Prof. Dr. Middelburg, Editor
Biogeosciences

Dear Prof. Middelburg,

Attached please find the comments from the reviewers, our detailed responses, changes made to the text, and where in the text the corresponding changes were made.

We feel that we answered the concerns of the reviewers, especially the concern that our observations might be explainable due to AOB/AOA activity.

Anonymous Referee #1

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 certainly leave a number of outstanding questions.

1. When calculating the initial thermodynamics of feammox, the authors describe the use of Fe(OH)3 as the structure for ferrihydrite. Why is the formula Fe2O3.0.5H2O subsequently used in the calculations/discussion?

Response:

$Fe(OH)_3$ is not used at all in this manuscript to describe the structure for ferrihydrite, nor is $Fe(OH)_3$ mentioned anywhere in the text.

Note: Many authors do use $Fe(OH)_3$ as a substitute for $Fe_2O_3.0.5H_2O$ in ΔG calculations involving ferrihydrite since it is unstable, and with a few exceptions (Majzlan *et al*., 2004), not many values for its ΔG_{0f} have been reported. Since we synthesized 6-line ferrihydrite for the incubation experiments discussed in this work, and a ΔG_{0f} value from a reputable group was available, we feel that using $Fe_2O_3.0.5H_2O$ for the ΔG calculations is preferable. By using $Fe_2O_3.0.5H_2O$, ΔG of the Feammox reaction, for the incubation conditions, is -145.8 $kJmol^{-1}$, it would be -90.3 $kJmol^{-1}$ using $Fe(OH)_3$. Both result in a negative ΔGr when NH_4^+ is oxidized to NO_2^- and Fe(III) reduced to Fe(II), not affecting any conclusions put forward in this manuscript.

Hence, no change was made to the text in response to this comment, and we believe we were actually doing what the reviewer asked for.

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: We have now included the methods of the 454 analyses and the construction of phylogenetic trees in the revised version of this manuscript (Supplemental Information section, SL81-104, L69-79).

3. Could the authors please explain further what happened in those batch experiments 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: We have added the following explanation to the discussion (L437-447):

'In the incubations to which ferric citrate was added as the Fe(III) source, Fe(III) was reduced rapidly 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), known to reduce iron under anaerobic conditions (Lin *et al*., 2007; Lentini *et al*., 2012). *Acidimicrobiaceae bacterium* A6 was not detected in these incubations. Since acidic conditions as well as minimal dissolved Fe(II) and NO_2^- concentrations are required to make the Feammox reaction energetically favorable as shown in Equation 1, the presence of iron oxides as the main Fe(III) source might may have helped to maintain the concentrations of Fe(II) in solution below the detection limit through the incubation since iron oxides can sorb Fe(II) and/or incorporate it into their structure.'

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: Figure S6, showing the stoichiometry between the Fe(II) production and ammonium consumption was added to the Supplemental Information. (SL199-207).

5. There are some discrepancies between the coupling of iron and ammonium in the 180-day main series of incubations. Following the spike of NH4Cl on day 125, ammonium is rapidly consumed. Across the time period 120-140 days, only a small increase in Fe(II) is observed. Between days 140-160 ammonium continues to be consumed, and 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.

Response: The discrepancies between the coupling of iron and NH_4^+ are due to: (i) incomplete Fe(II) extraction and anammox activity in the earlier incubations (extraction efficiency with weak HCl changes as the amount of Fe(II) increases and as there are iron-phase transformations); (ii) ammonium removal via anammox in the early incubations might also have contributed to less Fe(II) production per ammonium removed. We added several sections to the text to make this clear.

'Extraction efficiency of Fe(II) was affected by the HCl concentration and the extraction time. About 5-10 % more Fe(II) could be extracted with either 1N HCl extraction over 24 hours or with 0.5 N HCl over 36 hours as opposed to 0.5 N HCl over 24 hours. Furthermore, after more Fe(II) was produced in the system with increasing incubation time, the Fe(II) extraction efficiency improved. Only a 1-2% difference was observed in the Fe(II) extracted over 24 hours using 0.5N vs. 1N HCl towards the end of the incubation period. Clays, present in the soil incubations, typically sorb Fe(II) more efficiently when the total Fe(II) is low, furthermore ferrihydrite is slowly converted to magnetite, resulting in relatively different associations to different phases of the Fe(II) over the duration of the incubation. All of which leads to incomplete Fe(II) extractions, especially when the Fe(II) is low. Here we report Fe(II) data obtained via 0.5N HCl extractions over 24 hours to ensure that the methods and hence data are comparable to those reported by other researchers focusing on iron reduction and iron bioavailability.' (L151-164.)

Although we had previously discussed the effect of anammox activity early in the incubations on the NH_4^+: Fe(II) ratio. We added the following sentence:

'Although the discrepancies in the Feammox stoichiometry between iron and NH_4^+ are attributed in part to incompletely Fe(II) extraction, the influence of anammox activity in the earlier incubations would also have contributed to a lower Fe(II) produced to NH_4^+ removed ratio than the theoretical value of 1:6.' (L 479-483).

6. Trends in the abundance of the Acidimicrobiaceae A6 signal are similarly confusing. 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: We did discuss this in our original text. See L485-492:

'A parallel pathway to Feammox, such as anammox, could explain the lower stoichiometric ratio, especially at earlier incubation times. In the samples taken before the incubation, $0.17 \pm 0.05 \times 10^6$ copies g^{-1} dw of anammox rRNA gene were found, which decreased to $0.09 \pm 0.06 \times 10^5$ on day 130 (Fig. S4d). We postulate that anammox was responsible for some initial NH_4^+ and NO_2^- removal, and denitrification became more dominant for NO_2^- removal later during the incubation period (Fig. 5 and Fig. S4b, d).'

We added an additional sentence to make this clearer:

'NH_4^+ removal via Anammox in the early incubations may also explain why the observed NH_4^+ oxidation rates and the abundance of *Acidimicrobiaceae A6* did not change proportionally over the full incubation period.' (L492-494).

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: In response to this comment we conducted ^{15}N label isotope trace incubations. The results from these experiments are consistent with the findings of the non-labeled incubations but they unequivocally demonstrate that the ammonium removal is via oxidation.

The following sentences were added to the text:

'Finally, ^{15}N isotope tracer incubations were conducted using slurries collected form the stable Feammox membrane reactor. Five treatments (n = 3 per treatment) were conducted: (1) control with only anoxic DI water; (2) $^{15}NH_4Cl$ addition; (3) $^{15}NH_4Cl$ + Fe(III) addition; (4) $^{15}NH_4Cl$ and C_2H_2 addition; (5) $^{15}NH_4Cl$, C_2H_2, and Fe(III) addition. The headspace gas of each 50mL incubation vial was sampled every 24 hours for $^{15}N_2O$ analysis (Supplementary Information 1.4).' (L138-143).

'In the ^{15}N isotope tracer incubations, detectable ^{15}N-N_2O was only found in samples amended with both, $^{15}NH_4Cl$ and Fe(III), with ^{15}N- N_2O production rates 2.14±0.059 or 0.072±0.023 $\mu g\ g^{-1}\ d^{-1}$ in samples incubated with or without C_2H_2 treatment (Table S2).' (L369-371).

'$^{15}N\text{-}NH_4^+$ incubations, as an extension of C_2H_2 treatment, showed that $^{15}N\text{-}N_2O$ built up when $^{15}NH_4Cl$ was added as the NH_4^+ source (Table S2), demonstrating that NH_4^+ was oxidized during the Feammox process rather than be adsorbed or taken uptake by microorganisms in the system.' (L459-463).

'Furthermore, in the isotope tracer incubations, $^{15}N\text{-}N_2O$ was below the detection limit in samples to which Fe(III) was not supplied, showing again that NH_4^+ oxidation proceeded only when iron was being reduced.' (L474-476).

We have also added the methods of the $^{15}N\text{-}N_2O$ incubations/analyses in more detail and more detailed results to the supplemental materials. (SL120-135, Table S2)

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, have provided them in Table 2

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: this was reworded to read: ' % in total 16S rRNA gene sequences'. L307-308.

Anonymous Referee # 2

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 fully agree that the evidence we give here, that a novel *Actinobacterium* is linked to the Feammox process, is circumstantial, we have said so in the original manuscript, and we make it even clearer now. We do feel that the evidence is significantly stronger than the reviewer suggests and are providing detailed responses below. This is the first work that links ammonium oxidation under iron reduction to a specific bacterial community. Our goal was to describe how the enrichment culture was achieved, its characterization, that it could achieve Feammox (ammonium oxidation under iron reducing conditions) in a sable manner and for extended time periods while only adding ammonium and iron oxide sources. We feel that having achieved this goal and that is a significant contribution to the field.

We have made modifications to the text, discussing more clearly the link between the growth of *Acidimicrobiaceae* bacterium A6 and the active ammonium oxidation coupled to iron reduction described in this study.

A main concern of the reviewer was that oxygen leakage could explain the ammonium oxidation by AOB, which we also address in detail.

Briefly,

1) All incubations were prepared and conducted in an anaerobic hood, with resazurin as an indicator, where oxygen leakage can be ruled out, therefore it is very unlikely that AOB played a role in the ammonium oxidation in the incubations conducted here. (L82-84, L87-88, L382-383, L385-390)

2) 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 incubations with and without Fe(III) where otherwise identical, oxidation of ammonium via AOB/AOA should not have been inhibited by the absence of Fe(III) sources. (Fig S2, L393-397,) There was sufficient Fe present in trace quantities so it would not have been a limiting nutrient since other organisms were growing well in these incubations.

3) In the isotope tracer incubations, detectable $^{15}N\text{-}N_2O$ was only shown in samples amend with both, $^{15}NH_4Cl$ and Fe(III), which is consistent with the discussions in item 2 above (Table S2, L369-371).

4) The ammonium removal rate and Fe(III) reduction was not affected by the presence of acetylene. Ammonium oxidation by AOB would have been affected by acetylene. (L465-477)

5) Ammonium removal was only observed in samples where the presence of *Acidimicrobiaceae* bacterium A6 was detected, and where iron was being reduced, while any incubation where this *Acidimicrobiaceae* bacterium A6 was not detected exhibited any ammonium removal. (L393-397.)

6) In the continuous-flow membrane reactor, which had high NH_4^+ removal and Fe(III) reduction rates, this *Acidimicrobiaceae* bacterium A6 was enriched from 14.8% to 40.2% over 150 days of operation, while no other known NH_4^+ oxidizer (AOB or anammox) was detected (Fig. 4, L401-404).

Although we had previously ended the discussion with:

'Isolating the pure bacterial strain will allow to establish a direct link between *Acidimicrobiaceae* bacterium A6 and the Feammox process studied here.'

We have expanded this qualifier to read:

'Conclusive linkage between *Acidimicrobiaceae A6* and Feammox process requires the isolation of the strain and then conduct incubations with the pure strain. At this point, the observations (i) that ammonium removal only occurred in samples when the presence of *Acidimicrobiaceae* bacterium A6 was detected and when iron was being reduced, (ii) that *Acidimicrobiaceae* A6 numbers increased gradually after sequential ammonium and Fe(III) additions, and (iii) the results from the enrichment culture which was operated for an extended time period while only adding ammonium and iron oxide sources and during which *Acidimicrobiaceae A6* became the dominant while no other known NH_4^+ oxidizer (AOB or anammox) was detected after 150 days of operation, indicate that *Acidimicrobiaceae A6* is likely to play an important role of the oxidation of ammonium under iron reduction conditions.' (L502-512)

Detailed responses to each comment are given below:

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

Response: We have rephrased that statement to read:

'The most common removal of nitrogen from soil environments is mineralization (for organic nitrogen), followed by nitrification and then denitrification' (L29-30)

Line 11: What are these saturated with?

Response: We mean water saturated and clarified this in the revisions. (L31)

Lines 11-14: This sentence contradicts the preceding sentence. How can nitrification and denitrification occur if there is no O2 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 reworded 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 aerobic ammonium (NH_4^+) oxidation bacteria (AOB). Nitrate (NO_3^-) is mainly delivered by groundwater discharging into such systems or surface water infiltration, although some nitrification does occur, such as in the vicinity of roots, where there is O_2 leakage.' (L31-35.)

Results and 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 O2 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 O2 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 O2 to the bottles.

Response:

1. The experiments were conducted in an anaerobic chamber and we did state that in the method section:

'The vials were sealed tightly with rubber stoppers and were stored in an anaerobic glove box for 30 days.' (L82-84)

To make this clearer and also that any additions/sampling were done in the anaerobic chamber plus that the chamber was being monitored with an oxygen indicator, we now added to this statement that:

'All incubations, addition of reagents, and sampling was conducted in an anaerobic glove box with a solution of resazurin as the redox indicator.' (L87-88)

Therefore, O_2 leakage, if it occurred at all, should have been miniscule in these experiments.

2. AOA were not found in our incubations (L382-383). AOB existed in the system in the initial incubation, and *amoA* genes decreased with time to below the detection limit after 90 days of incubation (Fig S4e). We have expanded the text stating that:

'Even though a small amount of AOB would be enough for NH_4^+ oxidation in the presence of O_2 leakage, in our control samples without added Fe(III) no NH_4^+ consumption was detected (Fig. S2), indicating that NH_4^+ consumption in the presence of Fe(III) is not attributed to AOB. Moreover, the decrease in *amoA* gene at a time of increasing NH_4^+ oxidation also indicates that neither AOB nor acidophilic ammonia oxidizers were the drivers of the NH_4^+ oxidation in the later incubation times.' (L385-390).

Hence, oxygen leakage coupled to aerobic (or microaerophillic) ammonium oxidizers cannot explain the ammonium oxidation in our experiments.

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 *Actinobacterium* is the organism responsible for Feammox reaction. Using DGGE and pyrosequencing, we were able to show that after sequential ammonium and Fe(III) additions, and no further organic carbon addition, the abundance of *Actinobacteria* increased and that it became dominant in an enrichment culture that had a high ammonium remove rate coupled to iron reduction (Fig.4 and Fig. 6). In the continuous flow membrane reactor, which had a high NH_4^+ removal and Fe(III) reduction rate, this *Acidimicrobiaceae* bacterium was enriched from an initial 14.8% to 40.2% after 150 days operation, and no other known NH_4^+ oxidizers (AOB or anammox) were detected in that reactor (L401-404).

These results indicate that the dominant bacteria (*Acidimicrobiaceae* bacterium) in the incubation and reactor were most likely playing an important role (directly or indirectly) in the oxidation of ammonium under iron reduction condition.

As mentioned above, we now end the discussion with the following statement:

'Conclusive linkage between *Acidimicrobiaceae A6* and Feammox process requires the isolation of the strain and then conduct incubations with the pure strain. At this point, the observations (i) that ammonium removal only occurred in samples when the presence of *Acidimicrobiaceae* bacterium A6 was detected and when iron was being reduced, (ii) that *Acidimicrobiaceae* A6 numbers increased gradually after sequential ammonium and Fe(III) additions, and (iii) the results from the enrichment culture which was operated for an extended time period while only adding ammonium and iron oxide sources and during which *Acidimicrobiaceae A6* became the dominant bacterial species while no other known NH_4^+ oxidizer (AOB or anammox) was detected after 150 days of operation, indicate that *Acidimicrobiaceae A6* is likely to play an important role of the oxidation of ammonium under iron reduction conditions.' (L502-512).

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: We present rigorous data showing that ammonium oxidation only proceeded when iron was being reduced, and only when the *Acidimicrobiaceae* bacterium was present.

1. In the incubation experiments conducted (which included controls with only NH_4^+, only iron, autoclaved, and various Fe(III) sources), this *Acidimicrobiaceae* bacterium was only detected and growing in samples to which NH_4^+ was supplied as an electron donor, ferrihydrite was supplied as electron acceptor, and $NaHCO_3$ was supplied as a carbon source (Fig. 1, Fig.3, Fig.S1, Fig S2). (L393-397).

2. Results of $^{15}N\text{-}NH_4^+$ incubations, that we have now included, show an equivalent $^{15}N\text{-}N_2O$ buildup (in the presence of C_2H_2) to the $^{15}N\text{-}NH_4^+$ removed during Feammox, demonstrating the ammonium that was removed was oxidized.

We have now added the following sentences:

'$^{15}N\text{-}NH_4^+$ incubations, as an extension of C_2H_2 treatment, showed that $^{15}N\text{-}N_2O$ build up when $^{15}NH_4Cl$ was added as the NH_4^+ source (Table S2), demonstrating that NH_4^+ was oxidized during the Feammox process rather than be adsorbed or taken uptake by microorganisms in the system.' (L459-463).

'Furthermore, in the isotope tracer incubations, ^{15}N- N_2O was below the detection limit in samples to which Fe(III) was not supplied, showing again that NH_4^+ oxidation proceeded only when iron was being reduced.' (L474-476).

After discussing the observations that the Feammox activity increased after addition of bicarbonate we state:

'According to a phylogenetic comparison with similar clones from studies reported in the GenBank (Fig. 5), and taking into account its special growth characteristics (stimulated by inorganic carbon, oxidizing NH_4^+ coupled to Fe(III) reduction), also its gradual activity increase with increased Feammox activity, as well as a strong link between it and a Feammox enrichment reactor, this uncultured *Acidimicrobiaceae* bacterium A6 is probably a previously unreported species in the *Acidimicrobiaceae* family that might be either responsible or play a key role in the Feammox process described here. *Acidimicrobiaceae* bacterium A6 was more active and the Feammox pathway was faster in samples with higher $NaHCO_3$ amendments (Fig. 2 and 6), which, in addition to the fact that ΔG in Equation 1 is negative, indicates that if this *Acidimicrobiaceae* bacterium is actually responsible for conducting the Feammox reaction as depicted in equation 1, it may be an autotroph.' (L419-430).

Given the experimental results, we feel that a guarded statement as given above is reasonable. If the editor/reviewer disagrees we are willing to remove this statement or make it more conditional. Again, the main objective is to describe our Feammox enrichment culture. The next step is isolating this *Acidimicrobiaceae* bacterium, which would allow demonstrating unequivocally that it grows on this reaction.

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). For the different iron source incubations we have only DGGE data and they do show community differences for the different treatments. We could obtain pyrosequencing if so desired, but it would take an additional month and we do not see much gain in the main conclusions of this work. Pyrosequencing was done to show in more detail what microorganisms were in the system. Combining these two results, does help tracking the change of the microbial community during the incubations. We feel that this is important for us to explain which microorganism populations increased with time and became more dominant in the Feammox system. Therefore we feel that there is a value in showing the DGGE results, and we would prefer to do so in the manuscript. If the editor and/or reviewer insist that

we remove the DGGE results or move them to the supplemental materials, we will of course do so.

We agreed that an abundance of transcript does not necessarily mean that there is a functional protein with the same abundance. However, rRNA, is still a good indicator for bacterial metabolic activity. (Poulsen *et al*., 1993; Park *et al*., 2010). (L323-324). Therefore, we prefer to report the rRNA quantification results.

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 does inhibit denitrifiers and we used it for that specific purpose. Several researchers have used incubations with acetylene to gain 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 the ammonium removed was first converted to nitrite and then denitrified, or if a direct oxidation to 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 NO_2^- and N_2O (Fig.S5, L458-460).

Acetylene did not inhibit ammonium oxidation nor iron reduction; hence the acetylene incubations do not indicate that the two processes are uncoupled.

We added the following sentence:

'The fact that NH_4^+ oxidation was not affected by the presence of acetylene is a further indication that AOB are not responsible for this process since they would be affected by acetylene.' (L472-476).

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: We added the following sentence to address this comment:

'Because there was no initial nitrate or nitrite in the system, because all experiments were conducted under strict oxygen free conditions, and because of the rapid decrease of *amoA* genes, neither NO_3^- reduction nor aerobic NH_4^+ oxidation could be the reason for the formation of NO_2^- during the incubations. (L450-454).

We have measured the *nirK* abundance, which showed a similar trend to that of *nirS*, although the number of *nirK* gene was two orders lower than *nirS*. (L328-329). The *nirK* abundance has now been added to Fig S4c in the supplemental information.

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: The first part of this comment was already addressed above and the text states:

'Even though a small amount of AOB would be enough for NH_4^+ oxidation in the presence of O_2 leakage, in our control samples without added Fe(III) no NH_4^+ consumption was detected (Fig. S2), indicating that NH_4^+ consumption in the presence of Fe(III) is not attributed to AOB. Moreover, the decrease in *amoA* gene at a time of increasing NH_4^+ oxidation also indicates that neither AOB/AOA nor acidophilic ammonia oxidizers were the drivers of the NH_4^+ oxidation in the later incubation times.' (L385-390).

In terms of the acidophilic ammonium oxidizers, we looked for their presence but they were not detected. This is now included in the text:

'Through quantification of thaumarchaeal *amoA* genes, none of the acidophilic ammonia oxidizers was detected in our system' (L333-334.)

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: There is slowly released carbon in our system, in the early incubations probably from soil and, and later on and especially in the membrane reactor, which did not have soil nor any organic carbon in its influent, from cell turnover. This organic carbon is necessary to drive the denitrification, which reduces the nitrite that was produced during the ammonium oxidation. Over the entire incubation time, no organic

carbon source was added to any incubation, except for the citrate in the incubations with ferric citrate.

Since the Feammox activity increased after addition of bicarbonate we have speculated that this is an autotrophic process. We have rephrased this statement as follows:

'Acidimicrobiaceae bacterium A6 was more active and the Feammox pathway was faster in samples with higher $NaHCO_3$ amendments (Fig. 2 and 6), which, in addition to the fact that ΔG in Equation 1 is negative, indicates that if this *Acidimicrobiaceae* bacterium is actually responsible for conducting the Feammox reaction as depicted in equation 1, it may be an autotroph.' (L426-430)

This is based on the experimental evidence and the thermodynamics and we are making no definitive statement.

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 where we explain in detail what these incubations were used for and what they have shown. We believe that the results of the incubations with acetylene block provide insights into the Feammox process, strengthen the interpretations of the results, and provide further evidence that the ammonium removal is not due to AOB activity. We used the same techniques as were used by several other investigators for very similar purposes.

Moreover, the authors use the word "pathway" wrongly throughout the manuscript.

Response: We have changed pathway to process where appropriate. i.e. 'Feammox pathway' was changed to 'Feammox process'. (L54, L56, L90, L425, L449, L463, L503).