

Point-by-point response to the comments of the reviewer #2

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General comments

1. The authors studied abundance and population structures of AOA and AOB in the soil samples collected from the ecological experimental station. To investigate community diversification and population shifts of AOA and AOB in acidic soils, the authors selected maize and paddy rice fields receiving long-term experimental fertilization loadings as model ecosystems. The authors revealed that the fertilization processes can increase the numbers of AOA and AOB than the non-fertilized sites. The group 1.1a AOA dominated in paddy fields while the group 1.1b AOA were mainly found in upland soils. On the other hand, AOB populations were monotone and mainly occupied by the cluster 3 Nitrosospira. Although some aspects of the study might be of potential interest for readers, I have major concerns about the goal of this research, experimental designs, interpretation of data sets and discussions.

Title: ecosystem-specific selection in an acidic soil. The title of this manuscript is quite inscrutable. I think this title does not reflect the work performed in this study.

Reply: The title has been corrected as 'Conversion of aerobic upland to flooding paddy field alters community structure of archaeal ammonia oxidizers in an acid soil'

2. I see the mismatch between the research goals and actual experimental approaches. In other word, the experimental sets used in this study were not suitable to answer author's ecological questions. For example, the last sentence of the introduction, the authors say "Conversion of upland acid soil to paddy field can result in the depletion of oxygen in soil, leading to ecological pressure for the evolution of obligate aerobic AOA and AOB". I believe the controlled model experiments are necessary if the author's major interest is the shift (or evolution?) of AOA and AOB populations with the gradient of oxygen.

Reply: We appreciate the comments, while we are afraid that there might have sort of misleadings.

This experiment is not meant to monitor the real-time dynamics of evolutionary process of AOA communities. Rather, it is more like a molecular survey of *amoA* genes in two ecosystems. At present we still have no idea of how to run an evolutionary experiment in natural settings, which are central theme of life science. But I would like to conclude this has raised huge attentions by evolutionary microbiologist as following. Marx CJ (2013). Can You Sequence Ecology? Metagenomics of Adaptive Diversification. *PLoS Biol* **11**: e1001487.

In the meantime, we are really fascinated by the molecular survey data in this study. We find out that AOA within the soil group 1.1b dominated archaeal communities in aerobic upland soil, while marine 1.1a group AOA was found predominant in paddy soil after conversion from aerobic land for more than 100 years. (This result was further corroborated by our published data using pyrosequencing analysis). It is noteworthy that both soils are only ~100 meters away and of the same origin. Comes then the question why AOA communities are different.

Apparently, flooding is the most striking difference between upland and paddy fields, and it comes to our mind first in support of the difference in AOA communities between aerobic upland and paddy soils, despite other factors could not be excluded.

We strongly believe that the mechanisms shaping niche differentiation of AOA communities in natural environment represents one of the most important research fields. An enormous diversity of AOA has been shown in soils on Earth, but the mechanism why they are different across different soils remains elusive. The observation in this study is interesting, although the exact mechanism is not known.

3. Not only oxygen level but also various factors were different between maize and rice fields. Thus, it is almost impossible to conclude the difference of AOA communities found in maize and rice fields were actually created by the difference of oxygen level.

First, I must point out that the authors did not mention how the difference of two crops could influence the AOA and AOB populations. **Second**, the sampling time was different between these two sites. I believe the seasonal population changes must be considered. Moreover, paddy field sampling was done after harvesting of late rice. It indicates that the field was already dry and not anoxic. **Third**, the pH levels of upland and paddy fields were different each other (Table 1). Can the authors eliminate the possibility that the shift of AOA populations was mainly caused by the increase of pH? **Forth**, the interactions between ammonia oxidizers and other competitive and corporative microorganisms were unclear. I think paddy rice fields are

more likely eutrophic freshwater lake sediments while maize soils are typical soil environments.

In general, group 1.1a AOA are common in aquatic environments while group 1.1b AOA are common in soil environments. Without thinking the level of oxygen, the shift of AOA population might be explained by other environmental factors since these two soils are so different in many ways. Moreover, the surface of paddy field sediment is occasionally saturated by oxygen because of the photosynthesis of benthic eukaryotic algae and cyanobacteria. The authors collected the soil samples with the depth of 15 cm. I am afraid this rough soil sampling ruined the real depth profiles of microbial populations. I could not find the accession numbers of DNA sequences determined in this study.

Reply: We fully agree with the reviewer as quoted 'not only oxygen but also various factors were different between maize and rice fields. Therefore, it is almost impossible to conclude the difference of AOA communities found in maize and rice fields were actually created by the difference of oxygen level'. In fact, we think it is IMPOSSIBLE to link AOA community shifts with any environmental factors measured in this study.

However, there is an unequivocal evidence of AOA community difference between maize and rice field. This difference must come from niche specializations associated with the conversion of aerobic upland soil to flooding rice field. Flooding is the most striking difference between maize and rice field, while the possible role of other environmental changes was discussed in the revised version.

First, as for crop effect, we mentioned this in the revised version;

Second, for the seasonal population changes, we agree that abundance of AOA may show sort of seasonable variations, but we are afraid that phylogenetically different groups of AOA population could dominate the communities of archaeal ammonia oxidizers at different seasons within a year. For example, it may be impossible to have group 1.1a AOA at rice tillering stage, while group 1.1b AOA could be dominant at rice maturing stage.

Third, as for pH difference between upland and paddy field, we could not eliminate the possibility that the shift of AOA populations was mainly caused by the increase of pH, and this was discussed in the revised ms.

Fourth, as for the interactions between ammonia oxidizers and other competitive and corparative microorganisms, it was discussed in the revised ms.

As the reviewer mentioned, we agree that group 1.1a AOA are common in aquatic environments while group 1.1b AOA are common in soil environments. The shift of AOA population might be explained by other environmental factors than oxygen, since these two soils are so different in many ways. This was discussed in the revised ms.

Specific comments of the reviewer #1

1. P1718, lines 2-3. Please delete "and evolutions". I believe it is hard to discuss about the evolution of ammonia-oxidizing microorganisms with the experimental design of this manuscript. Line 9. Please spell out amoA. Line 21. : : :the marine group 1.1a AOA could be better adapted to low-oxygen environment : : :. As mentioned above, the experimental observation in this study cannot specify the influence of oxygen due to the inappropriate experimental settings.

Reply: deleted.

2. P1721, line 20. Nine different treatments. But 10 different treatments are seen in upland soils in Fig. 1.

Reply: Conversion of upland soil to paddy field generally leads to accumulation of soil organic matters. Therefore, one additional treatment of organic manure fertilization was established only for upland field, but not for paddy soil. This was described in materials and methods section.

3. P1723, line 4. Please remove "fresh". Line 6. "at speed 6.0 for 40s" is better. Line 18. Please specify the types of plasmid used, hopefully with accession numbers

Reply: done. pEASY-T1 plasmid vector was used. The accession number is EU233623.

4. P1724, lines 16-19. I believe the authors were at least able to use the nested approach including the first amplification with no GC clamp bacterial amoA primers then with GC clamp bacterial amoA primers. I believe it is almost impossible to compare AOA and AOB communities by using completely different two molecular techniques. The authors were able to analyze AOA communities by using clone library approaches as well as bacterial case.

Reply: We tried the strategy as suggested but failed to get the PCR products when attached with GC clamp. We agree that it is impossible to compare AOA and

AOB communities by using the two completely different techniques. Therefore, all relevant description was removed in the ms.

Yes, we were able to analyze AOA communities using clone library. However, this study is not meant for comparison between AOA and AOB, but to assess whether conversion of aerobic upland soil to paddy field could affect community structures of AOA and/or AOB in acid soil.

5. P1728, line 11. Which statistical analysis was used?

Reply: Spearman's correlation analysis was used and it was described in materials and methods section.

6. P1728, Lines 24-26. Was that dry or wet when the sampling was done?

Reply: fresh soil was collected.

7. P1732, 19-21. Not strong! Physiology studies using pure cultures or enrichment cultures are ideal to see the oxygen responses.

Reply: Corrected.

8. Table 1. Why we do not see the difference of nitrogen and carbon loadings between NPK and 2NPK treatments?

Reply: We speculate that nitrogen was assimilated due to the vigorous growth of crops. The removal of plant residues may leads to no difference of carbon loading between NPK and 2NPK treatments.

9. Fig. 2. Since the authors obtained beautiful DGGE profiles, the authors may apply clustering methods and diversity index calculation.

Reply: done.

10. Fig. 4. I think the authors should mention the original sources of AOB clustering.

Reply: done.