

## Response to Referee #1

At first, we would like to express our appreciations to you for your kind help and valuable comments about the revision of this manuscript (MS No.bg-2019-114). We have considered your valuable suggestions and carefully revised this manuscript.

**The detailed responses inserted into reviewer #1 comments are attached as follows:**

*Anonymous Referee #1*

*Received and published: 19 April 2019*

*The manuscript “Effects of Sea Animal Colonization on the Coupling between Dynamics and Activity of Soil Ammonia-oxidizing Bacteria and Archaea in Maritime Antarctica” investigated the abundance, diversity, community structure and bioactivity of ammonia oxidising bacteria and archaea in Antarctic maritime soils colonized by sea animals. The results found that soils colonized by seals and penguins exhibited higher AOB than AOA, as well as higher ammonia oxidizing rates than the control tundra soils. These findings suggest that AOB may play a more important role than AOA in driving ammonia oxidizing in penguin and seal colonized soils, while AOA more important in control tundra soils. The research provided very interesting findings, which contributes to understand the nitrogen cycling in Antarctic coastal soils. Here are my concerns:*

**Author response:** Thanks for your positive comments and valuable suggestions.

*1. Too many abbreviations for samples and sites, authors got PS, PL, MS, SS and BS for sample IDs and PTS, PLS, MS, STS and BS for sites. This is just too confusing to read.*

**Author response:** Thanks for your good suggestions. In the revised manuscript, we have used SS, PS, PL, MS, and BS for the samples and sites consistently to escape the ambiguity.

*2. Line 25, Nitrospira is an AOB, Nitrososphaera is an AOA, need to change their order in the sentence.*

**Author response:** The order has been changed in the sentence.

*3. Line 41, Comammox (COMplete AMMonia OXidiser) is an abbreviation, please provide its full name. 4. line 40-41, “Only recently...”, this sentence seems to be out of picture, **I would suggest to remove it.***

**Author response:** Thanks for your good suggestions. This sentence and

comammox has been removed in the revised manuscript.

5. Line 56, “However, there has been limited...”, I don’t think this sentence is correct, especially after the authors listed so many studies on ammonia-oxidisers in line 52-56.

**Author response:** This sentences has been removed in the revised manuscript.

6. Line 210 “mean pH range of 5.3-6.6”, The word mean and range seems contradict to each other, I guess the word “mean” here represents the mean of each sampling site. This is better to be clarified.

**Author response:** This has been corrected into “the soil mean pH ranged from 5.3 to 6.6 at each tundra patch”.

7. Line 211, “Penguin and seal colony tundra soils, PTS and STS, had lower TOC...” Firstly I couldn’t find the C:N ratio in Table 1;

**Author response:** The data about C:N ratios have been added in **Table 1**.

secondly the table 1 used id SS, PS, PL etc, but main text used PTS, STS etc, therefore lacking consistency,

**Author response:** In the revised manuscript, we have used id SS, PS, PL, MS and BS in **both Table 1 and the main text** for their consistency.

lastly, the TOC level of PS (PTS) site was not significantly different from the PLS, MS and BS sites. I think the lack of significance was due to large variations?

**Author response:** Yes. The lack of significance might be due to large variations of TC contents caused by high soil heterogeneity in each tundra patch. Generally, penguin or seal colonies and the active areas are devoid of vegetation due to toxic overmanuring and their trampling. Penguin and seal colony tundra soils, PS and SS, had lower TC contents and C:N ratios than the animal-lacking tundra soils (PL), tundra marsh soils (MS), and background tundra soils (BS).

8. Line 213, “as expected, soil nutrient levels...”, why is this expected? I could understand that TN may be higher with penguin guano and seal faeces input, but why TP and TS? Furthermore, there was no significant TN difference in BS with SS, PS and PL, similarly for TS, TP, and even ammonia. This greatly reduces the reliability of authors’ claim.

**Author response:** (1) According to food chains, krill, as main food for penguins, is rich in N, P and S, whereas penguin is one of main foods for seals. The N, P, and S are highly enriched in penguin guano, and they are typical elements for penguin guano (Sun et al., 2000; Sun et al., 2004; Zhu et al., 2013; Zhu et al., 2014). Therefore soil nutrients N, P and S are higher in penguin or seal colony soils due to the deposition of penguin guano or seal excrements in maritime Antarctica; (2) Generally, penguin or seal colonies and the active areas are devoid of vegetation due to toxic overmanuring and trampling (Tatur et al., 1997; Sun et al., 2004), whereas the animal-lacking tundra areas adjacent to penguin or seal colonies, with moderate amount of nutrients, is favorable for vegetation, such as mosses and algae, due to the volatilization and deposition of ammonia and sulfur-containing compounds from penguin guano or seal excreta. **The growth and nitrogen fixation of the vegetation, and the volatilization and deposition of ammonia and sulfur-containing compounds** increased soil TC, TN and TS contents in animal-lacking tundra soils (Zhu et al., 2011; Zhu et al., 2013). In penguin or seal colonies, the penguin or seal populations showed high inhomogeneous distribution, and this led to the large differences in soil TC, TN, TS, NH<sub>4</sub><sup>+</sup>-N contents. **Therefore, overall mean TC, TN and TS contents showed no significant differences between SS, PS, PL and BS (Table 1).**

The related references are as follows:

- Tatur, A., Myrcha, A., and Niegodzis, J.: Formation of abandoned penguin rookery ecosystems in the maritime Antarctic, *Polar Biology*, 17, 405–417, <https://doi.org/10.1007/s003000050135>, 1997.
- Sun, L. G., Xie, Z. Q., and Zhao, J. L.: Palaeoecology: A 3,000-year record of penguin populations, *Nature*, 407, 858, <https://doi.org/10.1038/35038163>, 2000.
- Sun, L. G., Liu, X. D., Yin, X. B., Zhu, R. B., Xie, Z. Q., and Wang, Y. H.: A 1,500-year record of Antarctic seal populations in response to climate change, *Polar Biology*, 27, 495–501, <https://doi.org/10.1007/s00300-004-0608-2>, 2004.
- Zhu, R. B., Liu, Y. S., Xu, H., Ma, D. W., and Jiang, S.: Marine animals significantly increase tundra N<sub>2</sub>O and CH<sub>4</sub> emissions in maritime Antarctica, *Journal of Geophysical Research: Biogeosciences*, 118(4), 1773–1792, <https://doi.org/10.1002/2013JG002398>, 2013.
- Zhu, R. B., Sun, J. J., Liu, Y. S., Gong, Z. J., and Sun, L. G.: Potential ammonia emissions from penguin guano, ornithogenic soils and seal colony soils in coastal Antarctica: effects of freezing-thawing cycles and selected environmental variables, *Antarctic Science*, 23(1), 78–92, <https://doi.org/10.1017/S0954102010000623>, 2011.
- Zhu, R. B., Wang, Q., Ding, W., Wang, C., Hou, L. J., and Ma, D. W.: Penguins significantly

increased phosphine formation and phosphorus contribution in maritime Antarctic soils, Scientific Reports, 4, 7055, <https://doi.org/10.1038/srep07055>, 2014.

*After a close inspection on the numbers provided in the table 1, it seems that the large ammonia in SS and PS was due to a single sample in each site, I don't know how far SS1 and SS2 are to generate such large differences. Furthermore, this may not make much sense, the SS1 has ammonia concentration of 650 mg/kg, the highest among all other SS samples and 35 times higher than SS2, but its total nitrogen was only 1.2%, even 0.4% lower than SS2. Similar unusual pattern was also in the ammonia concentration in PS2 sample I would strongly suggest the authors to recheck their measurements. As these environmental factors are the basis of many statistical analysis performed later, this would completely make authors conclusion invalid.*

**Author response:** We have rechecked the measurement results, and confirm that our data are right and valid. The reasons are as follows:

(1) We measured soil TC and TN concentrations again, **which are provided in the following Table**, and the results are similar to those in this study, and their concentrations still showed large differences at the each sites within penguin or seal colony; (2) We have measured soil physiochemical properties several times which were given in our previous published papers:

Zhu RB, Liu YS, Xu H, Ma DW, Jiang S. Marine animals significantly increase tundra N<sub>2</sub>O and CH<sub>4</sub> emissions in maritime Antarctica. Journal of Geophysical Research: Biogeosciences, 2013, 118: 1773–1792, doi:10.1002/2013JG002398.

Zhu RB, Liu YS, Ma ED, Sun JJ, Xu H, Sun LG. Nutrient compositions and potential greenhouse gas production in penguin guano, ornithogenic soils and seal colony soils in coastal Antarctica. Antarctic Science, 2009, doi:10.1017/S0954102009990204.

Soil chemical properties, especially NH<sub>4</sub><sup>+</sup>-N, NO<sub>3</sub><sup>-</sup>-N, P and S concentrations, also showed large differences due to effects of penguin or seal activities according to the two papers above.

Therefore we think that TC, TN, TS, TP, NH<sub>4</sub><sup>+</sup>-N and NO<sub>3</sub><sup>-</sup>-N levels showed high heterogeneity in penguin or seal colony tundra soils, PS and SS **due to the deposition of penguin or seal excreta, and the differences of tundra vegetation and soil texture caused by animal tramp.**

	Original No.	No. in the paper	Detection in 2015			Re-detection in 2019		
			N(mg/g)	C(mg/g)	C/N	N(mg/g)	C(mg/g)	C/N
Seal colony soils in western coast on Fildes Peninsula	SK1	SS1	12.12	48.67	4.02	9.99	54.52	5.51
	SK4	SS2	16.94	70.06	4.13	13.38	81.81	6.15
	SK6	SS3	0.87	5.56	6.37	1.51	10.34	6.85
	SK7		2.40	13.64	5.69	The sample is used up.		
	SK8	SS4	1.28	8.59	6.71	The sample is used up.		
	SK9		2.63	18.88	7.19	2.51	18.98	7.56
	SK10	SS5	1.30	11.54	8.87	The sample is used up, the same as below		
Penguin colony soils on Ardley Island	E1		10.54	50.58	4.8	8.68	55.83	6.43
	E2	PS1	14.55	84.65	5.82			
	E3		7.73	51.64	6.68	7.92	55.84	7.05
	E4	PS2	8.34	38.08	4.56			
	E5		15.07	89.71	5.95	13.48	92.33	6.85
	E6	PS3	17.90	120.76	6.75			
	E7		27.33	156.78	5.74	26.34	162.93	6.19
	E8	PS4	15.45	107.47	6.96			
	E9		9.99	73.10	7.31	8.87	79.72	8.99
	E10	PS5	7.97	45.82	5.75			
The middle tundra soils on Ardley Island	M1	PL1	11.53	117.64	10.2	9.88	124.91	12.64
	M2		13.61	138.41	10.17			
	M3	PL2	3.93	38.05	9.68	4.51	50.41	11.18
	M4		8.09	82.40	10.18			
	M5	PL3	25.30	302.52	11.96	23.94	301.93	12.61
	M6		20.19	222.45	11.02			
	M7	PL4	7.17	71.85	10.02	6.37	74.82	11.75
	M8		9.84	114.99	11.69			
	M9		11.47	110.65	9.65			
	M10		15.84	177.48	11.21	15.69	190.83	12.16
	M11		11.61	119.29	10.27			
	M12		4.34	44.40	10.23			
	M13		9.65	116.36	12.05			
	M14		3.33	30.13	9.04	2.77	30.49	11.01
	M15		12.95	147.59	11.39			
The tundra marsh soils in west of	W1	MS1	8.93	95.54	10.7	9.65	111.82	11.59
	W2		11.92	148.81	12.49			
	W3	MS2	15.89	193.95	12.2	14.35	191.57	13.35

Ardley Island (almost no animals)	W4		17.83	217.76	12.21			
	W5		12.93	141.64	10.95	10.79	136.73	12.67
	W6	MS3	19.79	226.90	11.46			
	W7		10.81	122.84	11.37	9.37	122.43	13.07
	W8	MS4	26.57	355.02	13.36			
	W9		21.88	254.01	11.61	20.87	257.11	12.32
	W10	MS5	23.51	292.00	12.42			
	adw-A		20.67	260.05	12.58	19.98	265.81	13.30
	adw-B		14.74	188.68	12.8			
	adw-C		17.29	235.79	13.63	17.76	252.1	14.19
The background tundra soils On Fildes Peninsula	GW1	BS1	4.76	56.72	11.91	4.81	56.89	11.83
	GW2	BS2	5.05	56.63	11.21	5.2	63	12.12
	GW3	BS3	4.30	47.69	11.09			
	gwc1		3.29	31.78	9.66	3.1	35.4	11.42
	gwc2		3.09	29.65	9.6			
	gwc3		2.41	24.03	9.96	2.5	28.3	11.32
	gwc4		2.37	24.39	10.29			

9. Line 219, “likewise, soil...”, which site is author referring here? PTS or PLS? Or stating a generally pattern from PTS, PLS to MS? Please clarify. As PTS is clearly not showing this pattern.

**Author response:** This only stated a general pattern from PS, PL sites to MS sites. Considering that PS sites do not show this pattern due to large spatial variations, this sentence was removed in the revised manuscript. The related description about soil chemical properties has been reorganized as follows:

PS and SS had generally lower C:N ratios than the penguin-lacking tundra soils (PL), tundra marsh soils (MS), and background tundra soils (BS). Soil mean TN, TS and  $\text{NH}_4^+\text{-N}$  levels were higher in PS, SS, PL, and MS than in BS. Soil  $\text{NH}_4^+\text{-N}$  contents were 1–2 orders of magnitude higher in PS and SS than in PL, MS, and BS, with the means of 176.9 and 137.6  $\text{mg NH}_4^+\text{-N kg}^{-1}$ , respectively. The highest  $\text{NO}_3^-\text{-N}$  contents occurred in SS. Phosphorus levels were significantly greater ( $p < 0.05$ ) in PS (10.6–32.9  $\text{mg g}^{-1}$ ) than in other types of tundra soils (mean  $< 6.0 \text{ mg g}^{-1}$ ). Overall, penguin or seal activities altered the local soil biogeochemical properties through the deposition of their excreta, leading to generally low C:N ratios.

10. Line 222, “therefore, the soil TP and  $\text{NH}_4\text{...}$ ” this is a very bold statement, and lacking proof. Something like linear regression would be required.

**Author response:** It is difficult to quantify **animal activity intensity**, therefore we do not use the phrase “**animal activity intensity**” to avoid ambiguity in the revised manuscript. This statement has been corrected as follows: **Overall, penguin or seal activities altered the local soil biogeochemical properties through the deposition of their excreta, leading to generally low C:N ratios in tundra soils.**

11. Line 229, (fig. 2), figure 2 has 3 parts (a, b and c), please specify which part of the figure 2 is referred to.

**Author response:** It is Fig. 2a. This has been added in the revised manuscript.

12. Line 229, “overall...” please provide a scatter plot to visualise this (can be put in supplementary)

**Author response:** A scatter plot (**Fig. S2**) has been provided to visualize this in Supplementary Material.

13. Line 231, “the archaeal amoA gene showed a heterogeneous distribution” what does heterogeneous distribution mean?

**Author response:** It means that the AOA amoA gene showed a heterogeneous distribution **in the abundance** among the different tundra patches. i.e. The AOA amoA gene abundances were two orders of magnitude lower in PS and SS relative to those in BS and MS. The maximal AOA amoA gene abundance appeared in BS, followed by MS and PL, whereas the PS and SS soils had the lowest AOA amoA gene abundances. This sentence has been reorganized in the revised manuscript.

14. Line 232, there was a mixed usage of AOA amoA and archaeal amoA in the manuscript, please make them consistent.

**Author response:** For consistency, we have used AOA *amoA* instead of archaeal *amoA* in the revised manuscript.

15. Line 237, “fig 3”, similar to a previous comment, there are 6 parts of figure 3, please specify which part does this refer to.

**Author response:** This figure related to sea animal activity intensity has been removed in the revised manuscript. We added **Fig. 3:** Effects of soil C:N alteration on AOA and AOB abundances, and potential ammonia oxidation rates (PAOR) at five tundra patches, to show effects of sea animal activities on AOA and AOB abundances and PAOR.

16. Line 235, “Soil AOA *amoA* gene abundances were significant...” This statement is inappropriate, I would agree that animal activity reduces archaeal *amoA* gene abundance, but the statement of increasing archaeal gene abundance with reduced animal activity need a better proof. A correlation analysis between the activity intensity index and archaeal *amoA* gene abundance would be required.

**Author response:** Thanks for your good suggestion. This figure related to sea animal activity intensity has been removed in the revised manuscript, and the corresponding statement of increasing archaeal gene abundance with reduced animal activity has also been deleted. We added **Fig. 3**: Effects of soil C:N alteration on AOA and AOB abundances, and potential ammonia oxidation rates (PAOR) at five tundra patches, to show effects of sea animal activities on AOA and AOB abundances and PAOR. The related statements are reorganized as follows:

Soil AOA *amoA* gene abundance showed a significant positive correlation ( $r=0.52$ ,  $p<0.001$ ) with C:N ratio (Fig. 3a), but a significant negative correlation with  $\text{NH}_4^+$ -N contents ( $r= -0.52$ ,  $P = 0.013$ ) (Table 2). Overall, penguin or seal activities significantly increased the abundance of soil AOB *amoA* genes, but reduced the abundance of AOA *amoA* genes, leading to very large ratios ( $1.5 \times 10^2$  to  $3.2 \times 10^4$ ) of AOB to AOA *amoA* copy numbers in PS and SS.

17. Line 240, “The soil AOB *amoA* gene abundances increased...” this is incorrect, author stated that the order of sampling reflected the intensity of seal activity (highest in SS1 and lowest in SS5) (line 123-127), but clearly the abundance of bacterial AOB gene reduced with reduced penguin or seal activity.

**Author response:** The related statements about animal activity intensity has been deleted. The related statements are reorganized as follows: The soil AOB *amoA* gene abundances showed a significant negative correlation with C:N ratio ( $r=-0.71$ ,  $P < 0.001$ ) (Fig. 3b), but significant positive correlation with  $\text{NH}_4^+$ -N ( $r=0.53$ ,  $P < 0.05$ ) and TP ( $r=0.47$ ,  $P < 0.05$ ) (Table 2). The ratios of AOB to AOA *amoA* copy numbers were strongly affected by animal activities, and were much higher in PS and SS than in PL, MS, and BS (Fig. 2b; Kruskal–Wallis test,  $\chi^2 = 18.2$ ,  $P = 0.01$ ). Their ratios showed significant positive correlation with  $\text{NH}_4^+$ -N contents ( $r=0.62$ ;  $P < 0.01$ ) and TP ( $r=0.43$ ,  $P < 0.05$ ) (Table 2), but significant negative correlation with the C:N ratios ( $r= -0.79$ ;  $P < 0.001$ )(Fig. 3c). Overall, penguin or seal activities significantly increased the abundance of soil AOB *amoA* genes, but reduced the abundance of AOA *amoA* genes, leading to very large ratios ( $1.5 \times 10^2$  to  $3.2 \times 10^4$ ) of AOB to AOA *amoA* copy numbers



**in PS and SS.**

18. line 242 “The ratios of AOB to AOA amoA...” please cite figure 2c for this sentence.

**Author response:** Fig. 2b has been cited for this sentence.

19. line 250, “ The PAOR was significantly higher in STS...” this is not fully correct, the PAOR of PS samples was not significantly different from the BS site.

**Author response:** This sentence has been reorganized as follows: The PAOR was slightly higher in SS (mean 76.1  $\mu\text{g N kg}^{-1} \text{h}^{-1}$ ) than in PS (mean 64.7  $\mu\text{g N kg}^{-1} \text{h}^{-1}$ ), but significantly higher than in PL, MS, and BS (mean 12.0–21.8  $\mu\text{g N kg}^{-1} \text{h}^{-1}$ ). Overall the PAOR was significantly higher in animal colony soils (mean 70.4  $\mu\text{g N kg}^{-1} \text{h}^{-1}$  for SS and PS) than in non-animal colony soils (15.7  $\mu\text{g N kg}^{-1} \text{h}^{-1}$  for PL, MS, and BS; Kruskal–Wallis test,  $\chi^2 = 11.6$ ,  $P = 0.02$ ).

20. Figure 3, again, archaeal results appeared before bacterial results, thus their figure should appear before bacterial figures.

**Author response:** The figures for AOA results have been moved before the figures for AOB in the revised manuscript.

21. Line 258, “Interestingly, the PAOR...” Please confirm this statement with a statistical analysis, as PAOR increased from SS3 to SS5.

**Author response:** This statement has been corrected and reorganized as follows: PAOR significantly negatively correlated with soil C:N ratios ( $r=0.73$ ,  $P<0.001$ )(Fig. 3d), but significantly positively correlated with TS contents ( $r=0.47$ ,  $P<0.05$ ) and TP contents ( $r=0.43$ ,  $P<0.05$ ) (Table 2).

22. Line 271, “Specifically, the AOA amoA gene...” please present these results as a table or a figure.

**Author response:** These results have been provided in Table S1.

23. Line 276 “Phylogenetic analysis showed that the AOA...” Why and how phylogenetic analysis was used to group sequences into OTUs? In addition, the entire sentence is confusing, please revise.

**Author response:** (1) The sequences with 97% identity were grouped into one OTU using the Mothur Program by the furthest neighbor approach (Zheng et al., 2014); (2)

The entire sentence has been reorganized as: **Phylogenetic tree showed that the AOA *amoA* sequences were grouped into 16 unique OTUs, representing 100% of all the AOA *amoA* OTUs identified, and these sequences were affiliated with two *Nitrososphaera* clusters (Fig. 5a)**

24. Line 289, “Phylogenetic analysis showed that AOB *amoA*...”Why and how phylogenetic analysis was used to group sequences into OTUs? In addition, the entire sentence is confusing, please revise.

**Author response:** (1) Phylogenetic analysis was used to find the evolutionary ties between species. The sequences were edited using DNASTAR (DNASTAR, Madison, WI, USA), and then aligned by muscle using the UPGMB clustering method with the ClustalX program. The sequences with 97% identity were grouped into one OTU using the Mothur Program by the furthest neighbor approach; (2) The entire sentence has been revised as follows: **Phylogenetic analysis showed that the AOB *amoA* sequences were grouped into 38 unique OTUs, representing 58.5% of all the AOB *amoA* OTUs identified, and they were grouped into four *Nitrosospira* clusters according to the evolutionary distance of the phylogenetic tree (Fig. 5b).**

25. Line 312, “The AOA richness and phylotypes were evidently inhibited: : :” what does this mean? The richness of AOA is indeed lower in STS and PLS, but this result has already been presented in line 269.

**Author response:** It means the AOA richness was lower in SS and PL because of seal or penguin activities. This results has been presented on line 269, **therefore here this sentences was deleted in the revised manuscript.**

26. Line 323 why RDA was used to investigate the correlation among *amoA* gene abundance, diversity and etc? I would think RDA is used to deal with matrix dataset, but all these variables are vector variable. If only correlations were required, Pearson or partial Pearson correlation would be sufficient. If the contribution of each variable is required, I would think VPA analysis would be a better option.

**Author response:** Thanks for your good suggestions. According to your suggestions, we deleted the description about the RDA analysis and results. Our data about environmental variables did not show normal distribution, therefore we used Spearman correlation analysis to show their relationships between *amoA* gene abundance, the ratios of AOB to AOA, PAOR and environmental variables, and the

results were given in Table 2.

27. Line 325, “The AOA *amoA* gene abundance: : :”, which type of correlation is this? Please report the *r* value, and may be also scatter plots in the supplementary. Furthermore, authors stated that both AOA *amoA* gene abundance and diversity were related to C:N ratio, but only one *P*-value was reported.

**Author response:** (1) The description about the RDA analysis and results has been deleted in the revised manuscript, and Spearman correlation coefficients and *P*-values were given in the text and Table 2; (2) The scatter plots about *amoA* gene abundance, the ratios of AOB to AOA, PAOR and C:N ratios have been provided in Fig. 3.

28. Table 1 need to provide full name of site, also the site codes do not match those in the main text.

**Author response:** The full name for the site has been given in Table 1, and all the site codes have been corrected for the consistency with the main text.

29. Figure 2. The order of figure need to change, Figure 2b appeared first in the manuscript, and they should appear first in figure 2.

**Author response:** The order of this figure has been changed in the revised manuscript.