Response to Reviewer #1

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 are in blue color as follows.

Anonymous Referee #1

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The study by Zhang et al., represents an important contribution to the understanding of the nitrification and denitrification processes in river estuaries. The authors have analyzed the abundance and diversity of the bacterial and archaeal *amoA* gene, and the dissimilatory nitrite reductase *nirS* gene of denitrifiers in a transect from the Yangtze river mouth to the open water, in spring and summer, surface and bottom water and, in some cases, also differentiating between particle-associated and free-living microbial communities. In addition, they have analyzed the spatial and temporal structure of these microbial communities by clustering analysis and analyze the possible environmental factors influencing these processes. The manuscript is well-written and experimentally supported. However, I have some comments I would like to see addressed before recommend it for publication:

-Abstract and throughout the manuscript: The fact that the abundance of archaeal AOA *amoA* gene is higher than AOB *amoA* gene DOES NOT imply that archaea have a more dominant role than AOB in the nitrification process (see for example Mußmann et al., 2011, PNAS). I recommend addressing this possibility and reviewing what is known about it. Same thing applies for the claim that denitrification is lower than nitrification based on the lower abundance of *nirS* respect to *amoA* gene. As the authors have indeed estimated nitrification and denitrification rates it is recommended to support your statement on the rate measurements rather than on the gene abundances.

Response:

(1) We agree the reviewer's suggestion. Mußmann et al. (2011) reported that abundance of AOA *amoA* genes greatly outnumbered AOB *amoA* genes, but AOB were most likely responsible for ammonia-oxidizing process in one of the wastewater treatment plants via FISH combined with microautoradiography, although a number of studies in oceans (especially coastal oceans and estuaries) showed that AOA *amoA* abundance were greater than AOB *amoA* abundance, so was active (Wuchter et al., 2006; Caffrey et al., 2007; De Corte et al., 2009).

We revised the relevant statements 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.*"

Reference

Caffrey, J. M., Bano, N., Kalanetra, K., and Hollibaugh, J. T.: Ammonia oxidation and ammonia-oxidizing bacteria and archaea from estuaries with differing histories of hypoxia, ISME J., 1, 660–662, 2007.

De Corte, D., Yokokawa, T., Varela, M. M., Agogué, H., and Herndl, G. J.: Spatial distribution of bacteria and archaea and *amoA* gene copy numbers throughout the water column of the Eastern Mediterranean Sea, ISME J., 3, 147–158, 2009.

Mußmann, M., Brito, I., Pitcher, A., Sinninghe Damsté, J. S., Hatzenpichler, R., Richter, A., Nielsen, J. L., Nielsen, P. H., Müller, A., Daims, H., Wagner, M., and Head, I. M.: Thaumarchaeotes abundant in refinery nitrifying sludges express *amoA* but are not obligate autotrophic ammonia oxidizers, P. Natl. Acad. Sci. USA, 108, 16771-16776, 2011.

Wuchter, C., Abbas, B., Coolen, M. J. L., Herfort, L., van Bleijswijk, J., Timmers, P., Strous, M., Teira, E., Herndl, G. J., Middelburg, J. J., Schouten, S., and Sinninghe Damsté, J. S.: Archaeal nitrification in the ocean, P. Natl. Acad. Sci. USA, 103, 12317–12322, 2006.

(2) We also revised the description on nitrification vs. denitrification potential according to the reviewer's suggestion.

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*".

-Material and methods: What was the water depths at "surface" and "deep" samples?

Response:

The water depths of the "surface" samples were 1-5 m and the "bottom" samples were 7-50 m. We supplied this information in the revised manuscript (Page 5, Line 7). We also supplied Table S2 for the detailed biogeochemical variables for each sampling point. Please refer to the end of this file.

Why is the phylogeny of the *nirS* gene based on protein sequences?

Response:

Phylogenetic diversity of the *nirS* gene was analyzed based on the DNA sequences. But the phylogenetic tree of the *nirS* gene was constructed based on the translated amino acid sequences. That is because the diversity of *nirS* gene was extraordinarily high while the protein sequences were more conservative than nucleic acids

sequences. So the phylogenetic tree based on the amino acid sequences was more suitable than based on the nucleic acids sequences. So far, almost all phylogenetic trees of the *nirS* gene in literatures were based on the amino acids sequences (Braker et al., 2000; Jayakumar et al., 2004; Castro-González et al., 2005; Hannig et al., 2006; Santoro et al., 2006; Tiquia et al., 2006; Falk et al., 2007; Ruiz-Rueda et al., 2007; Dang et al., 2009).

References:

Braker, G., Zhou, J., Wu, L., Devol, A. H., and Tiedje, J. M.: Nitrite reductase genes (nirK and nirS) as functional markers to investigate diversity of denitrifying bacteria in Pacific Northwest marine sediment communities, Appl. Environ. Microbiol., 66, 2096–2104, 2000.

Castro- González, M., Braker, G., Farías, L., and Ulloa, O.: Communities of nirS-type denitrifiers in the water column of the oxygen minimum zone in the eastern South Pacific, Environ. Microbiol., 7, 1298–1306, 2005.

Dang, H., Wang, C., Li, J., Li, T., Tian, F., Jin, W., Ding, Y., and Zhang, Z.: Diversity and distribution of sediment nirS-encoding bacterial assemblages in response to environmental gradients in the eutrophied Jiaozhou Bay, China, Microb. Ecol., 58, 161–169, 2009.

Falk, S., Hannig, M., Gliesche, C., Wardenga, R., Köster, M., Jürgens, K., and Braker, G.: nirS-containing denitrifier communities in the water column and sediment of the Baltic Sea, Biogeosciences, 4, 255–268, doi:10.5194/bg-4-255-2007, 2007.

Hannig, M., Braker, G., Dippner, J., and Jürgens, K.: Linking denitrifier community structure and prevalent biogeochemical parameters in the pelagial of the central Baltic Proper (Baltic Sea), FEMS Microbiol. Ecol., 57, 260–271, 2006.

Jayakumar, D. A., Francis, C. A., Naqvi, S. W. A., and Ward, B. B.: Diversity of nitrite reductase genes (nirS) in the denitrifying water column of the coastal Arabian Sea, Aquat. Microb. Ecol., 34, 69–78, 2004.

Ruiz-Rueda, O., Trias, R., Garcia-Gil, L. J., and Bañeras, L.: Diversity of the nitrite reductase gene nirS in the sediment of a free-water surface constructed wetland, Int. Microbiol., 10, 253–260, 2007.

Santoro, A. E., Boehm, A. B., and Francis, C. A.: Denitrifier community composition along a nitrate and salinity gradient in a coastal aquifer, Appl. Environ. Microbiol., 72, 2102–2109, 2006.

Tiquia, S. M., Masson, S. A., and Devol, A.: Vertical distribution of nitrite reductase genes (nirS) in continental margin sediments of the Gulf of Mexico, FEMS Microbiol. Ecol., 58, 464–475, 2006.

-Results: >Page 17828 line 20: Briefly describe “water column A” and “Water column B” classification by Francis et al., 2005

Response:

We supplied the description on “water column A” and “water column B” according to Francis et al. (2005) — “*All archaeal amoA sequences fell in the previously described sediments (160 sequences), water column A from the Black Sea and Monterey Bay (225 sequences), and water column B from the Eastern Tropical North Pacific (one sequence) clusters (Francis et al., 2005)*”. (Page 10, Line 12-15)

>Page 17829 line 8-9: this sentence belongs to the discussion

Response:

We deleted this sentence as suggested (Page 11, Line 4).

>Page 17830 line10-11: As mentioned above, higher abundance of AOA *amoA* gene does not imply higher contribution of AOA to the nitrification process. Besides, this is not part of the result section.

Response:

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

>Page 17830 lines 18-19: Same as above regarding the *nirS* gene and the denitrification potential

Response:

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

>Line 21-22-23: Same as above.

Response:

We deleted "*suggesting that the estuary bottom might favor the potentials for both nitrification and denitrification*". (Page 12, Line 3)

-Discussion: >Page 17833 line 18-20: Nitrification and denitrification data should support the higher abundance of *amoA* genes vs *nirS* genes as indicators of higher nitrification potential, not the other way around.

Response:

We revised this section — "*Although high levels of diversity were observed in the YRE, the nirS gene abundance was significantly lower than that of total amoA gene in the YRE (P = 0.006, unpaired t-test; P = 0.001, paired t-test). 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 1-5)

>Page 17834 lines 1-4: as mentioned above this is not always like this! In general, I think the authors should have also included some discussion on the differences between AOA/AOB ratio in the salinity transect and related to previous studies

Response:

We revised this sentence 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 11-13)

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).

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

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.

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 (℃)	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.