Interactive comment on

Biogeochemical evidence of anaerobic methane oxidation and anaerobic ammonium oxidation in a stratified lake using stable isotopes” by Florian Einsiedl et al.

We like to express our deep gratitude for the detailed and constructive feedback from two reviewers. Below, we have provided a detailed point-by-point list of answers and replies to the comments and suggestions raised by the reviewers. We have made every attempt to address the excellent suggestions and the numerous highly valuable recommendations where appropriate, and have provided detailed responses and explanations below.

Reviewer #1 agrees with our assertion, that links between nitrate-AOM and anammox have not been widely demonstrated in the literature, and that our study is an important step in developing an environmental understanding of this process. Reviewer #1 found the study well executed and the data of high quality. Reviewer #2 was more critical, while also stating that the authors did a good job in interpreting their data in an attractive way.

Response to general statement:

Reviewer #1 mentioned that the authors seem well aware of the limitations of their isotope results and temper their conclusions with an appropriate amount of the limitations of the presented data (with very few exceptions where a slight over-reach of data interpretation can be identified). In contrast, Reviewer #2 asked for a more cautious interpretation of the data.

We have settled in the revised manuscript on a compromise approach that is primarily based on opinion of Reviewer #1 that our original conclusions were well tempered, while we have also made several text additions in the revised manuscript that caution against an over-interpretation of our findings (for example L. 339).

Point-by-point response:

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Comments of Reviewer #1

The quality of the figures seems sub-par and some effort should be given to improve

on details such as text and symbol sizes and colors, axes labels and ticks, etc.

Response: 1 & 2: We agree that an improvement of the quality of the figures is necessary and we also have changed the expression of concentrations to mmol/L.

L137: What is the reasoning behind the two diffusion coefficients for methane? This is presented in an apparent attempt to bracket a range of acceptable flux estimates, but is not explained in the text.

Response: The modelling was already discussed in detail with Reviewer #2 and we made every attempt to address both recommendations. In the revised manuscript we have calculated the Kz for Lake Fohnsee, so it is not necessary to show two diffusion coefficients.

Something about Equation 1 seems incorrect. Are there meant to be two equalities here? Do the ‘x’ terms both refer to depth? In general I think ‘z’ is more frequently used for referencing to depths. Please confirm that the expression of this diffusion equation is properly written

Response: In the revised manuscript, we have corrected the equation after Clark (1975), have extended the used equations for clarification (see Rev. #2), and define in the manuscript that "x" represents the depth.

L310: I understand that in the presence of NO3-, sulfate reduction is not thermodynamically predicted to proceed, however some arguments have been made for processes occurring in micro-zones inside of particles, for example. How much anticipated change in sulfate would be predicted – and would the IC measurements actually be sensitive enough to this? The increasing presence/abundance of Deltaproteobacteria also lend some credence to the idea that at least some level of SO42- reduction could be occurring. Changing units into molarity would help readers with this comparison as I noted above.

Response: Sulfate concentrations were clearly above the detection limit of the IC and we observed a decrease of sulfate concentrations from 8 mg/L at a water depth of 21 m to around 7 mg/L close to the lake. This 14% decrease in sulfate concentration with increasing depth could be interpreted to indicate partial bacterial sulfate reduction in micro-environments of particles near the lake sediment surface as suggested by the reviewer or alternately, by mixing effects between sulfate-free water from the sediments, where methanogenesis may occur, and lake water. As we also found nitrate concentration at the same depth (22 m) where we observed decreasing sulfate concentrations we can only speculate what processes control decreasing sulfate concentrations.

To address this, we revised the manuscript to the following new text at the end of § 4.1:

Decreasing sulfate concentrations at the bottom of the lake and nitrate concentrations at the same water depth of less than 1 mg/L can be explained by partial bacterial sulfate reduction in micro-environments of particles near the lake sediment surface (Bianchi et al. 2028, Nature Geoscience volume 11, pages263–268(2018)) or by mixing effects between sulfate-free water from the sediments, where methanogenesis may occur, and sulfate-containing lake water.

The δ18O values of nitrite are reported, but nowhere in the text is it explained how these values were determined or calibrated. Further, given the low pH of lake water, the δ18O values of nitrite are very likely to be in isotopic equilibrium with the water, yet appear to fall around -4 to -6‰ which would be much too low. Given a lake water δ18O value of ~ -10‰ – the δ18O value for nitrite in equilibrium should fall closer to +4‰ (see Casciotti et al., 2007). Finally, the δ18O values of nitrite in this study are not mentioned or involved in aspect of the conclusions – and should probably be omitted for clarity (e.g., they aren't used to bring any new insight into the system as presented).

Response:

We used international nitrate standard with known isotopic composition ($\delta^{15}N$ & $\delta^{18}O$ values) and a lab-internal standard for $\delta^{15}N$ of nitrite but not for $\delta^{18}O$ of nitrite, while using the measurement gas N_2O. In the revised version of the manuscript, we will add the following information: "The isotopic composition of nitrite was determined using the azide method, similar to the analysis of nitrate. In order to ensure the proper reduction of nitrite to N_2O, in addition to the samples, internal laboratory standards for KNO2 were analyzed in each batch (Lb1, δ15N = -63‰ and Lb2, δ15N = +2.7‰). Corrections of the raw δ^{15}N values were made based on the known values of the nitrate and nitrite standards.

With respect to the observed δ^{18}O values of nitrite, the paper by Casciotti et al. demonstrates perfectly that there is an isotopic exchange between oxygen of the water and oxygen of nitrite. Once this exchange is achieved, an isotopic equilibrium is established depending on the isotopic fractionation. This fractionation leads to significantly higher δ18O values of nitrite compared to those of water. In Casciotti's study, the isotopic fractionation determined for freshwater is around -14‰. So based on the δ18O of the water in this study close to -10‰, the expected δ18O for nitrite should be +4‰, assuming there is only abiotic exchange. More recently, Sebilo et al. (2019) published a study based on isotope tracing during nitrite or nitrate reduction. This study revealed that the oxygen isotope shift was immediate and the authors attribute it primarily to denitrifying bacteria, given the rapidity of exchange. In this study, the δ18O of the water was close to -10‰ and the δ18O of the nitrite during its reduction was relatively constant, oscillating between 0 and -2‰, and hence displaying lower values than those expected with abiotic exchange alone.

The results obtained in the here discussed manuscript, with relatively constant δ18O values for nitrite close to -5‰ indicate that an isotopic exchange occurred between the oxygen of the water and the oxygen of nitrite occurred, and that the latter was predominately controlled by biotic reactions. Moreover, since denitrification alone should have resulted in a δ^{18}O value of the nitrite between -2 and 0‰, this discrepancy seems to confirm that another biotic process is taking place.

Minor revisions:

L15: “Nitrate dependent anaerobic methane oxidation and anaerobic oxidation of ammonium (anammox) have the potential...”

Response: Here it is not clear what changes the reviewer would like to see. We suggest to change the sentence as follows:

Nitrate-dependent anaerobic oxidation of methane and anaerobic oxidation of ammonium (anammox) are two recently discovered processes in the nitrogen cycle that can reduce nitrogen loading of aquatic ecosystems and to reduce methane emissions to the atmosphere.

All other minor revisions focusing more or less on awkward wording were accepted and have greatly improved the manuscript that now reads as follows:

L20: anammox does not require italics.

Response: was changed

L24: is the most parsimonious explanation”

L32: … contain significantly different microbial communities that include bacteria known to be involved in…”

L47: …coupled to nitrate…

L54: … ANME-2d lineage promotes/conducts the reduction of nitrate…

L57: Besides

L59: …related to Crenothrix also have the…

L60: Crenothrix “was corrected”.

L61: … may act as a driver…

L74: references do not need to be italicized “was corrected”

L86: …sequencing of 16S rRNA genes that provides evidence for the…

L115: … sterile filter, which was then kept frozen…

Response: …kept frozen with the filtered microbial biomass

L149: ... which represents the lower bound...

Response: Here we have re-written the modelling part and detailed answers can be found in our response to Reviewer #2

L153 – 157 – this should be moved to introduction or discussion.

Response: As a short introduction to the stable isotope section, we are of the opinion that this text fit well in the current section, and hence have made no changes.

Response: The suggested improvements of the reviewer were accepted and we made the following changes in the revised manuscript:

L168: ...(2007), respectively.

L168: Nitrite was converted to N2O using acetic acid buffer sodium azide.

L169: ... mixture of both nitrate and nitrite was reduced to N2O via azide.

L171: Can you provide some estimate of error propagation for this inverse mixing calculation?

Please also compare answer to Rev #2 (on page 6)

The calculation of the isotopic composition is based on the measurement of the isotopic composition of N_2O with an IRMS and the correction between the values obtained for the standards and the values measured by linear regression. For samples obtained from 14, 16, 18 and 20m depth, both nitrite and nitrate are present. However, taking into account the concentration ratios, the amount of nitrite represents at most 10% of the total concentration for the samples except for the 20m sample where the nitrite concentration is around 1 mg/L and the nitrate concentration is around 0.5 mg/L. For this point, taking into account the two molecules and calculating the nitrate $\delta^{18}O$ gives a value of 5.6‰ whereas it was 5.4‰ without correction.

L173: ... by buffered azide solution for subsequent analysis.

L181: How were the δ15N values of the nitrite standards determined – and to what level of precision? There is no mention of calibrated δ18O standards for nitrite. Yet δ18O of nitrite data are reported (albeit not discussed). Please clarify or omit.

Response: We have the revised the text as follows:

"The isotopic composition of nitrite was determined using the azide method, similar to the analysis of nitrate. In order to ensure the proper reduction of nitrite to N_2O, in addition to the samples, internal laboratory standards for KNO2 were analyzed in each batch (Lb1, δ15N = -63‰ and Lb2, δ15N = +2.7‰). Corrections of the raw δ^{15}N values were made based on the known values of the nitrate and nitrite standards.

L192: *Here it is unclear if the methane isotope analyses were conducted on the same bottles? Viamanualinjection? Wasthisafullbottlepurgeandtrapapproach? Wasthis automated? Were there standards included in this approach? How were the analyses standardized (e.g., extractions of methane of known composition from water?)?*

Response: As stated in the original text, "the concentrations and carbon isotope ratios of dissolved methane in the lake water samples were determined using the static headspace equilibrium technique (EPA, 2002) where 10% of the water sample in the capped bottles was replaced with helium followed by outgassing of the dissolved gases in the water sample into the headspace for 1 h at 25ºC.

In the revised text, we have now clarified that:

- methane concentration and C isotope ratios were determined from the same bottle;

- that only 10% of the bottle content was replaced with an inert headspace gas;

- that this process was not automated;

- standardization of the measurements was accomplished as follows: Instrument stability and linearity was ensured by daily measurements of an in-house methane mix of 5% CH4 (balance helium). Carbon isotope analyses of methane were standardized by measurements of Isometric Instruments (Victoria, BC, Canada) gases containing methane with known □13C values including the following: B-iso1 (δ13C = -54.5‰, δ2H = -266‰), L-iso1 (δ13C = -66.5‰, δ2H = -171‰), and H-iso1 (δ13C = -23.9‰, δ2H = -156‰);

- standard solutions with dissolved methane of know isotopic compositions were not available;

Response: The improvements suggested by the reviewer were accepted and we made the following changes in the revised manuscript:

L236: Aerobic conditions...

L237: The average concentration of nitrate...

L239: ...nitrate concentrations decreased...

L261: ... were too low for stable isotope analyses.

L298-300 – Rephrase. Awkward wording.

L301:... water depth below 16m, suggesting that only a very small fraction of methane can be oxidized with such trace amounts of oxygen.

Response: Here we have decided to remove the O_2 calculations and will present the results by using a numerical model in a different manuscript.

Response: The suggested improvements by the reviewer were accepted and we made the following changes in the revised manuscript:

L314: A stable isotope technique was used...

L327: We also present several lines of qualitative and quantitative evidence for the co-occurrence of anammox together with denitrification coupled to AOM towards the bottom of the NMTZ.

L339: Here the language reads as though AOM coupled to denitrification has been unequivocally demonstrated, which isn't exactly the case. I think here it is best to qualify this a bit more.

Repsonse: In fact, denitrification could also be coupled to more canonical heterotrophy at the same time. Hence we revised the text as follows

New: …. where *AOM* may affect microbial nitrate reduction, although more canonical heterotrophy could also occur.

L344: *Again – I would recommend softening and rephrasing:*

Old: This strongly suggests that the additional isotopic difference in $\delta^{15}N$ values between nitrate and nitrite of around +15‰ is likely the result of production of highly ^{15}N enriched nitrate derived from *anammox*.

Response: This is consistent with the additional isotopic difference in $\delta^{15}N$ values between nitrate and nitrite of around +15‰ arising as the result of production of highly ^{15}N enriched nitrate deriving from anammox."

L350: ...superimposed on 'normal' isotope effects...

L356: *As written this statement is incorrect. I would recommend restating:*

"*During anammox, when nitrite is reduced with ammonium as electron donor and nitrate is produced, one oxygen atom from water having a δ18O value of around -10‰ is incorporated into the newly formed nitrate." This incorporation of a new O atom is also most likely associated with a kinetic isotope effect – as has been demonstrated in nitrite oxidizing bacteria (see Buchwald and Casciotti, 2010).*

Response: We agree that our simplified statement was not correct since kinetic oxygen isotope fractionation was not considered. We have now revised this sentence as follows:

During anammox, when nitrite is reduced with ammonium as electron donor and nitrate is produced, one oxygen atom from water (δ18O value of around -10‰) is incorporated into the newly formed nitrate, with an additional kinetic oxygen isotope effect (Buchwald and Casciotti, 2010).

Response: The following suggested improvements of the reviewer were accepted and we made the following changes in the revised manuscript:

L359: ... the anammox process leads to δ18O values of nitrate remaining low, while...

L361: ...by an inverse isotope effect and values continue to increase.

L368: Although not mentioned, I am curious whether any nitrite oxidizing bacteria were detected in the genomic analyses? I assume from their omission that they were not. This could be a useful fact to mention if so.

Response: Because nitrate and nitrite reduction is such a widespread trait held by many facultative anaerobic bacteria, it is not possible to use our 16S rRNA gene sequence data to specifically show the abundance of 'normal nitrate and nitrite reducers' as the reviewer suggested. However, the Gammaproteobacteria are very abundant in our samples, and are well known to have many species that are capable of nitrate and nitrite reduction, a trait that is widespread throughout this class. Since the Gammaproteobacteria relative abundance increases with depth into the anoxic zone (Fig. 3b), it is likely that many of the Gammaproteobacteria in deeper waters of the lake are responsible for nitrate and nitrite reduction, and denitrification.

We will add a few lines to the revised manuscript.

L373: ... environmental conditions, helping any nitrate reducing ANME-2d (with lower doubling times) in the denitrification zone...

Was accepted

L380: ... the meaning behind this sentence is unclear...

Was reformulated (see below)

L382: ... potion? (see below)

Was deleted

Response: L 380 & L382 The presence of two separate populations of NC10 bacteria at a water depth between 12 and 22 m, in the region where also anaerobic oxidation of methane with denitrification may exist, suggest that this organism was also partially contributing to the anaerobic oxidation of methane with nitrite (*n-damo*). However, again it remains unclear whether *Crenothrix* that also peaked in this region completely reduced dissolved nitrate to N_2 or both, NC10 bacteria (NO_2^- reduction) and *Crenothrix* are involved in the N loss processes. In this context it is also worth mentioning that the highest abundance of NC10 bacteria in our and other studies is often observed at the oxic - anoxic interface (Ettwig et al., 2008) and it is controversially discussed whether *M. oxyfera* can also use external O_2 to oxidize methane near the oxycline. Therefore, the respective roles of NC10 and *Crenotrix* remain unclear in this study.

L398: ... as shown...

Was accepted