Response to Reviewer #2

We greatly thank both anonymous reviewers for the useful and detailed comments on the manuscript, based on which we have revised the manuscript. And the corresponding responses to the comments were in blue color as follows.

Anonymous Referee #2

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This study presents an interesting dataset on the abundance and diversity of bacterial and archaeal *amoA* gene, and the dissimilatory nitrite reductase *nirS* gene of denitrifiers along two transects in the Yangtze River Estuary during spring and summer in the case of the transect along a salinity gradient. In addition, during the summer cruise, both the particle-associated and the free-living microbial communities were analyzed. Overall, the paper provides valuable information on the diversity and distribution of nitrifiers and denitrifiers in estuarine ecosystems. Interestingly, the authors find an apparently higher coupling between potential nitrification and denitrification in the particle-associated compared to the free-living fraction, based on the abundance of *amoA* and *nirS* genes. Despite the overall quality and interest of the manuscript I have some comments which should be revised and addressed before publication. One aspect greatly hindering the conclusion of whether AOA or AOB may contribute more to nitrification is that abundance, as determined by quantification of *amoA* gene copy numbers, does not necessarily reflect an actual contribution to nitrification. The same holds for the conclusion of higher nitrification vs denitrification potential, based on gene copy numbers. Several recent studies found no direct correlation between AOA abundance and potential nitrification rates in both marine, estuarine and soil ecosystems. Therefore the author should carefully revise their statements and adequately discuss their results.

Response:

(1) We agree the reviewer’s suggestion. We revised the relevant statements on AOA vs. AOB throughout the manuscript.

Page 2, Line 13 (in the revised version; the same below): We deleted “*suggesting that the archaea might play a dominant role in nitrification in the YRE*”.

Page 11, Line 26: We deleted “*suggesting that the ammonia-oxidizing process* *might be contributed predominantly by the archaea in the YRE*".

Page 14, Line 14-16: We revised "*This suggested that compared to the surface water, higher potentials for both nitrification and denitrification might occur in the bottom water*" as "*These suggested that compared to the surface water, the bottom water might be more favorable for both nitrification and denitrification potentials*".

Page 14, Line 18-19: "*Thus, higher potentials in the bottom water*" was revised as "*Thus, higher gene abundances in the bottom water*".

Page 16, Line 11-13: We revised "*Archaeal amoA-type nitrifiers were suggestive of the dominant role in the ammonia-oxidizing process of the YRE, since the abundance of the archaeal amoA gene was significantly higher than that of the β-proteobacterial amoA gene (P = 0.001, both unpaired and paired t-test).*" as "*Notably, the qPCR analysis showed that the abundance of the archaeal amoA gene was significantly higher than that of the β-proteobacterial amoA gene (P = 0.001, both unpaired and paired t-test).*"

Page 16, Line 22: "*The dominant role played by AOA in nitrification*" was revised as "*The dominance of AOA in amoA-type nitrifiers*".

Page 18, Line 29; Page 19, Line 1: We revised "*Compared with the AOB, the AOA made a dominant contribution to the ammonia-oxidizing process in the YRE.*" as "*Compared with the AOB, the abundance of AOA are dominant in amoA-type nitrifiers in the YRE.*"

(2) We also revised the description on nitrification vs. denitrification potential based on ^{15}N rate data rather than on the gene abundances.

Page 2, Line 13-17: We revised "*Compared with the amoA gene, a distinctly higher level of diversity but lower gene copy numbers were found for the nirS gene suggesting lower denitrification than nitrification potential.*" as "*Compared with the amoA gene, a significantly higher level of diversity but lower gene copy numbers were found for the nirS gene. Nitrification and denitrification rates based on ^{15}N incubation experiments supported gene abundance data as denitrification rates were below detection limit, suggesting lower denitrification than nitrification potential.*"

Page 12, Line 1: We deleted "*suggesting that the denitrification potential was lower than that of nitrification in the region studied*".

Page 15, Line 8-21: The sentence of "*This suggested that higher potentials for both nitrification and denitrification might occur on the particles rather than in the water column.*" following the statement on gene abundance was deleted and placed in the end of this paragraph.

Page 16, Line 2-5: We revised "*suggesting lower denitrification than nitrification potential. This conclusion was supported by the ^{15}N-based nitrification and denitrification rate data. ……*" as "*This was supported by the ^{15}N-based nitrification and denitrification rate data as the denitrification rate was below the method detection limit. Taken together, ^{15}N-based rate and gene abundances suggested that the denitrification potential was lower than nitrification potential in the YRE.*"

Page 18, Line 22-23: "*both nitrification and denitrification potentials were higher at the estuary bottom than in the surface water*" was revised as "*the estuary bottom might be more favorable for both nitrification and denitrification potentials than the surface water*".

Specific comments

Page 2, lines 6-10. Please revise the order of words in this sentence.

Response:

This sentence was revised as "*We investigated the nitrifying and denitrifying microbial communities in the estuary of turbid subtropical Yangtze River (YRE), the largest river in Asia, by analyzing the ammonia monooxygenase gene amoA, including archaeal and bacterial amoA, and the dissimilatory nitrite reductase gene nirS using clone libraries and quantitative PCR (qPCR)*". (Page 2, Line 6-10)

Page 2. Line 15, 21. What do the authors mean by "distinctly"?. Throughout the text is not clear if they use this term as a synonym of "significantly".

Response:

We replaced "distinctly" with "significantly" throughout the manuscript. (Page 2, Line 14 and 20; Page 16, Line 1)

Page 2, lines 22-24. Please indicate which correlations.

Response:

We revised this sentence as "*Notably, positive correlations between the amoA and* *nirS gene abundances suggested potential gene-based coupling between nitrification and denitrification, ……*”. (Page 2, Line 21-23)

Page 8, lines 25-27 and page 9, lines 1-11. The description of the environmental conditions is very poor. It is not clear at which depth the samples for analysis were taken (the authors just mention surface and bottom waters). I suggest including at least a table with all the biogeochemical variables for each sampling point.

Response:

The detailed figures on environmental variables from the same cruises were published in Hsiao et al. (2013). Here, we added Table S2 in the supplementary material to present all the available biogeochemical variables for each sampling point (please refer to Table S2 in the end of the file). The relevant statements were added in the revised version — “*Detailed biogeochemical variables for each sampling point were showed in Table S2. For detailed figures on biogeochemical parameters, please refer to Hsiao et al. (2013).*” (Page 9, Line 10-12). We also added the information on sampling depth in the revised manuscript (Page 5, Line 7): “*In total, fifty samples were collected from the surface (1-5 m) and bottom (7-50 m) of each station (Table S2), except for site YE0, ……*”.

Reference:

Hsiao, S. S.-Y., Hsu, T.-C., Liu, J.-W., Xie, X., Zhang, Y., Lin, J., Wang, H., Yang, J.-Y. T., Hsu, S.-C., Dai, M., and Kao, S.-J: Nitrification and its oxygen consumption along the turbid Changjiang River plume, Biogeosciences Discuss., 10, 8685-8713, 2013.

Page 9, lines 9-11. The authors should clearly explain these patterns.

Response:

We deleted “*Ammonium and nitrite exhibited the biological mediated pattern,*”, since “*biological mediated pattern*” is just a speculation. We revised it as “*Ammonium and nitrite did not exhibit a gradient decreasing seaward, ranging from ……*”. Also, we added “*Detailed biogeochemical variables for each sampling point were showed in Table S2. For detailed figures on biogeochemical parameters, please refer to Hsiao et al. (2013).*” in the end of this paragraph. (Page 9, Line 8-12)

Page 11, lines 19-29. This description of the abundances is hard to follow. Also I do not think table 1 is the best way to represent these data. I think that a graphic presentation would help to compare between samples and periods.

Response:

We deleted the explanations of the samples in the revised manuscript for a clear description, since it does not influence understanding of the context. We also replaced Table 1 with a figure (please refer to the following figure). (Page 11, Line 20-29; Page 12, Line 1-7)

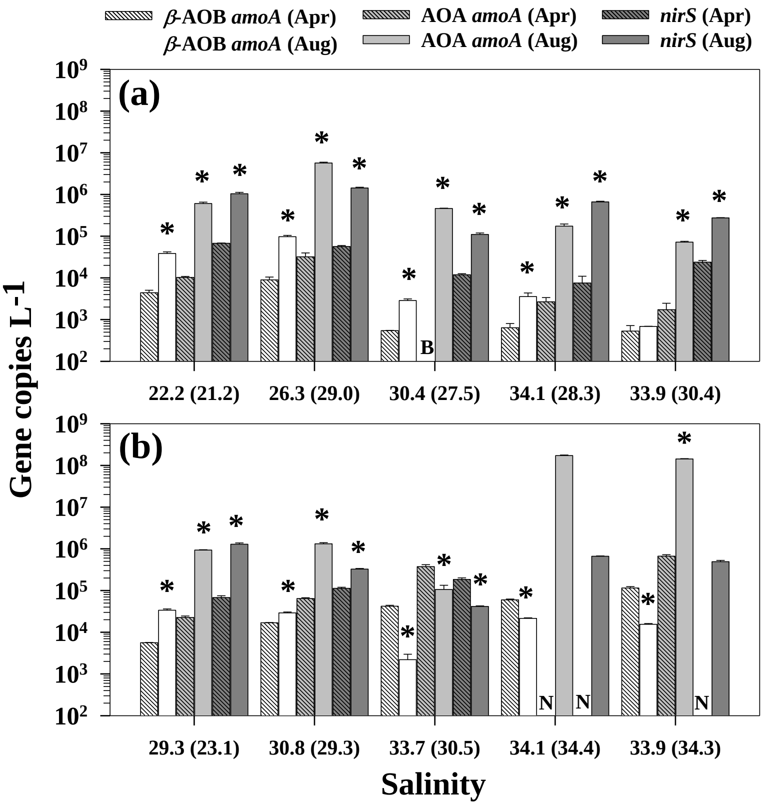

Fig. 7 (in the revised version) β-AOB and AOA *amoA* and *nirS* gene abundances from the free-living (0.2 ~ 3 μm) communities along the salinity transect in April and August. Error bars indicate standard deviation. Asterisks indicate statistically significant differences between the April and August samples. (a) and (b) represent the surface and bottom samples. B: below detection limit; N: not detected due to lack of enough environmental DNA.

Page 12, lines 1-19. There are several fragments here that should go in the discussion rather than in the results section.

Response:

Page 11, Line 26: We deleted "*suggesting that the ammonia-oxidizing process might be contributed predominantly by the archaea in the YRE*".

Page 12, Line 1: We deleted "*suggesting that the denitrification potential was lower than that of nitrification in the region studied*".
Page 12, Line 3: We deleted "*suggesting that the estuary bottom might favor the potentials for both nitrification and denitrification*".

Page 12, lines 22. The authors should include the error bars in figure 7.

Response:

Figure 7 (Figure 8 in the revised version) had already included error bars in the original version.

Page 13, lines 5-6. I do find figure 8 rather complicate and not much illustrative of what the authors try to show. I suggest just representing nitrification rates vs *amoA* gene abundance using different symbols for the particulate and the free-living fractions. Also I do not think that the regression equations make any sense.

Response:

We did analyze the correlations between nitrification rates and *amoA* gene abundances, but there were no significant relationships were found. Figure 9 in the revised version (Figure 8 in the original version) showed significant positive correlations ($P < 0.05$) between the ratios of particle-associated vs. free-living *amoA* gene copy numbers and the ratios of particle-associated vs. free-living ^{15}N-based nitrification rates. It suggested that ^{15}N-based nitrification rates were consistent with gene abundance in term of particle-associated vs. free-living samples and supported the finding that the particle-associated genes abundances were higher than the free-living ones at the sites with high TSM concentrations. We added the statement on the rates and the correlations in the revised manuscript for a better understanding (Page 12, Line 22-28) — "*The ^{15}N-based nitrification rates supported these findings. Higher rates were detected in the particle-associated samples than the free-living ones at the sampling points with high TMS concentrations (Hsiao et al., 2013). Also, the significant positive correlations ($P < 0.05$) were observed between the ratios of particle-associated vs. free-living amoA gene copy numbers and the ratios of ^{15}N-based nitrification rates (Fig. 9) despite no direct correlations between rates and gene copies.*" Also, we deleted the regression equations in Figure 9 according to the reviewer's suggestion (please refer to the following figure).

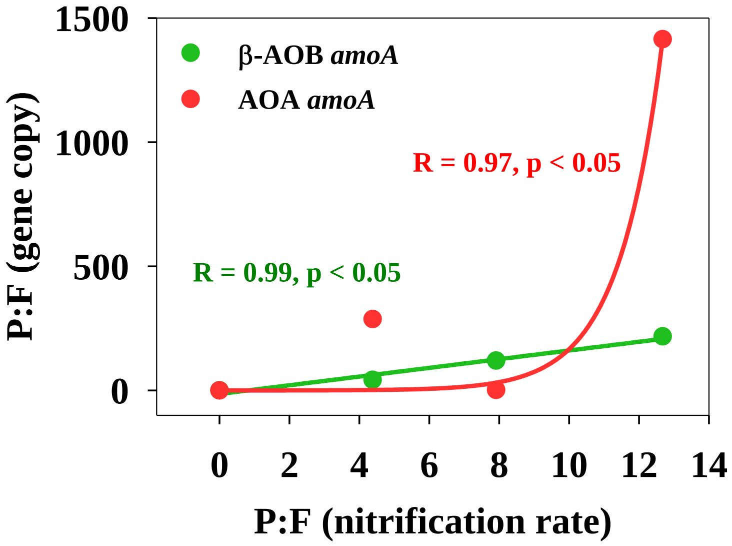

Fig. 9 (in the revised version) Correlations between the ratios of particle-associated vs. free-living *amoA* gene copy numbers and the ratios of ^{15}N-based nitrification rates.

Page 13, lines 7-10. Please revise this sentence, as it is, it makes not much sense. I guess the authors mean something like: “In the latitudinal transect, *amoA* and *nirS* genes in the free-living fraction mostly outnumbered those in the particle-associated fraction”.

Response:

We revised this sentence as “*In the latitude transect, however, β-proteobacterial amoA, archaeal amoA and nirS genes from the free-living communities mostly outnumbered those in the particle-associated fraction, ……*”. (Page 13, Line 1)

Page 13, lines 20-24. Again, a higher number of *amoA* or *nirS* copies does not mean higher nitrification or denitrification rates.

Response:

We revised this sentence as “*These suggested that compared to the surface water, the bottom water might be more favorable for both nitrification and denitrification potentials, ……*”. (Page 14, Line 14-16)

Page 13 lines 24-29. Please revise these sentences for English usage.

Response:

We revised this sentence as “*Thus, higher gene abundances in the bottom water might be a consequence of benefiting from environmental conditions of low DO and high*

TSM concentrations or simply caused by mix of gene from sediment, as revealed by our phylogenetic analysis." (Page 14, Line 18-21)

Page 14, lines 14-16. I would delete this part of the sentence ". . .and the advantages. . .outer estuary". In addition, this relationship is rather obvious, the more particles, the more number of gene copies in the particle-associated than in the free-living fraction. I wonder if the authors could provide the total abundance of prokaryotes in each sample and fraction.

Response:

We deleted this part as suggested (Page 15, Line 8). And we are sorry that we have no the data of total prokaryote abundances in the particle-associated and free-living fraction.

Page 14, lines 18-21. As mentioned above, I do not think that figure 8 is adequate as a correlation between ratios is rather complicate. I think that the authors should represent rates vs gene copies.

Response:

We did analyze the correlations between nitrification rates and *amoA* gene abundances, but there were no significant relationships were found. Figure 9 in the revised version (Figure 8 in the original version) showed higher rates in the particle-associated than free-living fraction, which was consistent with the gene abundances, although the correlations were between ratios. We revised this sentence as "*This finding was supported by* ^{15}*N-based nitrification rates (Hsiao et al., 2013) and the significant positive correlations (*$P < 0.05$*) between the ratios of particle-associated vs. free-living amoA gene copy numbers and the ratios of* ^{15}*N-based nitrification rates (Fig. 9).*" (Page 15, Line 8-11)

Page 14, lines 22-29. The authors could simplify this idea, and provide some more cites.

Response:

We deleted one possibility explaining that the microniche of suspended particulates could be beneficial to microbial activity — "*In addition, particles also offer a refuge to protect microorganisms from grazing (Tuomainen et al., 2003).*", since it is supported by few literatures. And we added some more cites in the revised manuscript

(Page 15, Line 13-20).

References

Crump, B. C., Baross, J. A., and Simenstad, C. A.: Dominance of particle-attached bacteria in the Columbia River estuary, USA, Aquat. Microb. Ecol., 14, 7-18, 1998.

Hanaki, K., Wantawin, C., and Ohgaki, S.: Nitrification at low levels of dissolved oxygen with and without organic loading in a suspended-growth reactor, Water Res., 24, 297-302, 1990.

Xia, X., Yang, Z., and Zhang, X.: Effect of suspended-sediment concentration on nitrification in river water: importance of suspended sediment-water interface, Environ. Sci. Technol., 43, 3681-3687, 2009.

Page 15, line 13-14. As already mentioned, the correlations should be between rates and gene copies, not between P:F ratios.

Response:

We deleted this sentence, and revised this part as "*This was supported by the* ^{15}*N-based nitrification and denitrification rate data as the denitrification rate was below the method detection limit. Taken together,* ^{15}*N-based rate and gene abundances suggested that the denitrification potential was lower than nitrification potential in the YRE.*" (Page 16, Line 2-5)

Page 15, lines 15-21. Please revise this sentence, it makes no sense. The authors should better explain the undetectable denitrification rates.

Response:

We revised this part as "*This was supported by the* ^{15}*N-based nitrification and denitrification rate data as the denitrification rate was below the method detection limit. Taken together,* ^{15}*N-based rate and gene abundances suggested that the denitrification potential was lower than nitrification potential in the YRE. Denitrifiers are reported to be often capable of several different respiratory pathways, including oxygen respiration (Santoro, 2010). Thus, we speculated that diverse denitrifiers were not actively conducting denitrification in the YRE water; instead, heterotrophic metabolism with* O_2*-respiration is more likely since DO concentrations were not low enough during our cruise periods.*" This speculation was just our explanation for the undetectable denitrificatin rates. (Page 16, Line 2-10)

Page 15, lines 22-25. The authors could calculate the AOB/AOA ratio and see if the ratio changes in relation to environmental factors (such as ammonium concentration).

Response:

The AOA/β-AOB *amoA* ratios ranged from 0 to 9243, which was consistent with the previous studies in the ocean (Wuchter et al., 2006; Mincer et al., 2007; Moin et al., 2009; Beman et al., 2010). But there was no a significant changing trend of the ratios along the salinity gradient (please refer to the following tables). We also tried to analyze the correlations between the AOA/β-AOB *amoA* ratios and the available environmental variables according to the reviewer's suggestion. Only phosphate and N_2O concentrations were positively correlated with **particle-associated** AOA *amoA*: β-AOB *amoA* ratios, respectively (please refer to the following figure).

Table 1. AOA/AOB *amoA* gene abundances ratios from the surface free-living communities along the salinity transect in April. S: surface; F: free-living; BDL: below detection limit.

Sample	AprY1	AprY2	AprY3	AprY4	AprY5
	SF	SF	SF	SF	SF
Salinity	22.2	26.3	30.4	34.1	33.9
AOA/AOB	2.3	3.6	AOA BDL	4.2	3.3

Table 2. AOA/AOB *amoA* gene abundances ratios from the bottom free-living communities along the salinity transect in April. B: bottom; F: free-living; N: not detected due to lack of enough environmental DNA.

Sample	AprY1	AprY2	AprY3	AprY4	AprY5
	BF	BF	BF	BF	BF
Salinity	29.3	30.8	33.7	34.1	33.9
AOA/AOB	4	3.8	8.9	AOA N	5.8

Table 3. AOA/AOB *amoA* gene abundances ratios from the surface free-living and particle-associated communities along the salinity transect in August. S: surface; F: free-living; P: particle-associated; BDL: below detection limit.

Sample	AugY0		AugY1	AugY2	AugY3		AugY4	AugY5	
	SP	SF	SF	SF	SP	SF	SF	SP	SF
Salinity	0.2		21.2	29.0	27.5		28.3	33.9	

AOA/AOB	752.3	111.7	15.7	58.3	0.1	161.6	48.7	AOB BDL	104.9

Table 4. AOA/AOB *amoA* gene abundances ratios from the bottom free-living and particle-associated communities along the salinity transect in August. B: bottom; F: free-living; P: particle-associated.

	AugY0		AugY1	AugY2	AugY3		AugY4	AugY5	
	BP	BF	BF	BF	BP	BF	BF	BP	BF
Salinity	0.2		23.1	29.3	30.5		34.4	34.3	
AOA/AOB	17.7	1123.3	27.7	45.1	312.7	48.1	7972.9	279.7	9243.0

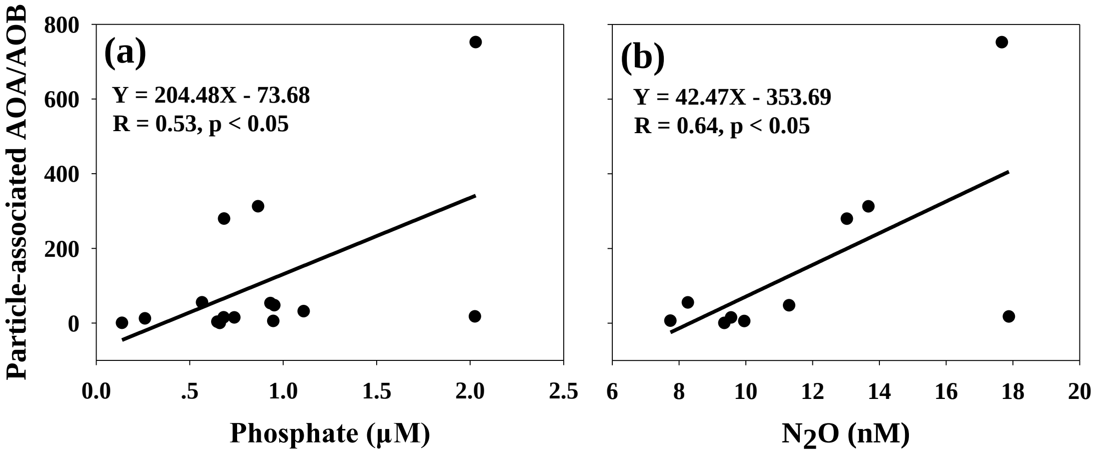

Figure. Correlations between the particle-associated AOA/*β*-AOB *amoA* ratios and phosphate and N_2O concentrations

References:

Beman, J. M., Sachdeva, R., and Fuhrman, J. A.: Population ecology of nitrifying archaea and bacteria in the Southern California Bight, Environ. Microbiol., 12, 1282–1292, 2010.

Francis, C. A., Roberts, K. J., Beman, J. M., Santoro, A. E., and Oakley, B. B.: Ubiquity and diversity of ammonia-oxidizing archaea in water columns and sediments of the ocean, P. Natl. Acad. Sci. USA, 102, 14683–14688, 2005.

Mincer, T. J., Church, M. J., Taylor, L. T., Preston, C., Karl, D. M., and DeLong, E. F.: Quantitative distribution of presumptive archaeal and bacterial nitrifiers in Monterey Bay and the North Pacific Subtropical Gyre, Environ. Microbiol., 9, 1162–1175, 2007.

Moin, N. S., Nelson, K. A., Bush, A., and Bernhard, A. E.: Distribution and diversity

of archaeal and bacterial ammonia oxidizers in salt marsh sediments, Appl. Environ. Microbiol., 75, 7461-7468, 2009.

Page 16, lines 11-29, page 17, lines 1-7. This section should be revised for clarity. Moreover, the authors should explain figure 9 in the results section. I think that the authors could simplify figure 9 just representing total *amoA* gene abundances vs *nirS* gene abundances. It has been already stated that *β-amoA* gene abundance was very low. On the contrary, it would be interesting to simultaneously show such relationship in the particle-associated vs the free-living fraction, in order to show the higher potential coupling between both processes in particles.

Response:

We revised Figure 9 (in the original version) according to the reviewer's suggestion (please refer to the following figure), and explain the figure in the result section (Page 13, Line 5-8). Accordingly, we also revised this discussion section for clarity (Page 16, Line 28-29; Page 17, Line 1-18).

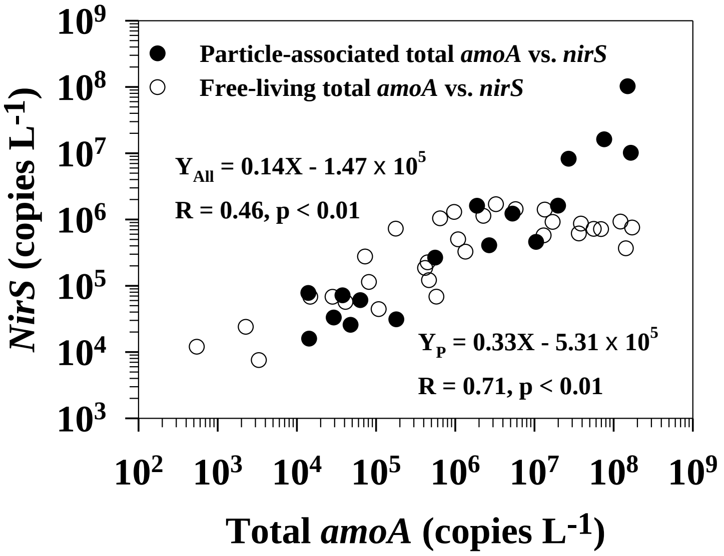

Fig. 10 (in the revised version) Analysis of relationships between the total *amoA* (sum of β-AOB and AOA *amoA*) and *nirS* gene abundances.

Page 17, lines 9-29, and page 18, lines 1-23. As for the previous section, figures 10 and 11 should also be explained in the result section. I do not find that figure 11 is necessary; the authors can provide the correlation coefficients in the text. Also figure 11c is redundant, the authors have already discussed that there *amoA* and *nirS* genes are more abundant in the particle-associated than in the free-living fraction.

Response:

The description and explanation on Figure 10 and 11 of the original version were moved to the result section (Page 13, Line 9-29; Page 14, Line 1-6). Figure 11 of the original version was deleted in the revised version according to the reviewer's suggestion. Accordingly, we also revised this discussion section for clarity (Page 17, Line 19-29; Page 18, Line 1-16).

Table S2. The biogeochemical variables for each sampling point. Apr: April; Aug: August.

Samples	Longitude (°E)	Latitude (°N)	Max depth (m)	Sampling depth (m)	Salinity	Temperature (°C)	DO (mg L^{-1})	Nitrate (μM)	Nitrite (μM)	Ammonium (μM)	Phosphate (μM)	Silicate (μM)	TSM (mg L^{-1})
AprY1	122.378	31.107	13.0	1.0	22.18	N	9.89	26.81	0.98	0.68	0.51	29.00	N
				11.0	29.30	N	9.30	76.43	0.31	0.16	1.15	14.71	N
AprY2	122.600	31.000	18.0	1.0	26.28	N	9.59	44.19	1.12	0.55	0.79	22.28	N
				16.0	30.75	N	9.13	22.22	0.64	0.37	0.56	13.80	N
AprY3	122.999	30.836	46.6	1.0	30.39	N	11.74	10.64	0.73	B	0.02	5.67	N
				44.0	33.70	N	8.34	8.87	0.19	B	0.34	7.43	N
AprY4	123.510	30.513	59.0	1.0	34.07	N	10.11	1.49	0.05	0.14	0.06	2.79	N
				55.0	34.14	N	8.50	6.76	0.29	B	0.40	7.64	N
AprY5	123.999	30.368	49.0	1.0	33.91	N	9.56	4.22	0.26	0.58	0.23	6.83	N
				45.0	33.91	N	8.86	5.72	0.29	0.10	0.35	7.84	N
AugY0	121.731	31.322	10.0	3.0	0.20	29.21	6.15	132.94	0.45	B	2.03	126.57	170.86
				7.0	0.20	29.21	6.15	132.52	0.46	0.35	2.03	125.10	261.78
AugY1	122.328	31.015	12.0	3.0	21.22	25.83	5.81	53.63	0.30	0.75	1.24	52.50	216.03
				8.0	23.06	25.96	5.87	52.15	0.30	1.22	1.21	55.49	216.19
AugY2	122.599	30.984	20.0	3.0	29.00	24.30	4.68	29.45	0.41	0.22	0.91	30.63	13.29
				16.0	29.31	23.99	4.60	26.59	0.32	1.02	0.89	29.31	87.44
AugY3	122.826	30.839	23.0	3.0	27.46	26.14	4.73	26.05	1.33	0.63	0.66	25.05	4.56
				20.0	30.55	22.88	4.26	24.56	0.31	0.38	0.87	31.52	50.72
AugY4	123.498	30.508	56.0	3.0	28.33	26.03	7.44	10.55	0.55	1.27	B	9.73	3.39
				50.0	34.39	19.68	3.78	12.66	0.15	0.55	0.92	21.63	9.66
AugY5	124.005	30.351	51.0	3.0	30.41	27.37	7.96	3.87	0.36	1.06	B	5.37	10.51
				46.0	34.30	21.49	4.31	9.12	0.18	0.22	0.68	16.60	4.46

AugYE5	122.834	30.001	48.0	5.0	29.36	25.74	4.48	22.00	1.02	B	0.68	23.84	3.08
				43.0	33.38	21.00	3.35	16.29	0.80	0.06	0.95	24.93	27.20
AugYE4	122.833	30.496	42.0	5.0	28.69	25.09	6.46	24.86	1.19	0.07	0.74	30.68	2.54
				38.0	32.92	21.07	3.25	19.06	0.57	0.29	1.11	25.97	60.87
AugYE3	122.834	31.008	33.0	3.0	24.93	26.52	4.87	32.64	0.98	0.17	0.95	30.15	5.37
				29.0	32.70	21.77	2.80	20.88	0.24	0.19	0.65	24.47	13.53
AugYE2	122.836	31.337	47.0	3.0	27.83	25.53	7.02	14.45	0.90	1.68	0.26	20.10	2.49
				43.0	33.25	21.30	2.60	17.75	0.33	B	0.93	26.49	3.88
AugYE1	122.836	31.662	34.0	3.0	24.06	26.32	6.00	24.25	0.95	0.44	B	18.42	6.87
				30.0	31.90	22.69	3.90	17.18	0.35	B	0.57	20.44	6.79
AugYE0	122.827	32.006	36.0	3.0	29.83	23.76	5.87	11.53	0.93	0.85	0.14	15.36	8.26

B, below detection limit;

N, not detected due to lack of sample.