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# ***Interactive comment on “Characterization of incubation experiments and development of an enrichment culture capable of ammonium oxidation under iron reducing conditions” by S. Huang and P. R. Jaffé***

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We want to thank the reviewer for the insightful comments and are providing our answers below:

In this manuscript the authors attempt to link geochemical signals suggestive of anaerobic ammonium oxidation coupled to Fe(III) reduction (feammox) to changes in the microbial community structure across a range of batch and flow-through enrichments. While the results are certainly interesting, the linkages are not completely clear, and

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certainly leave a number of outstanding questions.

1. When calculating the initial thermodynamics of feammox, the authors describe the use of  $\text{Fe}(\text{OH})_3$  as the structure for ferrihydrite. Why is the formula  $\text{Fe}_2\text{O}_3 \cdot 0.5\text{H}_2\text{O}$  subsequently used in the calculations/discussion?

Response: Ferrihydrite is unstable, and with a few exceptions (Majzlan et al., 2004), not many values for its  $\Delta G^\circ_f$  have been reported. Hence, many authors use  $\text{Fe}(\text{OH})_3$  as a substitute for  $\Delta G$  calculations involving ferrihydrite. Since we synthesized 6-line ferrihydrite for the incubation experiments discussed in this work, we feel that using  $\text{Fe}_2\text{O}_3 \cdot 0.5\text{H}_2\text{O}$  for the  $\Delta G$  calculations is more accurate. By using  $\text{Fe}_2\text{O}_3 \cdot 0.5\text{H}_2\text{O}$ ,  $\Delta G$  of the Feammox reaction, at the incubation conditions, is  $-145.8 \text{ kJ mol}^{-1}$ , it would be  $-90.3 \text{ kJ mol}^{-1}$  using  $\text{Fe}(\text{OH})_3$ . Both result in a negative  $\Delta G_r$  when  $\text{NH}_4^+$  is oxidized to  $\text{NO}_2^-$  and  $\text{Fe}(\text{III})$  reduced to  $\text{Fe}(\text{II})$ .

$\text{Fe}(\text{OH})_3$  is not used at all in this manuscript to describe the structure for ferrihydrite, nor is  $\text{Fe}(\text{OH})_3$  mentioned in the text. If we had used  $\text{Fe}(\text{OH})_3$ , neither the iron/ammonium stoichiometry nor any conclusions would have been affected.

2. A range of molecular tools were used in the analysis of enrichments cultures and flow-through reactors. Few details are provided to describe the 454 analyses, or the construction of phylogenetic trees. Please add details on the pipelines used for 16S gene analyses, and the tools used for tree generation.

Response: Methods for the 454 analyses and the construction of phylogenetic trees were used as described in the references we cite, and mentioned in Supplemental Information, section 1.2. We will be happy to summarize these methods in the Supplemental Information in the revised version of this manuscript. Similarly, details of the sampling pipeline are given in the methods, but we will give a graphical pipeline in the revised supplemental information.

3. Could the authors please explain further what happened in those batch experiments

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with ferric citrate, where Fe(III) reduction occurred rapidly but no ammonium oxidation was observed. What was driving Fe(III) reduction in these instances? In the discussion the authors mention that energetics of the reaction are only favorable when Fe(II) is removed from solution via sorption. From this, are we supposed to infer that the lack of Fe(II) sorption is the major reason for absence of Feammox in cultures with soluble Fe(III) sources?

Response: In the batch incubations amended with ferric citrate, Fe(III) reduction must have been conducted by dissimilatory iron reducers, using organic carbon as electron donor. The DGGE results for incubations with ferric citrate (Fig. 3, lane 7) show that the most dominant species was an Actinobacterium (Table S1), which are known to be iron reducers under anaerobic conditions (Lin et al., 2007; Lentini et al., 2012). Acidimicrobiaceae bacterium A6 was not detected in these incubations.

So far, we don't know whether the aqueous Fe(II) that accumulates in solution with soluble Fe(III) sources inhibits the Feammox reaction (as posed by the reviewer), if an oxide is required as the oxygen source for the NO<sub>2</sub><sup>-</sup> formation (postulated as one possible mechanism by Yang et al., 2012), or if Feammox bacteria can't reduce dissolved/complexed Fe(III), as is the case for several iron reducers. We found that in our incubations, only samples incubated with iron oxides showed the Feammox reaction. Fe(III) selectivity and its bioavailability for the Feammox process certainly requires further study.

4. An additional chart showing the stoichiometry between Fe(II) production and ammonium consumption across all the 180-day incubation time points would be beneficial. This ratio is discussed for a few select time points (page 12310) currently. This would enable readers to track the linkage between iron and ammonium in these experiments, without having to refer to multiple graphs.

Response: We agree and will do so. Figure S6 showing the stoichiometry between the Fe(II) production and ammonium consumption will be added to Supplemental Informa-

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tion in the revised version of this manuscript.

5. There are some discrepancies between the coupling of iron and ammonium in the 180-day main series of incubations. Following the spike of  $\text{NH}_4\text{Cl}$  on day 125, ammonium is rapidly consumed. Across the time period 120-140 days, only a small increase in  $\text{Fe(II)}$  is observed. Between days 140-160 ammonium continues to be consumed, and  $\text{Fe(II)}$  concentrations increase rapidly. It would be helpful for the authors to address these discrepancies in geochemical data, as it detracts for the idea of a 'tight couple' between ammonium oxidation and iron reduction.

We attribute some of the discrepancies, as will be described in detail below and also in response to comment # 6 to incomplete  $\text{Fe(II)}$  (less efficient when  $\text{Fe(II)}$  was lower) extraction and anammox activity in the earlier incubations.

Response: The discrepancies between the coupling of iron and  $\text{NH}_4^+$  on days 125-140 are due to incompletely  $\text{Fe(II)}$  extraction. About 5-10 % more  $\text{Fe(II)}$  can be extracted with 1N HCl extraction over 24 hours or with 0.5 N HCl over 36 hours. When more  $\text{Fe(II)}$  is produced in the system with increasing incubation time, the  $\text{Fe(II)}$  extraction efficiency improves. Only a 1-2% difference was observed in the  $\text{Fe(II)}$  extracted using 0.5N vs. 1N HCl with 24 hour extractions towards the end of this incubation period. There are clays in the system that will sorb  $\text{Fe(II)}$  early on, furthermore ferrihydrite is slowly converted to magnetite. All of which leads to incomplete  $\text{Fe(II)}$  extractions, especially when the  $\text{Fe(II)}$  is low.

Here we report  $\text{Fe(II)}$  data obtained via 0.5N HCl extractions over 24 hours. As the extraction efficiency improves towards the end of the experiment, the ratio of  $\text{NH}_4^+$  consumption to  $\text{Fe(II)}$  production is 1:5.3, which is close to the stoichiometry of 1:6. See also the response to the question below, anammox numbers became negligible later in the incubation, meaning that earlier in the incubations more ammonium may have been oxidized via anammox, which would not have contributed to  $\text{Fe(II)}$  accumulation.

6. Trends in the abundance of the Acidimicrobiaceae A6 signal are similarly confusing.

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Similar ammonium oxidation rates can be identified at a number of points on figure 2b, such as after day 60, and following day 125. Despite similar ammonium oxidation rates, the abundances for Acidimicrobiaceae bacterium A6 are completely different at these two time points, further clouding the role of this species in catalyzing the feammox process.

Response: The presence of anammox bacteria is shown in the pyrosequencing results (Fig. 4) and qPCR analysis (Fig. S4c). Anammox abundance decreased from  $0.17 \pm 0.05 \times 10^6$  copies/g to  $0.09 \pm 0.06 \times 10^5$  copies/g over 130 days of incubation, which suggests that anammox was a parallel pathway for ammonium oxidation during the earlier incubation times, and decreased gradually in importance. This is also consistent with the fact that at earlier incubation times the ratio of Fe(II) produced to  $\text{NH}_4^+$  oxidized was lower and approached the stoichiometry for Femmox 6:1 towards the end of the incubations.

The linkages presented here between abundances of certain bacteria, and geochemical trends are too loose, and would be strengthened considerably by a tracking technique (stable isotopes?) to conclusively demonstrate the role of Acidimicrobiaceae A6 in the feammox process.

Response: We have done stable isotope experiments in our pervious study, using sediments from the same location and very similar incubation techniques (Shrestha et al., 2009). Using labeled  $^{15}\text{NH}_4^+$  in the incubations, resulted in the production of  $^{15}\text{N}_2$ , which conclusively showed that ammonium-N was converted to nitrogen gas in these sediments under iron reducing conditions. Conclusive linkage between Acidimicrobiaceae A6 and Feammox process requires the isolation of the strain and then conduct incubations with the pure strain. Therefore at this point, the gradual increase in Acidimicrobiaceae A6 numbers after sequential ammonium and Fe(III) additions, and more importantly the results from the enrichment culture are the strongest link between Acidimicrobiaceae A6 and Feammox. No other known  $\text{NH}_4^+$  oxidizer (AOB or anammox) was detected in the Feammox membrane reactor after 150 days of operation,

indicating that the dominated bacteria, which was Acidimicrobiaceae A6 is likely to play an important role of the oxidation of ammonium under iron reduction condition.

7. The paper needs editing, either by the authors or a technical editor. There are multiple spelling mistakes throughout the manuscript (e.g. page 12300, lines 5 (through), 14 (from), and 25 (column)).

Response: Done

Minor comments:

Page 12301, line 12: please provide forward and reverse primer sequences, rather than just the target region.

Response: Agreed, we will provide them in a tabular form in the revised manuscript.

Page 12302, line 4: please change rDNA to rRNA (also page 12305, line 15)

Response: Done

Page 12305, line 18: What do you mean by 'in terms of cell numbers'? I was under the impression that cell counts were not performed, so a different phrase should be used here to describe % of community

Response: All are cell counts per g dry soil.

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