

The current manuscript addresses one of the remaining mysteries of the nitrogen cycle: ferric iron dependent ammonium oxidation. Unfortunately the current manuscript only presents circumstantial evidence, which is not convincing.

Response: We thank the reviewer for the detailed comments. We fully agree that the evidence we given here, that a novel *Actinobacterium* is linked to the Feammox process, is circumstantial and we have said so in the manuscript. We do feel that the evidence is significantly stronger than the reviewer suggests and are providing detailed responses below. We agree that isolation of the bacterium is required for proof that it is responsible for the process described, and hope that this work, including providing the primers, will be a stepping-stone towards achieving this goal. This is the first work that links ammonium oxidation under iron reduction to specific bacterial communities. Our goal was to describe how the enrichment culture was achieved and its characterization.

Briefly,

All work was done in an anaerobic hood, where oxygen leakage can't be ruled out completely, but should have been minimum. The membrane reactor was run under a positive N<sub>2</sub> pressured headspace and all influents were purged with N<sub>2</sub>. DO in the influents and reactor was measured and was below detect.

Given the amount of Fe(II) produced in the incubations, small amounts of oxygen, if there had been leakage, should have been consumed rapidly, making sustained ammonium oxidation by AOB unlikely.

Although we agree that not detecting AOB cannot rule out their presence and activity, we did not have any ammonium removal in incubations to which no Fe(III) was added. Since the flasks were otherwise identical, oxidation of ammonium via AOB should not have required the presence of Fe(III).

In the incubations with acetylene, there was very similar ammonium removal and iron production with and without acetylene amendment. Acetylene was effective in blocking the conversion of N<sub>2</sub>O to N<sub>2</sub> and should have affected nitrification by AOB if that process was important in the removal of ammonium in our incubations.

Again, this is circumstantial, but we had no incubations where ammonium was oxidized and where the *Actinobacterium* was not detected. While in all of the incubations where this *Actinobacterium* was not detected, ammonium was not oxidized.

Introduction: Line 9: what do the authors mean by “conventional removal of nitrogen”?

Response: We refer to ammonium-N conversion via nitrification, denitrification and anammox to produce N<sub>2</sub>. We can certainly rephrase that.

Line 11: What are these saturated with?

Response: We mean water saturated and will clarify this in the revisions.

Lines 11-14: This sentence contradicts the preceding sentence. How can nitrification and denitrification occur if there is no O<sub>2</sub> and/or oxidized nitrogen species? Furthermore, it is incorrect. The presence of compounds does not mean much, what is important is fluxes. Nitrite hardly occurs in high amounts in oxygen minimum zones and in wastewater, but microorganisms that convert nitrite are very important in nature and form the basis of wastewater treatment.

Response: What we meant, and we will reword it to avoid confusion, is that in water-saturated sediments, such as wetland sediments and benthic sediments, there is little oxygen for significant nitrification by AOB. Nitrates are still delivered into such systems by groundwater discharging into them or surface water infiltration. Some nitrification does of course occur, such as in the vicinity of roots, where there is O<sub>2</sub> leakage.

## Results and Discussion:

I do not agree with most of the discussion.

1)The observed ammonium oxidation activity can also be explained by oxygen leakage to the used system. The ammonium oxidation rates are so low that a small amount of O<sub>2</sub> leakage would be enough to establish a small AOA or AOB community that would be able to convert the same amount of ammonium. The experiments are conducted in anoxic bottles; however, the authors cannot exclude O<sub>2</sub> leakage because the incubations were not conducted in an anaerobic chamber. It is conceivable that every time the authors sampled their batch incubations, they introduced O<sub>2</sub> to the bottles.

Response:

1. All experiments were conducted in an anaerobic chamber, including the incubations, sampling, and addition of ammonium, bicarbonate, or ferrihydrite. O<sub>2</sub> leakage, if it occurred at all, should have been miniscule in these experiments. This is explained clearly in the main text (P12298, L15; P12300, L16).

2. As mentioned above, Fe(II), which accumulated in the vials, does react quickly with oxygen, even if the Fe(II) is sorbed.

3. AOB existed in the system in the initial incubation, *amoA* genes decreased with time to below the detection limit after 90 days. We agree that this does not rule out the presence of AOB in small amounts at later times, but indicates that these incubations were not supporting the growth of AOA/AOB, even though ammonium oxidation became faster as the incubation time progressed.

4. In the control samples to which we did not add any Fe(III), while maintaining all other conditions the same, no detectable ammonium decrease was observed. If some minute amount of O<sub>2</sub> leakage had occurred so as to allow AOB to oxidize ammonium in our

experiments, the absence of Fe(III) should not have prevented AOB/AOA from oxidizing ammonium in these incubations.

2) The employed methods and the presented data set do not allow the identification of any microorganism that performs this reaction. Based on phylogenetic inferences and intensities of DGGE bands, one cannot establish or exclude the involvement of any of the detected microorganisms in the observed reaction. There is no evidence linking the activity to the presence of the detected microorganisms. There is no reason to believe that the increase in the population of Acidobacteria, Actinobacteria and betaproteobacteria is not merely coincidental. These microorganisms are found in all natural ecosystems and there are many members of these groups that can perform a multitude of different reactions.

Response: Again, we have not said that the *Actinobacteria* identified here is the organism responsible for Feammox reaction. Using DGGE and pyrosequencing, we were able to show that after sequential ammonium and Fe(III) additions, the abundance of *Actinobacteria* increased and that it became a dominant species in an enrichment culture that had a high ammonium remove rate coupled to iron reduction. No other known  $\text{NH}_4^+$  oxidizer (AOB, anammox, or acidophilic ammonium oxidizer [see response 7]) was detected in the Feammox membrane reactor after 150 days of operation, indicating that the dominant bacteria in this reactor, which belongs to *Actinobacteria* is likely to play an important role (directly or indirectly) in the oxidation of ammonium under iron reduction condition.

3) Even if the authors presented conclusive evidence, which they do not, for the iron-dependent ammonium oxidation activity, this would still not mean that these microorganisms are growing on this reaction. It could well be a side reaction of any microorganism.

Response:

1. The reaction as described in the text, and also given by others in the literature, shows a negative  $\Delta G$ , which indicates that there is a potential for bacteria to grow while oxidizing ammonium under iron reduction. Still, we are not saying that bacteria are growing on that reaction.

2. We had a series of controls in this study, including incubations with only ammonium, only iron, autoclaved, and various Fe(III) sources. The Feammox reactions proceeded only in samples where the *Actinobacteria* were growing, and when iron oxides as well as  $\text{NH}_4\text{Cl}$  were supplied (Fig. 1, Fig.S1, Fig S2).

3. The membrane reactor was operated for over 150 days (in the dark and anaerobically), adding only a trace nutrient solution, ferrihydrite, ammonium, bicarbonate, and maintaining the pH at  $\sim 4.5$ . During this time the biomass in the reactor increased, especially that of *Actinobacteria*. Given the compounds added and the products formed

(see also next point), there are not many other possible reactions that could yield energy for biomass growth.

4.  $^{15}\text{N}$  label isotope trace incubations have just been conducted (after the manuscript was submitted).  $^{15}\text{NH}_4\text{Cl}$  was added at a final concentration in the vials of  $0.5 \text{ mmol L}^{-1}$ . After 10 days of incubation,  $0.133 \text{ mmol L}^{-1}$  of  $^{15}\text{NO}_2^-$  was detected in samples with Feammox activity, while no  $^{15}\text{NO}_3^-$  was produced. We will be happy to add these results to the supplemental materials in the revised text.

All results presented here link the *Actinobacteria* activity to the Feammox process, either directly or indirectly. Although it appears so, we did not say that in this manuscript that the bacteria are growing on this reaction. Ideally, to make such a statement requires having the pure strain.

4) I do not see the point of DGGE. It is a very crude method with so many drawbacks that there is no place to list here. Decreases and increases in DGGE bands and their intensities do not mean anything. Furthermore, rRNA or mRNA amount does not mean that organisms with more RNA are more active. There is no direct correlation between RNA, levels of protein expression and activity. I would remove the whole DGGE section.

Response: DGGE was conducted to get an initial sense of the changes in the microbial communities after sequential additions of ammonium and Fe(III), and between samples incubated with different Fe(III) sources (Fig. 3). Pyrosequencing was done to show in more detail what microorganisms were in the system.

We agreed that an abundance of transcript does not necessarily mean that there is a functional protein with the same abundance. However, RNA, is still a good indicator for bacterial metabolic activity. (Poulsen *et al.*, 1993; Park *et al.*, 2010).

5) The authors use acetylene to inhibit ammonium oxidation. Acetylene inhibits both anaerobic and aerobic ammonium oxidizing microorganisms, methane oxidizers and denitrifiers. The effect of acetylene that the authors describe could well be due to the inhibition of the denitrifying community. The authors state that acetylene did not affect Fe(II) production, which strongly suggests that this activity is uncoupled from ammonium oxidation. If the organisms in the incubation were converting Fe(III) coupled to organic acid oxidation, indeed they would not be inhibited by acetylene.

Response: Yes, we agree that acetylene did inhibit the denitrifiers in our incubations since  $\text{N}_2\text{O}$  did build up. Several researchers have used incubations with acetylene for gaining insights into the Feammox process (Yang *et al.*, 2012; Ding *et al.*, 2014).

We used acetylene incubations at the same concentrations as was used in the Feammox studies by the researchers cited above. Our goal was to determine if all ammonium was first converted to nitrite and then denitrified, or if a direct oxidation to  $\text{N}_2$  might proceed in parallel (as suggested by Yang *et al.*, 2012). In the results presented here, the decrease

in ammonium was equal to the accumulation of  $\text{NO}_2^-$  and  $\text{N}_2\text{O}$ . This is different than the findings by Yang *et al.*, 2012.

Crucial here for the discussions about the potential effect of AOB, is that acetylene did not affect the decrease of ammonium (or production of Fe(II)). At the levels acetylene was supplied, it should have affected ammonium removal by AOB. Hence, it is hard to see how AOB could have contributed significantly to the removal of ammonium in our incubations.

6) The authors suggest that the *nirS* is increasing due to nitrite produced through ammonium oxidation coupled to iron reduction. Of course, nitrite could have been produced via nitrate reduction or normal aerobic ammonium oxidation. The authors should also measure *nirK* abundance.

Response: Since initially there was neither nitrate nor nitrite in the system, we don't think nitrite came from nitrate reduction in these incubations. Again, due to the oxygen free conditions of these incubations and the rapid decrease of *amoA* genes during the incubation, we do not believe that the nitrite produced was from aerobic ammonium oxidation. We have measured the *nirK* abundance, which showed a similar trend to that of *nirS*, although the number of *nirK* gene was an order of magnitude lower than *nirS*. We will be happy to show the *nirK* abundance in the revised version of this manuscript.

7) The decrease in the *amoA* gene does not mean that AOA or AOB are not responsible for ammonium oxidation activity. As I stated before, only a small amount of AOA or AOB would be enough for this activity. Further, I wonder if the authors considered checking their samples for acidophilic ammonium oxidizers. Surely, in their samples there is no free ammonia, but only ammonium (due to low pH).

Response: Agreed, a small amount of AOA or AOB would be enough for ammonium oxidation. As we discussed already above, in our control samples to which no Fe(III) were added, we did not detect ammonium consumption. AOB/AOA do not require the presence of Fe(III), which was the only difference in the incubations. Hence, it appears that the ammonium oxidation observed in study was not driven by AOA or AOB.

Yes, we checked for acidophilic ammonium oxidizers. Quantification of thaumarchaeal *amoA* genes was performed using primer CrenamoA23f and CrenamoA616r (Torna *et al.*, 2008), and none of these acidophilic ammonia oxidizers was detected in our system. We will make a statement to that effect in our revised version. Furthermore, the same arguments as above for AOB will also apply to acidophilic ammonium oxidizers, which do not need the presence of Fe(III) to oxidize ammonium.

8) The bicarbonate-amended samples have marginally higher rates. Furthermore, the authors cannot exclude the fact that there are still slowly released organic compounds in their samples.

Response: Beside bicarbonate-amended incubation, we have just conducted a  $^{13}\text{C}$  labeled  $\text{CO}_2$  amendment. 5% (vol.)  $^{13}\text{CO}_2$  was injected to a Feammox enrichment culture and the  $^{13}\text{C}$  in cells increased from 1.80% to 10.3% after 10 days incubation. Hence carbon is being fixed in these experiments by autotrophs and will leak into solution.

This carbon is necessary to drive the denitrification, since in all of our incubations and the membrane reactor, nitrite is rapidly reduced. Over the full incubation time, we did not add any further organic carbon source. So while there could have been some remaining degradable organic carbon in the early incubations containing the stabilized sediments, there was no source of organic carbon in the membrane reactor, and nitrifiers that were active in the reactor would have used carbon that leaks from autotrophs and from cell turnover.

9) Please remove all the speculation based on acetylene experiments. Acetylene is a very crude inhibitor and inhibits many, many reactions. Furthermore, without any genomic or biochemical data one cannot speculate on the pathway of any reaction.

Response: Please see Response 5.

Moreover, the authors use the word “pathway” wrongly throughout the manuscript.

Response: We will change the usage of pathway where appropriate.