

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.

Reviewer #1

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 Reviewer #1. 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 these processes. Reviewer #1 found the study well executed and the data of high quality.

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 acknowledgement 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 partially based on opinion of Reviewer #1 that most of our original conclusions were well tempered, while we have also made several text additions in the revised manuscript that further caution against an over-interpretation of our findings (L 419, and Ls 330, 362, 426).

Point-by-point response:

Point-by-point response:

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 was necessary and we also have changed the expression of concentrations to mmol/L. (Figs. 1-4 were improved as suggested).

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 reason was that we have not calculated the site-specific K_z value in our first draft of the manuscript and used the upper and lower ranges of literature data. The modelling was discussed in detail by Reviewer #2 and we made every attempt to address both

recommendations. In the revised manuscript we have calculated the turbulent diffusion coefficient K_z for Lake Fohnsee as suggested by Reviewer #2, and have improved the modelling section by fitting the methane profile only.

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 diffusion equation after Clark (1975), have extended the used equations for clarification (see Rev. #2), and define in the manuscript that “z” represents the depth. (from Line 121)

L310: I understand that in the presence of NO_3^- , 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 SO_4^{2-} reduction could be occurring. Changing units into molarity would help readers with this comparison as I noted above.

To address this, we revised the manuscript to the following new text:

L 313

Response: Sulfate concentrations were clearly above the detection limit of the IC and we observed a decrease of sulfate concentrations from 0.08 mmol/L at a water depth of 21 m to around 0.07 mmol/L close to the lake sediments. 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 measurable nitrate concentrations at the same depth (22 m) where we observed decreasing sulfate concentrations we can only speculate what processes control decreasing sulfate concentrations.

The $\delta^{18}\text{O}$ 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 $\delta^{18}\text{O}$ 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 $\delta^{18}\text{O}$ value of $\sim -10\text{‰}$ – the $\delta^{18}\text{O}$ value for nitrite in equilibrium should fall closer to +4‰ (see Casciotti et al., 2007). Finally, the $\delta^{18}\text{O}$ 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).

From line 164 and from line 354

Response:

We used international nitrate standard with known isotopic composition ($\delta^{15}\text{N}$ & $\delta^{18}\text{O}$ values) and a lab-internal standard for $\delta^{15}\text{N}$ of nitrite but not for $\delta^{18}\text{O}$ of nitrite, while using the measurement gas N_2O .

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 KNO_2 were analyzed in each batch (Lb1, $\delta^{15}\text{N} = -63\text{‰}$ and Lb2, $\delta^{15}\text{N} = +2.7\text{‰}$). Corrections of the raw $\delta^{15}\text{N}$ values were made based on the known values of the nitrate and nitrite standards.

With respect to the observed $\delta^{18}\text{O}$ values of nitrite, the paper by Casciotti et al. (2007) 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 $\delta^{18}\text{O}$ 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 $\delta^{18}\text{O}$ of the water in this study close to -10‰ , the expected $\delta^{18}\text{O}$ for nitrite should be $+4\text{‰}$, 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 $\delta^{18}\text{O}$ of the water was close to -10‰ and the $\delta^{18}\text{O}$ 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 $\delta^{18}\text{O}$ values for nitrite close to -5‰ indicate that an isotopic exchange occurred between the oxygen of the water and the oxygen of nitrite and that the latter was predominately controlled by biotic reactions. Moreover, since denitrification alone should have resulted in a $\delta^{18}\text{O}$ value of the nitrite between -2 and 0‰ , this discrepancy may suggest that another biotic process is taking place or the extent of isotope fractionation depends on the microorganisms controlling this process.

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:

The nitrite (n-damo)/ nitrate dependent anaerobic methane oxidation and the anaerobic oxidation of ammonium (anammox) represent two microbially-mediated processes that can reduce nitrogen loading of aquatic ecosystems and associated 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 throughout the manuscript

L24:*most likely explanation*...

Response: ...is the most parsimonious explanation

L30: ... *that consist of bacteria known to be involved in*...

Response: The associated methane concentration and stable isotope profiles indicate that some of the denitrification may be coupled to AOM, an observation supported by an increased concentration of bacteria known to be involved in n-damo/ denitrification with AOM (NC10 and Crenothrix) and anammox ('Candidatus Anammoximicrobium')....

L46: ...*coupled to nitrate*...

Response: misspelling was improved

L54: ... ,whereas ANME-2d lineage uses methane to reduce nitrate to nitrite (Raghoebarsing et al., 2006).

L56: *Beside*

Response: was deleted

L59: ...*related to Crenothrix also have the*...

Response: related to *Crenothrix* also have

L56: *Crenothrix may likely act as driver*

Response: ... Crenothrix may act as a driver...

L70: *references do not need to be italicized* "was corrected"

Response: (Shen et al., 2014; Zhu et al., 2018) was improved

L86: *sequencing of 16S rRNA genes that provides effidence for the*...

Response: Our findings show that microbially mediated linkages between n-damo/ denitrification with AOM and anammox have the potential to constitute an important sink of both dissolved nitrogen (NO₃⁻, NO₂⁻, NH₄⁺), and methane (CH₄), in stratified freshwater ecosystems.

L109: ... *sterile filter, which was then kept frozen*...

Response: ... The filter including the microbial biomass was kept frozen at

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

Response: Here we have re-written this § and detailed answers can be found in our response to Reviewer #2 and the manuscript (**from line 122**)

L150 *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:

L166: ...(2007), respectively.

Response: was added

L166: N₂O ...using sodium azide.

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

L170: the mixture of both nitrate and nitrite was reduced to N₂O.

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

L175: Can you provide some estimate of error propagation for this inverse mixing calculation?

Response: Please also compare answer to Rev #2

The calculation of the isotopic composition is based on the measurement of the isotopic composition of N₂O 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 $\delta^{18}\text{O}$ gives a value of 5.6‰ whereas it was 5.4‰ without correction.

L177: ...an azide buffer for subsequent analysis.

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

L181: How were the $\delta^{15}\text{N}$ values of the nitrite standards determined – and to what level of precision? There is no mention of calibrated $\delta^{18}\text{O}$ standards for nitrite. Yet $\delta^{18}\text{O}$ 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 N₂O, in addition to the samples, internal laboratory standards for KNO₂ were analyzed in each batch (Lb1, $\delta^{15}\text{N} = -63\text{‰}$ and Lb2, $\delta^{15}\text{N} = +2.7\text{‰}$). Corrections of the raw $\delta^{15}\text{N}$ values were made based on the known values of the nitrate and nitrite standards (Lines 167-176)

L192: Here it is unclear if the methane isotope analyses were conducted on the same bottles? Via manual injection? Was this a full bottle purge and trap approach? Was this automated? Were there standards included in this approach? How were the analyses standardized (e.g., extractions of methane of known composition from water?)?

From Line 190

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 25°C.

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 $\delta^{13}\text{C}$ values including the following: B-iso1 ($\delta^{13}\text{C} = -54.5\text{‰}$, $\delta^2\text{H} = -266\text{‰}$), L-iso1 ($\delta^{13}\text{C} = -66.5\text{‰}$, $\delta^2\text{H} = -171\text{‰}$), and H-iso1 ($\delta^{13}\text{C} = -23.9\text{‰}$, $\delta^2\text{H} = -156\text{‰}$);
- standard solutions with dissolved methane of known isotopic compositions were not available;

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

L245: Aerobic redox conditions....

Response: Aerobic conditions...

L248: The average concentrations of nitrate ...

Response: *nitrate concentrations decreased...*

L265: .. too low for stable carbon isotope analyses.

Response ... were too low for stable isotope analyses.

L298-300 – Rephrase. Awkward wording.

From Line 295

Response: Here we have decided to remove the O₂ calculations because the use of an analytical model has too many uncertainties for a solid evaluation of the effect of micro-aerobic methane oxidation within the oxycline. Instead we focus our interpretation of the difference of observed and modelled methane concentration profiles using the calculated

turbulent diffusion coefficient K_z and calculated a first order rate constant k for AOM with nitrate and compared it to literature.

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

L321: *A stable isotope technique was used...*

Response: Stable isotope data was used

L327: *We also present several lines of qualitative and quantitative evidence that anammox has occurred with AOM coupled with denitrification at the bottom of the NMTZ of the lake.*

Response: Several lines of qualitative and quantitative evidence indicate the co-occurrence of anammox, denitrification, and AOM towards the bottom of the NMTZ.

L361: *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.*

Response: We observed a difference of $\delta^{15}\text{N}$ values ($\Delta\delta^{15}\text{N}$) of nitrate and nitrite of around 11‰ in the NMTZ at depths of 16 and 18 m, where we suggest the microbial linkage of AOM and denitrification maybe via n-damo. But again, it is also possible that some of the denitrification is coupled to heterotrophic nitrate and nitrite reduction

L370: *This strongly suggests that the additional isotopic difference in $\delta^{15}\text{N}$ 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 $\delta^{15}\text{N}$ values between nitrate and nitrite of around +15‰ arising as the result of production of highly ^{15}N enriched nitrate deriving from anammox ($\Delta\delta^{15}\text{N}$ of +31‰).

L375: *...superimposed on 'normal' isotope effects...*

Response: was superimposed on "normal" isotope effects

L382: *As written this statement is incorrect.*

During anammox when nitrite is reduced with ammonium as electron donor to nitrate, one oxygen molecule from water with a $\delta^{18}\text{O}$ value of around -10‰ is incorporated into the newly formed nitrate. Additionally, $\delta^{18}\text{O}$ values of nitrite are lowered due to rapid oxygen isotope exchange with water oxygen (Buchwald and Casciotti, 2010; Casciotti et al., 2007; Casciotti et al., 2010) and remained, therefore, constant over the water depth of 16 m to 20m

Response: We have added the statement of an additional isotope effect to the sentence.

During anammox, when nitrite is reduced with ammonium as electron donor and nitrate is produced, one oxygen atom from water having a $\delta^{18}\text{O}$ 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 for nitrite oxidizing bacteria (see Buchwald and Casciotti, 2010) (Fig. 2c). As a result, the anammox process leads to $\delta^{18}\text{O}$ values of nitrate remaining low, while $\delta^{15}\text{N}$ of the remaining nitrate is affected by an inverse nitrogen isotope effect and values continue to increase.

L385: *As a result, the anammox process facilitates $\delta^{18}\text{O}$ values of nitrate remain low,*

Response: ... the anammox process leads to $\delta^{18}\text{O}$ values of nitrate remaining low, while...

L361: *..by an inverse isotope effect*

Was added... nitrate is affected by an inverse nitrogen 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.

Now Lines 426

L399: *...and may outcompete the nitrate reducing ANME-2d with lower doubling times in the denitrification zone of stratified lakes (Deutzmann et al., 2014).*

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

Was accepted

L408: *... the meaning behind this sentence is unclear...*

Response: 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* in nitrite reduction and nitrate reduction, respectively, linked with AOM remains unclear in this study.

L425: *...as shown...*

Response: ... as shown...

We agree with Reviewer #1 that a simple mass balance approach cannot be used for disentangling analyses of oxygen isotopes of nitrate and nitrite combinations without nitrite reference materials. Therefore, we have followed the suggestion of Reviewer #1 and have removed the O isotope data of nitrite in the revised manuscript, especially since they provided limited usefulness for arriving at the major conclusions of this manuscript.

Removal of the oxygen isotope data of nitrite required the following changes in the revised manuscript:

Line 104 now reads: Samples for isotope analysis of nitrite ($\delta^{15}\text{N}$), nitrate ($\delta^{15}\text{N}$, $\delta^{18}\text{O}$) ...

Line 166: $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ values of nitrate and $\delta^{15}\text{N}$ values of nitrite and ammonium were obtained

Line 189: The precision for $\delta^{15}\text{N}$ values of nitrate and nitrite was $\pm 0.5\text{‰}$ and for $\delta^{18}\text{O}$ of nitrate $\pm 0.8\text{‰}$.

Line 256: Simultaneously, $\delta^{15}\text{N}$ of nitrite increased from 0.1‰ to 18.7‰ concurrently with increasing $\delta^{15}\text{N}$ values of nitrate (Fig. 2C).

From line 256 onwards, the following text was deleted:

The $\delta^{18}\text{O}$ values of nitrite were near -5‰ . According to Casciotti et al. (2007), the here measured values are 9‰ lower than expected according to a situation where $\delta^{18}\text{O}$ values of nitrite was established in equilibrium with lake water with a $\delta^{18}\text{O}$ of -10‰ . However, Sebilio et al. (2019) found that the $\delta^{18}\text{O}$ values of nitrite are lower ($< +4\text{‰}$), as observed in our study, when the oxygen exchange reaction is controlled by biotic exchange processes compared to those suggested by Casciotti et al. (2007), who studied abiotic exchange reactions between nitrite and water-oxygen.

Reviewer #2

We like to express our gratitude for the detailed feedback from Reviewer #2. 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 had every attempt to address all suggestions and the numerous highly valuable recommendations where appropriate, and have provided detailed responses and explanations below.

Response to general comments:

Reviewer #1 mentioned that the authors seem well aware of the limitations of their isotope results and temper their conclusions with an appropriate acknowledgement 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.

Therefore, we have settled in the revised manuscript on a compromise approach.

To address this comment, we acknowledge in the revised version of the manuscript the limitation of isotope and microbial community data when activity indicators, for example derived from NanoSIMS analyses (L 419), are missing (for example: Ls 330, 362, 426, ..).

We also agree with reviewer #2 that microcosm and incubation experiments are excellent approaches to evaluate which processes govern isotope profiles similar to those observed in our study or to determine degradation rates. We are in fact pursuing such experiments (e.g. Kuloyo, 2020). However, it is also known that isotopic fractionation factors especially for transformation processes in the nitrogen cycle observed in the laboratory are not always transferrable to field sites (e.g. Brunner et al. 2013), and hence are a useful complement but not a replacement of field studies. Similarly, incubation experiments would also show a potential for processes (rates) that could be occurring *in situ*.

Reviewer #2 stated that the paper is prepared with a “certain degree of carelessness with a lot of typos and word adding”, but marked only a few typos within the manuscript. We have addressed all typos and stylistic improvements recommended by Reviewer #1 and #2 and tried to eliminate any remaining spelling and grammatical mistakes, in order to address the concerns of Reviewer #2.

Reviewer #2 expressed some concerns with the modelling portion of the manuscript. The modelling component in the original manuscript constituted only a minor part of our study and was rather used to develop the hypothesis. We agree that the application of a rather simple model demands a more cautious interpretation of the modelling results (micro-aerobic methane oxidation) and we have modified the revised manuscript accordingly. For instance, the conclusion that micro-aerobic methane oxidation will only occurring to a very limited extent, must be verified with a numerical model including concentration and isotope profiles that will be published elsewhere (see point-by-point answers)

In this context Reviewer #2 asked whether “steady-state conditions” can be assumed and mentioned that our modelled concentrations will be way off by using K_z of 0.1 to 2.1 m^2/d . We now explicitly state in the “Method” part of the revised manuscript that the studied ecosystem is a hydraulically static system, where we assume that advection or mixing do not occur. However, diffusion has to be modelled dynamically, in order to reflect system dynamics adequately and “steady-state conditions” cannot be assumed. Now it is stated in the revised manuscript (§2.4) (L119), that we assumed static conditions that means that there is no mixing and advection. These conditions can be assumed for lake Fohnsee between June to September and were therefore valid for our sampling campaign.

Reviewer #2 also commented on the use of K_z from literature data. This issue is addressed in detail in the point-by-point responses. In short, the suggested K_z value by the reviewer of 0.004 cm^2/s (0.03 m^2/d) is a factor of ten too small compared to (typical) literature data (e.g. Oswald, 2015 and others). If we calculate the K_z that is valid for the investigated lake (as suggested by Rev. #2) than the new K_z value fits perfectly with literature data (0.1 m^2/d), and the value that was used in the original draft of our manuscript (see also point-by point-answer). As also observed by Reviewer #2, we agree that field data and modelling results do not fit, and hence this supports our conclusion that only diffusion cannot describe the depth-profile of methane concentrations. Consequently, we have also calculated the degradation of methane using the fitting parameter k which represents the first order rate constant.

Reviewer #2 also commented that the statement in the abstract “that it is an exaggeration to state that nitrate-dependent methane oxidation has the potential is not really convincing”, while stating that “it does not happen; we know that”. Unfortunately, no references were provided that would conclusively document that this process is not occurring at any study site.

In contrast, there are a few studies demonstrating by genome analysis and anaerobic experiments with enrichment cultures that *Crenothrix* and other microbes can reduce nitrate with methane to N₂O (Oswald et al. 2017, Mustakhimov et al. 2013, Naqvi et al. 2018 etc). Therefore, it is worthwhile to discuss the occurrence and potential of this process to reduce nitrate loading in aquatic environments. These previous publications suggested that this newly discovered process (AOM with nitrate/ nitrite linked with anammox) could have environmental relevance. In this regard, we believe that our study and new data provide a valuable contribution to the literature on this topic.

Below we provide a point by point response to the comments of reviewer #2 below. Our responses appear in regular font while the original comments by the reviewers appear in italic font.

Comments of Reviewer #2

Some of the comments of Reviewer #2 were already discussed at the beginning of our point-by-point answers.

Main points:

Abstract: In light of much more important N-loss reactions (denitrification anammox) I think it is an exaggeration to state that nitrate-dependent methane oxidation has the potential to reduce nitrate loading. It just does not happen, we know that. I doubt that AOM-denitrification-anammox process really is an overlooked process...it simply is less important than canonical denitrification and aerobic methane oxidation.

Response: (see discussion above)

L 15: The nitrite (n-damo)/ nitrate dependent anaerobic methane oxidation and the anaerobic oxidation of ammonium (anammox) represent two microbially-mediated processes that can reduce nitrogen loading of aquatic ecosystems and associated methane emissions to the atmosphere.

There is no information on the site/lake. The name and location of the lake should at least be mentioned

Response: In the revised version of the manuscript, the name of the lake has been added to the abstract and we have re-written the first sentence as follows:

L 17: Here, we report vertical concentration and stable isotope profiles of CH₄, NO₃⁻, NO₂⁻ and NH₄⁺ in the water column of Lake Fohnsee (Southern Bavaria, Germany) that may indicate linkages between denitrification, anaerobic oxidation of methane (AOM) and anammox.

L56: Why is nitrate reduction more important in lakes than nitrite reduction, just because there is more nitrate than nitrite? Nitrite is an intermediate and assuming that the most important N

loss pathway is complete denitrification, nitrite reduction has to balance nitrate reduction, if nitrite does not accumulate.

Response: We have deleted this sentence

L88: "...coupled the diffusion model with a degradation term to clarify the effect of dissolved oxygen on methane oxidation. The observed coupled process has the potential to constitute an important sink of dissolved nitrogen (NO₃⁻, NO₂⁻, NH₄⁺) and methane(CH₄) in freshwater environments." What exactly is coupled? What coupled process are the authors referring to? This is not clear at this point of the article what they did in the model and how O₂ thresholds are integrated. Even if an explanation will follow in the method section, this needs to be clarified (or moved to the more detailed sections on the model parametrization).

Model: There is not enough explanation of the model. Obviously, it is not a real reaction-diffusion model, but it also is not just a diffusion model, right? What are there action parameters, how are they set? I am not an expert in modelling, but it remains unclear how the modelling works, apparently, a purely diffusive part and a reaction part is combined, but the coupling of the model components is unclear. Most importantly, how well constrained is turbulent diffusion? The results (modelled concentration profiles and isotope ratios in water column) will be highly sensitive to the choice of the D, and adopting values for D (by the way D is used usually for molecular diffusion only) from other lakes may not be appropriate. In fact, the authors seem to have a very limited knowledge of modelling turbulent diffusion in lakes. Firstly, it seems that their choice of what they call D (or K in the literature) is at least two orders of magnitude higher than would be expected for a stratified lake. They cite Oswald et al. from which D was adopted. But looking into the paper by Oswald, I saw that their choice of K_z was 4x10⁻³ cm² s⁻¹, which corresponds to approx. 0.035 m² d⁻¹. If the authors really used D/K_z values between 0.1 and 2.1, their modelled concentrations will be way off. Finally, assuming different turbulent diffusion coefficients for O₂ and CH₄ is non-sense. Turbulent diffusion is not solute-specific (in contrast to molecular diffusion), it is a hydrodynamic property of the flow field. As for the first-order methane oxidation rate coefficient, how can the authors just assume a value adopted from other studies? This parameter will change significantly between ecosystems, and has to be estimated based on fitting of the model to the observational data.

Response: We have re-written this part of the Methods section and have already given our view to this comment at the very beginning of the point-by-point answers.

In addition, we have written K_z instead of D, have calculated K_z for Lake Fohnsee and have clarified that the newly calculated K_z fits with literature data. We have already mentioned above that the reviewer's data from the literature seem not appropriate for this case study.

In the revised text we now use the 1D diffusion model with a degradation term to find some evidence that the observed concentration profile of methane cannot only be explained by

diffusion. Subsequently, we have fitted the theoretical methane concentration depth-profile to the field data using the estimated K_z value for the lake and the rate constant k as fitting parameters and compared the fitted k -value with literature data. The k -value is a characteristic parameter for sites with similar environmental conditions and may give a first hint of AOM with denitrification but does not provide compelling evidence, as outlined in our revised manuscript (from L 119; from L 295)

Furthermore, there is no difference in using a k -value as fitting parameter, as mentioned by the Reviewer, or using literature data for k that are characteristic for the studied redox process and evaluate the quality of fitting.

New:

From lines 299

Nitrate/nitrite isotope measurements: The authors write: Nitrogen and oxygen isotope ratios of nitrate were calculated by measuring nitrite alone as well as the mixture of nitrite and nitrate in a sample and using an inverse mixing calculation to determine the isotopic ratios of nitrate alone. First of all, there seems to be a duplication in this sentence. I think I understand what the authors did. They measured the isotopic composition of nitrite, and then the isotopic composition of the mixture. Based on mass balance calculation, they then calculate the isotopic ratios of nitrate alone. This works for $d15N$, but does it work for $d18O$? I am pretty sure that it does not. In a sample that contains nitrite and nitrate, O isotope fractionation during the conversion to N_2O is different for nitrite and nitrate. Hence the $d18O$ of the N_2O cannot simply be standardized, because the O -isotope offsets will be different for nitrite and nitrate. In other words, the $d18O$ of a NO_x sample is probably meaningless, and so will be the calculated $d18O$ nitrate values. The nitrate $d18O$ should have been measured after removal of the nitrite. Could the changes in $Dd15N$ (nitrate-nitrite) be an artifact that is simply the result of this effect and changing nitrite/nitrate concentration ratios?

Response: (from L164)

Regardless of the method used (bacterial reduction, reduction with cadmium or most recently with titanium), the objective is the production of N_2O . The method used for this study was the reduction of nitrate to nitrite on an activated cadmium column and then the conversion of nitrite to N_2O with an azide buffer. This method has the advantage of being able to test the conversion yields at each stage. For each step, international standards are used. In very rare cases in the environment, a significant amount of nitrate and nitrite may be present. Our approach is based not on the addition of an additional reagent, which could also create a bias, but on the conversion of nitrite to N_2O and the conversion of the nitrate+nitrite mixture to N_2O . Details of the calculations were recently published by Sebiló et al. (2019) in Scientific Reports and we now refer to this publication.

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 for unknowns (e.g. samples) by linear regression. For

samples obtained from 14, 16, 18 and 20m depth, both nitrite and nitrate were 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 depth, taking into account the two molecules and calculating the nitrate $\delta^{18}\text{O}$ gives a value of 5.6‰ whereas it was 5.4‰ without correction.

L188: How was complete outgassing of CH₄ assured before headspace analysis? Was brine/NaOH added? Concentrations were calculated based on Henry's Law, but what about the $\delta^{13}\text{C}$? Is there an isotope shift between CH₄ in the headspace and the CH₄ dissolved? If so, was that considered?

Response: (from L 190)

- outgassing was not complete since, since we followed the headspace equilibration technique by EPA (2002);

- following this EPA technique, we did not add either a salt solution or NaOH to the sample solution;

- We assumed negligible C isotope fractionation between dissolved methane and methane in the headspace (e.g. Feux 1980) and therefore report the measured $\delta^{13}\text{C}$ values for headspace methane.

Results: I am a bit disappointed by the low number of data points/analyses. As a consequence, isotope gradients are not well resolved (and their interpretation is hence complicated), and the profiles are not replicated for several time points. Do the authors assume steady state conditions? How relevant is this for the model fitting?

Response: We agree that a depth resolution of 0.5 m or even lower would have been better, but we sampled up to 2L of lake water for each depth (water samples for IC, isotopes, DOC and microbiology) and we wanted to exclude mixing of water from different depths by sampling.

Figure 3c is very difficult to read? Why not showing profiles (connected symbols) for the most relevant OTUs. It is almost impossible to see the vertical structure.

Response: We now present Figure 3c with the symbols connected for the most relevant OTUs to make it easier to see the vertical structure.

Discussion: It is not clear to me what the arguments are that allow the authors to exclude oxic methane oxidation. I agree that the concentration profile suggests reaction below the redoxcline, but you do not need to model this to come to this conclusion. At the same time, do the authors assume steady state? Apparently, the lake undergoes seasonal fluctuations, so that

the curvature of the concentration profiles may represent a non-steady state, and its interpretation with regards to where reaction takes place and where not is biased.

Response: We agree that a more complex model is needed to perform flux estimation and to evaluate if micro-aerobic methane oxidation has occurred near the oxycline. In addition, it will make sense as suggested by Reviewer #2 to incorporate the isotope compositions of methane in the modelling part, but this was out of scope for this paper and the former manuscript (L 303).

We have improved the modelling part and have shown the effect of diffusion and degradation on the depth-concentration profile of methane (L 295) and have rewritten this part of the manuscript.

L 303: However, because the oxic-anoxic transition zone is in close proximity to the nitrate reduction zone, numerical modelling studies are required that link the stable isotope ratio and concentration profiles of methane to study the effect of micro-aerobic methane oxidation near the oxycline at lake Fohnsee. (Fig. 4).

Can the authors explain why a 90% decrease in ammonium is associated with a 15N shift of only 4‰

L335-7: The authors say that above 20 m water depth, there is no evidence for ammonium oxidation. Why? Because the $\delta^{15}\text{NH}_4$ values do not increase? But they also do not increase much below that depth, where the authors suggest that anammox occurs. And most strikingly, the ammonium profile is essentially linear all the way up to the oxycline. To me this suggests that not much ammonium oxidation is taking place at this depth, and essential all NH_4 is oxidized at the oxycline.

Response: We do not understand that Reviewer #2 mentioned a 90% decrease in ammonium concentration that is linked to a small N isotope effect in the remaining ammonium. We have observed a decrease of ammonium concentration from 1 mg/L at the bottom of the lake to 0.8 mg/L at a depth of 20 m and simultaneously an increase of $\delta^{15}\text{N}$ of ammonium of 4 ‰. Above this depth $\delta^{15}\text{N}$ values of ammonium remain constant. Above a depth of 20 m, ammonium concentrations decrease from 0.06 mmol/L to below the detection limit at a water depth of 12 m, probably by diffusion where no significant isotope fractionation is expected and micro-aerobic NH_4 -oxidation.

To explain the small nitrogen isotope fractionation effects, Wunderlich et al. (GCA 2018) have suggested a transport limitation model, that is outlined in lines 345 to 353.

340-345: The authors cite the anammox isotope effect study by Brunner and colleagues. But they mix up equilibrium and kinetic N isotope effects between nitrite and nitrate. The inverse kinetic N isotope effect, which applies to active nitrate production from nitrite by anammox, is much lower than the -61‰ mentioned.

Response: Right, the equilibrium N isotope effect between nitrate and nitrite is $-60.5 \pm 1.0\%$ whereas the inverse kinetic N isotope fractionation associated with the oxidation of nitrite to nitrate is $-31.1 \pm 3.9\%$. The latter fits very well to our field data. The explanation for small nitrogen isotope effects during anammox is also given in L 345-355.

The authors should explain better why anammox could produce a $d18O$ vs $d15N$ NO_3 relationship of 0.5. Is this slope consistent with nitrate production from nitrite with the incorporation of O atoms from water? Such slopes in $d18O$ vs $d15N$ NO_3 plots have been observed in several ground/freshwater studies. Does this imply that in all these environments anammox was the main N-loss pathway?

Response: Indeed, slopes lower than 1 can also be observed at aerobic/ anaerobic interfaces such as groundwater systems, the oxycline of stratified lakes or as outlined in Wunderlich et al. (2018 in GCA) by specific organisms that re-oxidize nitrite to nitrate and do not imply that anammox is the main N-loss pathway in all environments. Please note that we consider a system that may be controlled by anaerobic redox conditions. This was also clearly stated in the manuscript to demonstrate that in a $\delta^{18}O$ vs. $\delta^{15}N$ plot for nitrate a slope lower than 1 is a powerful indicator for the occurrence of anammox in an anoxic environment.. Furthermore, we developed several lines of evidence to come to the conclusion that anammox may have occurred.

From L 335

The comment whether the slope is consistent with nitrate production from nitrite with the incorporation of O atoms is somehow exaggerated because we think that the reviewer knows that this depends on the enzymes involved etc. Here we relegate to the paper of Granger and Wankel (2016) in PNAS.

*What is the relative abundance of “normal” nitrate and nitrite reducers compared to $NC10$ and *Crenothrix*?*

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 requests. However, the Gamma proteobacteria 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 Gamma proteobacteria relative abundance increases with depth into the anoxic zone (Fig 3b), it is likely that many of the Gamma proteobacteria in deeper waters of the lake are responsible for nitrate and nitrite reduction, and denitrification. We will add an explanation to the revised manuscript (from line 426).

Some minor points:

These few suggestions were accepted and improvements were made in the revised manuscript.

I am wondering about the fact that there was not an appropriate $d18O_NO2$ standard used.

Response: We agree and have deleted the $\delta^{18}O$ values of nitrite from the revised manuscript following the suggestion of both reviewers.

I am still not convinced by the way they sell their data set (evidence for true AOM is weak), and I still think there are some issues, but I guess that this is part of the scientific exchange. We do not need to all agree. It is definitely a nice data set, certainly worth to be published.

Response: We agree with Reviewer #2 that a more refined depth resolution of the samples and the subsequently obtained data would have been beneficial for further strengthening the evidence for AOM; as pointed out previously in the manuscript, this was, however, not possible and, therefore, we have very carefully worded this section by stating “some evidence” in lines 294, 326, and 333

The authors tone down in their response the value of incubation experiments. I do not agree. Experiments, even if not directly reflecting the natural conditions, would have helped to calibrate the observed isotopic signatures, and to gain confidence in their interpretation. I was not referring to isotope effect experiments, and I am aware that rate measurements will deliver potential rates only, but clearly, rate measurements would have helped to indicate the potential for the use of specific substrates, and thus would have helped to support (or not) the claimed links between different biogeochemical reactions (anammox and AOM).

Response: We thank the reviewer for this clarification. Fact is that we have not conducted any complementary laboratory experiments but we agree that laboratory experiments could help to study the potential for the use of specific substrates.

The authors state that there is barely any mixing during summer (“no mixing, no advection”); I assume they mean no advective mixing, because turbulent diffusive mixing, even if sluggish will take place.

Response: This is an excellent point and we have modified the manuscript accordingly. Now line 134 reads as follows: This corresponds to the period where stagnant conditions for lake water are assumed to prevail (no advective mixing).....

As for the choice of a value for K_z : In my first review; I was not suggesting a value of 0.03 m^2/d . I was referring to a paper by Oswald et al. from Lake La Cruz (Oswald et al. 2016), for which this value was reported. I mixed up the different studies/lakes investigated by Oswald et al., and values from Lake Rotsee, as well as values for Lake Lugano seem to be higher, indeed. But the broad range of K_z values observed for the different meromictic lakes highlights that there is not “a typical literature value”, and that just assuming a value for K_z is difficult.

Response: Thank you for this clarification.

As for the interpretation of the NH_4 concentration versus the $d15NH_4$ profiles: I see a more or less steady decrease from 1 to <0.1 mgN/l between 22 and 12 m, without a strong overall $15N$

enrichment. From these patterns, I find it quite difficult to pinpoint anaerobic ammonium oxidation in the deep water column. Within the error of the $\delta^{15}\text{N}_{\text{NH}_4}$ analyses, the isotope profile looks almost straight to me....an observation that could most plausibly be explained by aerobic ammonium explanation at the redox transition zone, which serves as an efficient NH_4 sink without much N isotope fractionation.

Response: We clearly stated from line 342 to 344 where ammonium concentrations decreased and $\delta^{15}\text{N}_{\text{NH}_4}$ increased:

The decrease in ammonium concentration with decreasing water depth is accompanied by an enrichment of ^{15}N in the remaining ammonium shifting the $\delta^{15}\text{N}_{\text{NH}_4}$ values from 7.9‰ to 11.6‰ between 22 and 20 m water depth (Fig. 2C), suggesting that ammonium is oxidized anaerobically while enriching the remaining substrate in ^{15}N .

We also like to point out that we did not interpret the N isotope ratios and concentrations of ammonium in isolation, but considered the following observations in combination:

- both nitrite and ammonium concentration trends;
- $\Delta\delta^{15}\text{N}$ between nitrate and nitrite increased from 11‰ in NMTZ to > 26‰ at the water depth of 20 m, where $\delta^{15}\text{N}$ values of ammonium increased while NH_4^+ concentrations decreased (Fig. 2C);
- the deviation of the slope of $\delta^{18}\text{O}$ versus $\delta^{15}\text{N}$ values on a dual isotope plot (2D plot) for nitrate from the expected value of 1 for microbial denitrification is a powerful tool to identify anammox.
- This, together with the presence of ‘*Candidatus Anammoximicrobium*’ within the strictly anaerobic water column, is in our view sufficient to come to the conclusions that these combined observations may indicate that anammox has occurred at Lake Fohnsee between a water depth of 20-21 m.

Reviewer 1 also expressed the opinion that the statements about the key findings are reasonably tempered in the revised manuscript.

I am still confused with regards to the $\delta^{18}\text{O}_{\text{NO}_x}$ values. Again, in a sample that contains nitrite and nitrate, O isotope fractionation during the conversion to N_2O (independent of the method of conversion) must be different for nitrite and nitrate (via nitrite), simply because one versus two O atoms will be plucked off during the fractionating transformation, respectively. Hence it remains unclear to me how the $\delta^{18}\text{O}$ of the combined NO_3+NO_2 sample is standardized. The problem is not the yield, the problem is that the N_2O from the nitrate will likely have a much higher $\delta^{18}\text{O}$ than the $\delta^{18}\text{O}$ of the N_2O generated from the NO_2 .

Response: As mentioned in our response to Reviewer #1, we agree with the major limitation of the $\delta^{18}\text{O}$ values of nitrite as no reference material was used, which also impacts mixing calculations. However, for most of the nitrate samples (14m to 18m water depth) nitrite represents at most 15% of the total nitrate+nitrite concentration. Recalculations using a value of +4‰ for $\delta^{18}\text{O}$ of nitrite revealed that the $\delta^{18}\text{O}$ values of nitrate are between 2 and 3‰ lower compared to those calculated with the previously reported $\delta^{18}\text{O}$ values of nitrite (ca. -4 to +1‰). These changes had no consequences for our conclusions reported in the earlier version of the manuscript.

In the revised manuscript we have re-calculated the $\delta^{18}\text{O}$ values of nitrate.

Now from line 175 the revised manuscript reads: For $\delta^{18}\text{O}$ values of nitrate we performed a mass-weighted isotope mass balance calculation assuming that at a pH of 7 the $\delta^{18}\text{O}$ of nitrite is in equilibrium with water with a value close to +4‰.

Figs 2C and 5 were corrected using the new data set of $\delta^{18}\text{O}$ of nitrate and additional information was given for Fig. 2C.

L915: established is misspelled

Response: This part was deleted

In summary, we have made every attempt to address the suggestions and the highly valuable recommendations by both reviewers, and have provided detailed responses and explanations above. We hope that the revised manuscript can now be accepted for publication.

References:

- KULOYO, O., RUFF, S. E., CAHILL, A., CONNORS, L., ZORZ, J. K., HRABE DE ANGELIS, I., NIGHTINGALE, M., MAYER, B. & STROUS, M. (2020): Methane oxidation and methylotroph population dynamics in groundwater mesocosms. *Environmental Microbiology*, 22(4): 1222-1237 (early access February 2020; published April 2020). <https://doi.org/10.1111/1462-2920.14929>
- SEBILO, M., ALOISI, G., LAVERMAN, A. M., MAYER, B., PERRIN, E., VAURY, V., MOTHET, A. & LAVERMAN, A. M. (2019): Controls on the isotopic composition of nitrite ($\delta^{15}\text{N}$ and $\delta^{18}\text{O}$) during denitrification in freshwater sediments. - *Scientific Reports*, 9: 19206. [Doi.org/10.1038/s41598-019-54014-3](https://doi.org/10.1038/s41598-019-54014-3); published December 16, 2019.
- WUNDERLICH, A., HEIPIEPER, H.J., ELSNER, M. AND EINSIEDL, F. (2018) Solvent stress-induced changes in membrane fatty acid composition of denitrifying bacteria reduce the extent of nitrogen stable isotope fractionation during denitrification. *Geochimica et Cosmochimica Acta* 239, 275-283.