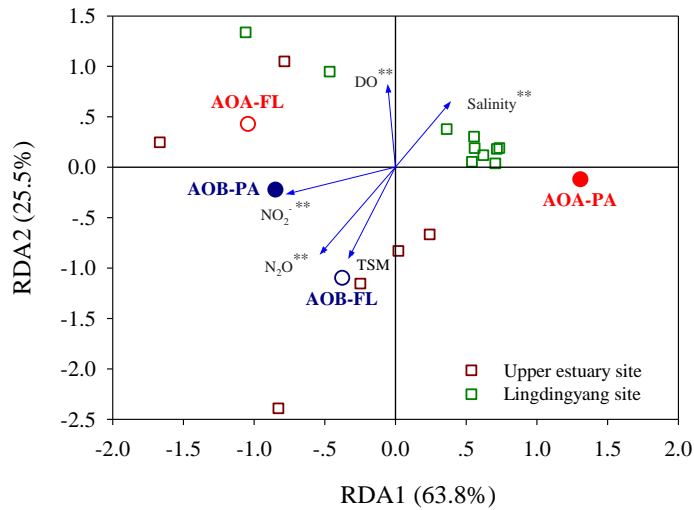
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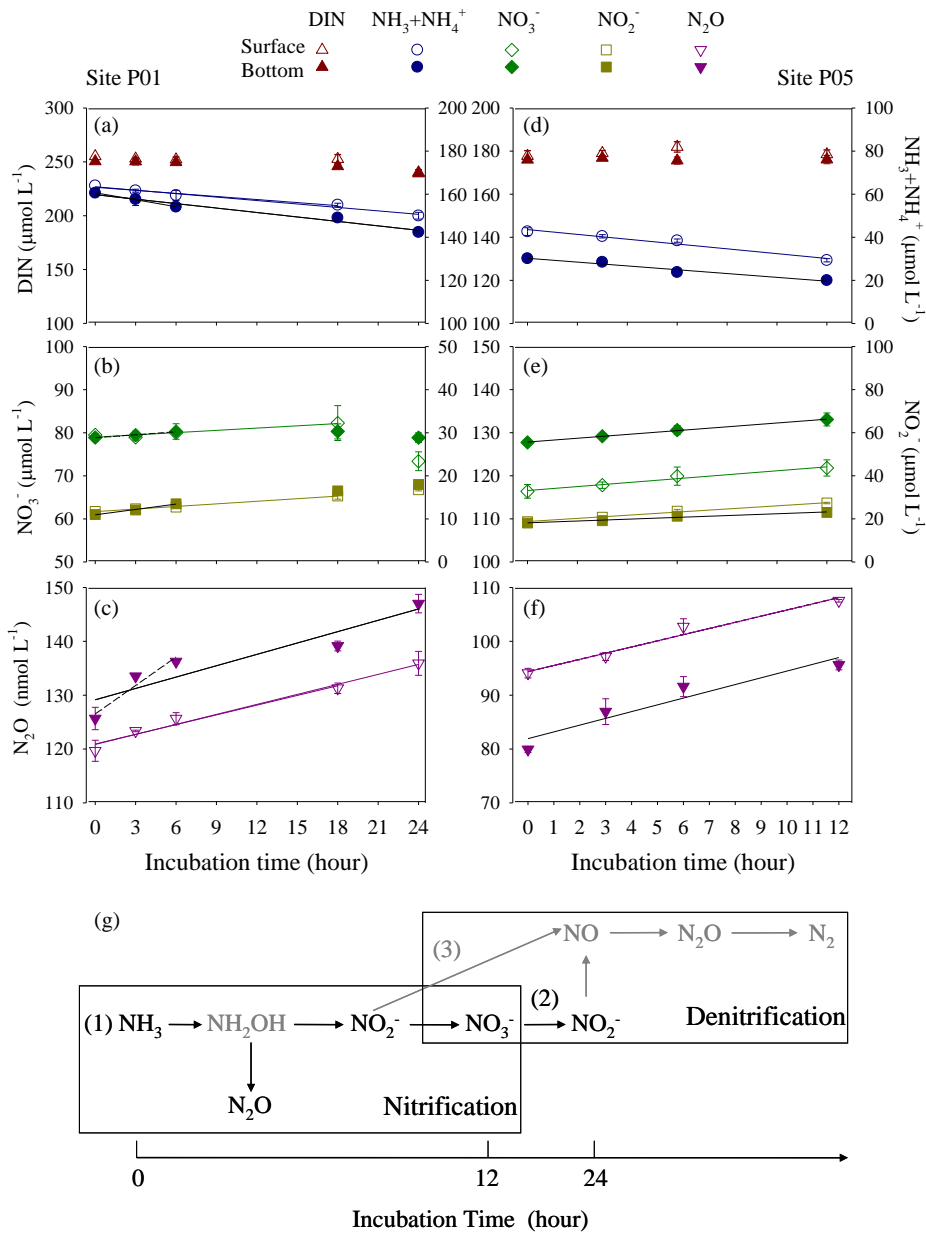


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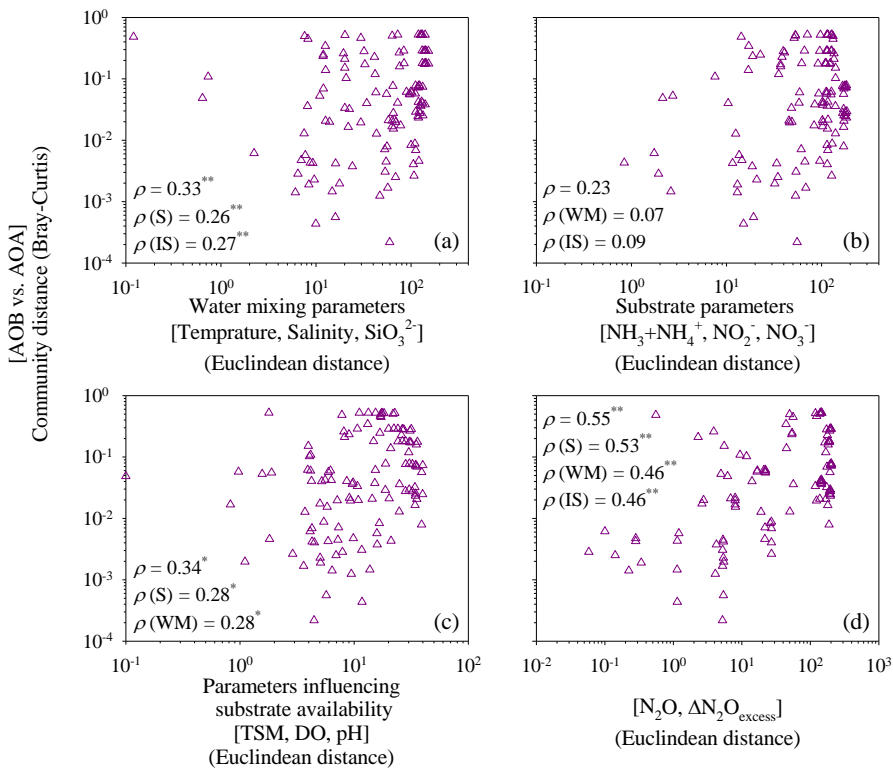
2 **Figure 4:** The redundancy analysis of the relative abundance of AOA *amoA* and AOB *amoA* under  
 3 biogeochemical constraints. PA, particle-associated; FL, free-living. Each square represents an  
 4 individual sample. Vectors represent environmental variables. \* $P < 0.05$ , \*\* $P < 0.01$  (Monte Carlo  
 5 permutation test).

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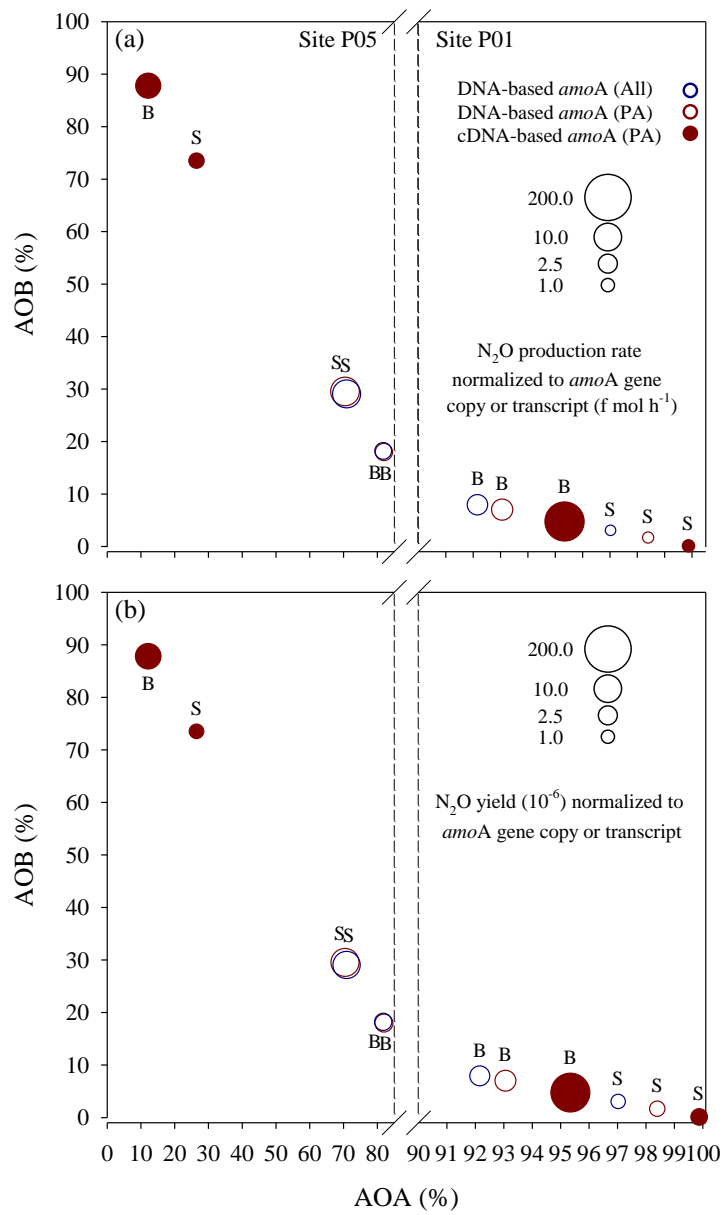


1 **Figure 5:** Variations in nitrogen compounds and N<sub>2</sub>O concentrations at sites P01 and P05 during the  
2 incubation experiments in surface (open symbols) and bottom (closed symbols) waters. (a, d) Total DIN  
3 (brown triangles) and NH<sub>3</sub>+NH<sub>4</sub><sup>+</sup> (blue circles), (b, e) NO<sub>3</sub><sup>-</sup> (green diamonds) and NO<sub>2</sub><sup>-</sup> (dark yellow  
4 squares), (c, f) N<sub>2</sub>O (purple inverted triangles). Linear regressions depend on whether variations in DIN  
5 concentration against time retain “mass balance” in a closed incubation system. The linear regressions  
6 of ammonia were used to estimate ammonia oxidation rates in (a) P01 over 18 and 24 h (surface water,  
7 blue lines) and 6 and 24 h (bottom water, black lines), and (d) P05 over 12 h (surface, blue line; bottom,  
8 black line). The linear regressions of nitrate estimated nitrite oxidation rates in (b) P01 over 18 h  
9 (surface water, green line) and 6 h (bottom water, black line), and (e) P05 after 12 h (surface, green line;  
10 bottom, black line). The nitrite linear regressions after 18 h (surface water, dark yellow line) and 6 h  
11 (bottom water, black line) in P01 and 12 h (surface, dark yellow line; bottom, black line) in P05 are also  
12 shown, but do not indicate oxidation rates. The N<sub>2</sub>O linear regressions were used to estimate N<sub>2</sub>O  
13 production rates in (c) P01 after 18 and 24 h (surface water, purple lines) and 6 and 24 h (bottom water,  
14 black lines; dashed line, no statistical significance test), and (f) P05 after 12 h (surface, purple line;  
15 bottom, black line). All regression equations,  $R^2$ , and  $P$  values are shown in Table 2. (g) A diagram  
16 showing transformations of nitrogen compounds and N<sub>2</sub>O production during incubation experiments.  
17 Nitrification (1) occurred during the entire P01 and P05 incubations and denitrification (2 and/or 3) may  
18 be present in the end phase of the P01 incubation. The gray arrows indicate the pathways of nitrogen  
19 loss unanalyzed here, and the gray compounds indicate the unmeasured nitrogen compound.



1  
2 **Figure 6:** Correlations between the relative abundance of AOB versus AOA and (a) water mixing  
3 parameters (temperature, salinity, and silicate), (b) substrate parameters (ammonia/ammonium, nitrite,  
4 and nitrate), (c) parameters influencing substrate availability (TSM, DO, and pH), or (d) N<sub>2</sub>O  
5 parameters (N<sub>2</sub>O and ΔN<sub>2</sub>O). The ammonia oxidizers matrix was calculated according to the relative  
6 AOA and AOB abundances. Dissimilarity matrices of the relative abundance of AOB *amoA* and AOA  
7 *amoA* were based on Bray-Curtis distances and environmental factors were based on Euclidean  
8 distances between samples. Standard and partial Mantel tests were run to measure the correlation  
9 between two matrices. Spearman correlation coefficient ( $\rho$ ) values are shown for standard (first value)

1 and partial Mantel (second, third, and fourth) tests. The  $P$  values were calculated using the distribution  
2 of the Mantel test statistics estimated from 999 permutations. \* $P < 0.05$ ; \*\* $P < 0.01$ .



1 **Figure 7:** N<sub>2</sub>O (a) production rates and (b) yields normalized to total *amoA* gene copy or transcript  
2 numbers of AOA and AOB in a given sample. They are presented along the x-y axes that represent the  
3 relative contributions of AOA and AOB to the total *amoA* gene or transcript pools. S, surface; B,  
4 | bottom. All, sum of free-living and particle-associated communities; PA, particle-associated  
5 | communities.

1 **Table 1** Rho ( $\rho$ ) values for the relationships between nitrifier and denitrifier gene abundances and biogeochemical parameters in the Pearl River  
 2 Estuary.

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Biogeochemical parameters	PA + FL			PA ( $> 0.8 \mu\text{m}$ )			FL ( $0.22\text{--}0.8 \mu\text{m}$ )		
	AOA- <i>amoA</i> (n = 16)	AOB- <i>amoA</i> (n = 16)	<i>nirS</i> (n = 16)	AOA- <i>amoA</i> (n = 16)	AOB- <i>amoA</i> (n = 14)	<i>nirS</i> (n = 16)	AOA- <i>amoA</i> (n = 16)	AOB- <i>amoA</i> (n = 16)	<i>nirS</i> (n = 16)
Temperature	-0.694*	0.359	0.085	-0.676*	0.303	0.165	-0.438	0.358	0.229
Salinity	0.644*	-0.339	-0.018	0.604*	-0.270	-0.047	0.403	-0.351	-0.356
SiO <sub>3</sub> <sup>-</sup>	-0.541*	0.559*	0.206	-0.497	0.503*	0.282	-0.350	0.481	0.238
TSM	-0.109	0.668*	0.047	-0.097	0.612*	0.194	0.191	0.565*	-0.071
pH	0.381	-0.656*	0.157	0.316	-0.615*	0.088	0.377	-0.605*	-0.059
DO	-0.074	-0.771**	-0.026	-0.121	-0.729**	-0.144	0.009	-0.697*	0.218
NH <sub>3</sub> /NH <sub>4</sub> <sup>+</sup>	-0.482	0.646*	0.068	-0.482	0.571*	0.196	-0.325	0.587*	0.000
NO <sub>3</sub> <sup>-</sup>	-0.485	0.359	-0.138	-0.444	0.353	-0.112	-0.588*	0.213	0.115
NO <sub>2</sub> <sup>-</sup>	-0.588*	0.447	0.126	-0.556*	0.356	0.212	-0.421	0.288	0.265
N <sub>2</sub> O	-0.421	0.641*	-0.194	-0.356	0.606*	-0.121	-0.385	0.490	0.047
$\Delta\text{N}_2\text{O}_{\text{excess}}$	-0.527*	0.559*	-0.160	-0.480	0.517*	-0.081	-0.369	0.504	0.096
N <sub>2</sub> O flux <sup>a</sup>	-0.190 (n = 8)	1.000** (n = 8)	-0.524 (n = 8)	-0.143 (n = 8)	1.000** (n = 8)	-0.310 (n = 8)	-0.571 (n = 8)	0.657 (n = 6)	-0.524 (n = 8)

1 | <sup>a</sup>Surface data; \*False discovery rate-adjusted  $P < 0.05$ ; \*\* False discovery rate-  
2 | adjusted  $P < 0.01$ .  
3 | PA, particle-associated communities; FL, free-living communities.

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1 **Table 2** Linear regressions of ammonia, nitrite, nitrate, and N<sub>2</sub>O concentrations against time and N<sub>2</sub>O yields during incubation experiments.

Site_Layer	Time (hour)	$\Delta(\text{NH}_3+\text{NH}_4^+)$ ( $\mu\text{mol L}^{-1} \text{h}^{-1}$ )			$\Delta\text{NO}_2^-$ ( $\mu\text{mol L}^{-1} \text{h}^{-1}$ )			$\Delta\text{NO}_3^-$ ( $\mu\text{mol L}^{-1} \text{h}^{-1}$ )			$\Delta\text{N}_2\text{O}$ ( $\text{nmol L}^{-1} \text{h}^{-1}$ )			N <sub>2</sub> O yield (%)
		Equation	R <sup>2</sup>	Rate <sup>a</sup>	Equation	R <sup>2</sup>	Rate <sup>a</sup>	Equation	R <sup>2</sup>	Rate <sup>a</sup>	Equation	R <sup>2</sup>	Rate <sup>a</sup>	
P01_S	18	$y = -0.47x + 163.20$	0.96*	0.47	$y = 0.20x + 11.69$	1.00**	0.20	$y = 0.18x + 78.98$	0.90*	0.18	$y = 0.60x + 120.93$	0.96*	0.60 <sup>b</sup>	0.26 <sup>b</sup>
	24	$y = -0.53x + 163.44$	0.98**	0.53	–	–	–	–	–	–	$y = 0.62x + 120.85$	0.98**	0.62	– <sup>c</sup>
P01_B	6	$y = -1.08x + 160.65$	1.00*	1.08	$y = 0.42x + 10.95$	1.00*	0.42	$y = 0.23x + 78.84$	0.98	0.23	$y = 1.61x + 127.04$	0.98	1.61 <sup>b</sup>	0.30 <sup>b</sup>
	24	$y = -0.69x + 159.76$	0.96**	0.69	–	–	–	–	–	–	$y = 0.70x + 129.14$	0.86*	0.70	– <sup>c</sup>
P05_S	12	$y = -1.12x + 43.58$	0.96*	1.12	$y = 0.73x + 18.78$	1.00**	0.73	$y = 0.46x + 116.58$	0.98**	0.46	$y = 1.15x + 79.79$	0.98**	1.15 <sup>b</sup>	0.21 <sup>b</sup>
P05_B	12	$y = -0.89x + 30.25$	0.96*	0.89	$y = 0.42x + 18.17$	0.96*	0.42	$y = 0.44x + 127.83$	1.00**	0.44	$y = 1.41x + 81.57$	0.96*	1.41 <sup>b</sup>	0.32 <sup>b</sup>

2 <sup>a</sup>These rates are net rates since  $\Delta(\text{NH}_3+\text{NH}_4^+)$  is the net consumption and  $\Delta\text{NO}_2^-$ ,  $\Delta\text{NO}_3^-$ , and  $\Delta\text{N}_2\text{O}$  is the net production during incubation.

3 <sup>b</sup>These rates and yields (when only nitrification occurred) were used to calculate the average *amoA* gene copy-specific N<sub>2</sub>O production rates and  
4 N<sub>2</sub>O yields in Figure 7.

5 <sup>c</sup>No estimation of N<sub>2</sub>O yield was made due to nitrification and denitrification may occur concurrently and DIN was not in balance.

6 \*  $P < 0.05$ ; \*\*  $P < 0.01$ .

7 –No regression analysis or no estimation made due to DIN was not in balance.

## Supplement of

### **Major role of ammonia-oxidizing bacteria in N<sub>2</sub>O production in the Pearl River Estuary**

Li Ma<sup>1,2</sup>, Hua Lin<sup>1,2,3</sup>, Xiabing Xie<sup>1</sup>, Minhan Dai<sup>1,2</sup>, Yao Zhang<sup>1,2</sup>

<sup>1</sup>State Key Laboratory of Marine Environmental Science, Xiamen University, Xiamen 361101, China

<sup>2</sup>College of Ocean and Earth Sciences, Xiamen University, Xiamen 361101, China

<sup>3</sup>Key Laboratory of Marine Ecosystem and Biogeochemistry, State Oceanic Administration, Second Institute of Oceanography, Ministry of Natural Resources, Hangzhou 310012, China

*Correspondence to:* Yao Zhang (yaozhang@xmu.edu.cn)

**Table S1** In situ biogeochemical parameters at the incubation experiment sites.

Site_Layer	Temperature (°C)	DO ( $\mu\text{mol L}^{-1}$ )	$\text{NH}_4^+$ ( $\mu\text{mol L}^{-1}$ )	$\text{NO}_3^-$ ( $\mu\text{mol L}^{-1}$ )	$\text{NO}_2^-$ ( $\mu\text{mol L}^{-1}$ )
P05_S	30	61.3	40.1	126.1	20.5
P05_B	30	54.7	33.3	123.5	24.5
P01_S	29	30.9	167.2	84.0	11.9
P01_B	29	30.0	166.5	82.0	11.6

S, surface; B, bottom.

1 **Table S2** Isotopic composition of N<sub>2</sub>O during bacterial and archaeal ammonia oxidation, bacterial nitrifier-denitrification, and bacterial  
 2 denitrification.

Pathway	Microorganisms	Species	Substrate	$\delta^{15}\text{N-N}_2\text{O}$	References			
Ammonia oxidation			NH <sub>4</sub> <sup>+</sup>	-68--60‰	Yoshida, 1988; Toyoda et al., 2017			
			<i>β</i> -proteobacteria	<i>Nitrosomonas europaea</i>	NH <sub>4</sub> <sup>+</sup>	-46.9--46.1‰	Sutka et al., 2006	
					NH <sub>4</sub> <sup>+</sup>	-19.88±0.39‰	Jung et al., 2014	
				<i>Nitrosomonas marina</i> C-113a	NH <sub>4</sub> <sup>+</sup>	-54.9--15.2‰ (0.5% O <sub>2</sub> ) <sup>a</sup>	Frame and Casciotti, 2010	
			NH <sub>4</sub> <sup>+</sup>		-13.6--6.7‰ (20% O <sub>2</sub> ) <sup>a</sup>			
			<i>γ</i> -proteobacteria	<i>Methylomonas methanica</i>	NH <sub>4</sub> <sup>+</sup>	-39.4‰	Mandernack et al., 2009	
				CN25 (marine)	NH <sub>4</sub> <sup>+</sup>	6.3-10.2‰	Santoro et al., 2011	
				MY1 (soil)	NH <sub>4</sub> <sup>+</sup>	-13.53±2.12‰	Jung et al., 2014	
			Archaea		MY2 (soil)	NH <sub>4</sub> <sup>+</sup>	-16.96±1.81‰	Jung et al., 2014
					MY3 (soil)	NH <sub>4</sub> <sup>+</sup>	-16.49±2.18‰	Jung et al., 2014
					JG1 (soil)	NH <sub>4</sub> <sup>+</sup>	-15.32±0.16‰	Jung et al., 2014
	AR (marine sediment)	NH <sub>4</sub> <sup>+</sup>		-12.91±1.50‰	Jung et al., 2014			

		CS (acid mine)	NH <sub>4</sub> <sup>+</sup>	-35.54±0.89‰	Jung et al., 2014
			NH <sub>2</sub> OH	-38.1--20.4‰	Sutka et al., 2003
		<i>Nitrosomonas europaea</i>	NH <sub>2</sub> OH	-5.5-5.1‰	Sutka et al., 2006
	$\beta$ -proteobacteria		NH <sub>2</sub> OH	-34.0--13.8‰	Yamazaki et al., 2014
		<i>Nitrospira multiformis</i>	NH <sub>2</sub> OH	-3.9-1.7‰ (Average -0.3±2.9‰)	Sutka et al., 2006
Hydroxylamine oxidation <sup>b</sup>		<i>Nitrosomonas marina</i> C-113a	NH <sub>2</sub> OH	-6.7‰	Frame and Casciotti, 2010
		<i>Nitrosococcus oceani</i>	NH <sub>2</sub> OH	-17.9--5.8‰	Yamazaki et al., 2014
	$\gamma$ -proteobacteria		NH <sub>2</sub> OH	-0.3-1.7‰ (Average 0.0±1.2‰)	Sutka et al., 2003
		<i>Methylococcus capsulatus</i>	NH <sub>2</sub> OH	1.3-5.2‰ (Average 3.4±1.9‰)	Sutka et al., 2006
		<i>Nitrosomonas marina</i> C-113a	NO <sub>2</sub> <sup>-</sup>	-57.6±4.1‰	Frame and Casciotti, 2010
Nitrifier-denitrification	$\beta$ -proteobacteria	<i>Nitrosomonas europaea</i>	NO <sub>2</sub> <sup>-</sup>	-39.1--31.0‰ (Average -34.8±2.7‰)	Sutka et al., 2003
		<i>Nitrospira multiformis</i>	NO <sub>2</sub> <sup>-</sup>	-24.2--21.5‰ (Average -22.9±0.6‰)	Sutka et al., 2006
	$\gamma$ -proteobacteria	<i>Pseudomonas fluorescens</i>	NO <sub>3</sub> <sup>-</sup>	-37.2--14.9‰	Toyoda et al., 2005
Denitrification	$\alpha$ -proteobacteria	<i>Paracoccus denitrificans</i>	NO <sub>3</sub> <sup>-</sup>	-20.0--7.9‰	Toyoda et al., 2005

1 <sup>a</sup>O<sub>2</sub> conditions of the incubation experiments.

- 1 <sup>b</sup>Although the  $\delta^{15}\text{N-N}_2\text{O}$  when using  $\text{NH}_2\text{OH}$  as a substrate are listed here, the isotopic composition of  $\text{N}_2\text{O}$  only when using  $\text{NH}_4^+$  as a substrate
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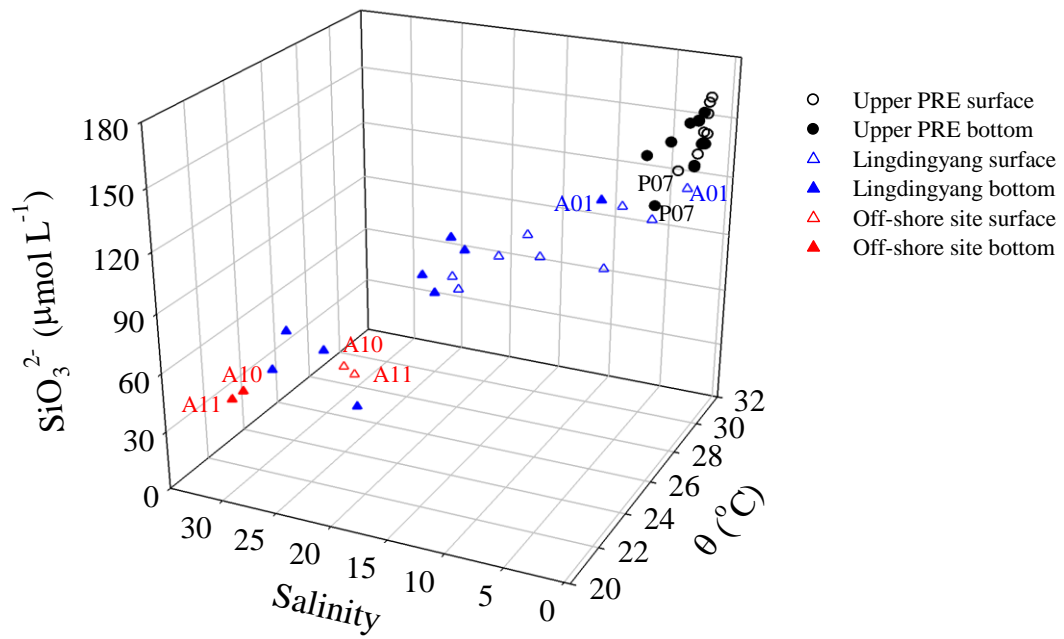
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1 **Table S3** The abundances of DNA- and cDNA-based *amoA* gene and the N<sub>2</sub>O production net rates and yields normalized to total *amoA* gene  
 2 copy or transcript numbers of AOA and AOB in a given sample at the incubation experiment sites.

Site_ Layer	DNA-based AOB (All) (copies L <sup>-1</sup> )	DNA-based AOA (All) (copies L <sup>-1</sup> )	N <sub>2</sub> O production rates (All) (fmol cell <sup>-1</sup> h <sup>-1</sup> )	N <sub>2</sub> O yields (All) (10 <sup>-6</sup> )	DNA-based AOB (PA) (copies L <sup>-1</sup> )	DNA-based AOA (PA) (copies L <sup>-1</sup> )	N <sub>2</sub> O production rates (PA) (fmol cell <sup>-1</sup> h <sup>-1</sup> )	N <sub>2</sub> O yields (PA) (10 <sup>-6</sup> )	cDNA-based AOB (PA) (copies L <sup>-1</sup> )	cDNA-based AOA (PA) (copies L <sup>-1</sup> )	N <sub>2</sub> O production rates (PA) (fmol cell <sup>-1</sup> h <sup>-1</sup> )	N <sub>2</sub> O yields (PA) (10 <sup>-6</sup> )
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

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



1

2 **Figure S1:** Three-dimensional scatter plot of potential temperature ( $\theta$ ) ( $^{\circ}\text{C}$ ), salinity,  
 3 and silicate ( $\text{SiO}_3^{2-}$ ) concentration. PRE, Pearl River Estuary.