Comment on

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

Rev. #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 Kz 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 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.

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 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).

From line 164 and from line 354

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. 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 $\delta^{15}N$ values were made based on the known values of the nitrate and nitrite standards.

With respect to the observed $\delta^{18}O$ 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 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 and that the latter was predominately controlled by biotic reactions. Moreover, since denitrification alone should have resulted in a $\delta^{18}O$ 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: N_2O …using sodium azide.

Response: Nitrite was converted to N_2O using acetic acid buffer sodium azide.

L170: *the mixture of both nitrate and nitrite was reduced to N_2O.*

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 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.

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

Response:... 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 KNO*2* were analyzed in each batch (Lb1, δ15N = -63‰ and Lb2, δ15N = +2.7‰). Corrections of the raw $\delta^{15}N$ 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? 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?)?*

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 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 δ 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:

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 O_2 calculations because the use of an analytical model has to 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.*

Repsonse: We observed a difference of δ15N values (Δδ15N) 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 $\delta^{15}N$ 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 δ15N values between nitrate and nitrite of around +15‰ arising as the result of production of highly 15N enriched nitrate deriving from anammox (Δδ15N 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 $\delta^{18}O$ value of around -10‰ is incorporated into the newly formed nitrate. Additionally, $\delta^{18}O$ 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 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 for nitrite oxidizing bacteria (see Buchwald and Casciotti, 2010) (Fig. 2c). As a result, the anammox process leads to δ18O values of nitrate remaining low, while δ15N 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 $\delta^{18}O$ values of nitrate remain low,*

Response: ... the anammox process leads to $\delta^{18}O$ 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 O2 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 sown*…

Response: ... as shown...

Rev. #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 Kz 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 Kz from literature data. This issue is addressed in detail in the point-by-point responses. In short, the suggested Kz 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 Kz 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 N_2O (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.

345

Comments of Reviewer #2

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

350 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 355 *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.

360 *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 CH_4, NO_3^-, NO_2^- and NH_4^+ in the water column of Lake Fohnsee (Southern Bavaria, Germany) that may indicate linkages between 365 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.

370

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 375 *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 sectionson 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 partis combined, but the coupling of the model components is unclear. Most importantly ,how well constrained is turbulent diffusion? The results (modelled concentration pro-files 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 Kz was 4x10-3 cm2 s-1, which corresponds to approx. 0.035 m2 d-1. If the authors really used D/Kz 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), itis 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 Kz instead of D, have calculated K_z for Lake Fohnsee and have clarified that the newly calculated Kz 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 Kz 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 N2O is different for nitrite and nitrate. Hence the d18O of the N2O cannot simply be standardized, because the O-isotope offsets will be different for nitrite and nitrate. In other words, the d18O of a NOx 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?

Reponse: (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 Sebilo 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 $\delta^{18}O$ gives a value of 5.6‰ whereas it was 5.4‰ without correction.

L188: How was complete outgassing of CH_4 assured before headspace analysis? Was brine/NaOH added? Concentrations were calculated based on Henry's Law, but what about the d13C? 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 $\delta^{13}C$ 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 d15NH4 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 NH4 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 $\delta^{15}N$ of ammonium of 4 ‰. Above this depth $\delta^{15}N$ 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 ± 1.0‰ whereas the inverse kinetic N isotope fractionation associated with the oxidation of nitrite to nitrate is −31.1 ± 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 NO3 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 NO3 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.

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Biogeochemical evidence of anaerobic methane oxidation and anaerobic ammonium oxidation in a stratified lake using stable isotopes

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Abstract. Nitrate pollution of freshwaters and methane emissions into the atmosphere are crucial factors in deteriorating the quality of drinking water and in contributing to global climate change. 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. Here, we report vertical concentration and stable isotope profiles of CH_4, NO_3^-, NO_2^- and NH_4^+ in the water column of Lake Fohnsee (Southern Bavaria, Germany) that may indicate linkages between denitrification, anaerobic oxidation of methane (AOM) and anammox. In a water depth from 12 to 20 m, a methane-nitrate transition zone (NMTZ) was observed, where $\delta^{13}C$ values of methane and $\delta^{15}N$ and $\delta^{18}O$ of dissolved nitrate markedly increased in concert with decreasing concentrations of methane and nitrate. These data patterns, together with the results of a simple 1D diffusion model linked with a degradation term show that the non-linear methane concentration profile cannot be explained by diffusion, and that microbial oxidation of methane coupled with denitrification under anaerobic conditions is the most parsimonious explanation for these data trends. In the methane zone at the bottom of the NMTZ (20 m to 22 m) $\delta^{15}N$ of ammonium increased by 4‰, while ammonium concentrations decreased. In

addition, a strong ^{15}N enrichment of dissolved nitrate was observed at a water depth of 20 m, suggesting that anammox is occurring together with denitrification. The conversion of nitrite to N_2 and nitrate during anammox is associated with an inverse N isotope fractionation and may explain the observed increasing offset ($\Delta\delta^{15}N$) of 26 ‰ between $\delta^{15}N$ values of dissolved nitrate and nitrite at a water depth of 20 m compared to the $\Delta\delta^{15}N_{nitrate\text{-}nitrite}$ of 11 ‰ obtained in the NMTZ between a water depth of 16 m and 18 m. 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’) whose concentrations were highest in the methane and ammonium oxidation zones, respectively. This study shows the potential for a coupling of microbially mediated nitrate dependent methane oxidation with anammox in stratified freshwater ecosystems, which may be important for affecting both methane emissions and nitrogen concentrations in lakes.

1 Introduction

Methane is a more potent greenhouse gas than CO_2 and is responsible for 20% of global warming (Change, 2001). Bastviken et al. (2004) have shown that lacustrine ecosystems may be responsible for 6 to 16 % of natural methane emissions. However, the variability in methane emissions and the lack of knowledge about their main environmental controls contribute large uncertainties into the global CH_4 budget (Sabrekov et al., 2017).

Methane is abundantly formed in anaerobic lake sediments by methanogenesis (Borrel et al., 2011; Conrad et al., 2007; Norði et al., 2013) and diffuses upwards through the water column toward the oxycline of often nitrate-containing seasonally stratified lakes. With the discovery of the anaerobic oxidation of methane (AOM) coupled to nitrate or nitrite reduction more than 10 years ago a new process was suggested that has the potential to reduce emissions of greenhouse gases of lacustrine environments by oxidizing CH_4 to CO_2 under anoxic conditions (Ettwig et al., 2010; Haroon et al., 2013; Raghoebarsing et al., 2006). Under controlled laboratory conditions, experiments showed that that *n-damo* (nitrite dependent anaerobic oxidation of methane) bacteria that are members of the candidate phylum NC10 use nitrite for the anaerobic oxidation of methane (Ettwig et al., 2010), while archaea such as ANME-2d prefer nitrate as electron acceptor (Haroon et al., 2013). Evidence of archaeal AOM coupled with bacterial denitrification was first reported from culture experiments with two microorganisms, ‘*Candidatus* Methylomirabilis oxyfera*’* which belongs to the phylum NC10 and reduces nitrite to N_2, whereas ANME-2d lineage uses methane to reduce nitrate to nitrite (Raghoebarsing et al., 2006).

Filamentous methane oxidizing bacteria related to the genus *Crenothrix* (Gammaproteobacteria) also use nitrate as a terminal electron acceptor (Kits et al., 2015; Naqvi et al., 2018; Oswald et al., 2017). Therefore, *Crenothrix* may act as a driver for methane oxidation in nitrate-containing stratified lakes, where environmental and redox conditions can often change over seasonal periods. A few environmental studies have documented the presence of NC10 like bacteria in lake sediments, which are thought to have a similar metabolism to ‘*Ca*. M. oxyfera’. Via micro-sensor measurements and molecular biological

analysis it was postulated that '*Ca. M. oxyfera'* is responsible for *n-damo* in the sediments of Lake Constance (Deutzmann et al., 2014), while others found some evidence of *n-damo* in the sediments of a lake in Japan (Kojima et al., 2012).

It has been speculated that denitrification can co-occur with anammox at oxic-anoxic interfaces (Strous and Jetten, 2004; Thauer and Shima, 2008). In the late 1980s, microorganisms driving the anammox reaction were first discovered in a wastewater pilot plant (Francis et al., 2007; Mulder et al., 1995). Subsequently, the significance of the anammox process in the nitrogen cycle of freshwater systems was shown in numerous studies (e.g. Schubert et al., 2006) and it was suggested that the process is of key environmental significance (Kuypers et al., 2003). The coexistence of heterotrophic denitrification, *n-damo,* and anammox was clearly demonstrated in bioreactor studies supplied with nitrate, methane and ammonium (Haroon et al., 2013; Hu et al., 2015; Luesken et al., 2011; Shi et al., 2013).

By comparison, the number of studies demonstrating the co-occurrence of *n-damo* and anammox processes in natural aquatic environments is limited (e.g. Shen et al., 2014; Zhu et al., 2018). More information is needed on the connection of these processes in the natural environment, in order to obtain an accurate estimation of methane fluxes to the atmosphere and to identify the factors driving and limiting the reduction of nitrate and its intermediates in lacustrine environments. Stable isotope fractionation has often been used to identify microbial transformation processes affecting nitrogen and carbon including denitrification and AOM (e.g. Wunderlich et al., 2012). Recently, Granger and Wankel (2016) showed that displaying the isotope compositions of nitrate in a 2D isotope plot ($\delta^{18}O$/ $\delta^{15}N$) enables the distinction between denitrification and anammox. In addition, aerobic and anaerobic methane oxidation was often documented by increasing $\delta^{13}C$ values in the remaining methane (Eller et al., 2005; Feisthauer et al., 2011). However, the separation of aerobic and anaerobic oxidation of methane based on calculated isotope enrichment factors of methane may fall short because of overlapping carbon isotope enrichment factors (Feisthauer et al., 2011).

Here we report chemical and isotopic evidence together with quantitative PCR (qPCR) and high-throughput Illumina sequencing of 16S rRNA genes to provide evidence for the co-occurrence of n-damo/ denitrification with AOM and anammox in a natural freshwater habitat. We also applied a simple 1D-diffusion model and coupled the diffusion model with a degradation term to test the hypothesis that methane oxidation with nitrate was microbially mediated. 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 (NO_3^-, NO_2^-, NH_4^+), and methane (CH_4), in stratified freshwater ecosystems.

2 Material and Methods

2.1 Field Site

The Ostersee lakes are located in Southern Germany and consist of a series of lakes that are hydrologically connected (Braig et al., 2010). The chain of lakes was formed after the rapid disintegration of the last ice sheet at the end of the Pleistocene. Lake Fohnsee which was sampled in 2016 is one of the Ostersee lakes. The lake is circa 22 m deep, fed by groundwater and

is stratified during summer with an oxic zone (epilimnion) near the surface and an anoxic redox zone (hypolimnion) below a water depth of approximately 12 m.

2.2 Sampling

A field campaign at lake Fohnsee was performed in summer 2016 to obtain depth-resolved water samples throughout the water column of the lake to a depth of 22 m. During the field campaign a submersible probe with sensors for temperature and oxygen content was used. Dissolved oxygen concentrations and lake water temperatures with a depth resolution of 1 m were measured on site. Water samples were taken with a discrete 2 L sampling unit ("Ruttner bottle") with a depth-resolution between 1 and 2 m. The detection limit of the oxygen-sensor FDO 925, WTW, Xylem, Germany) was < 0.625 µmol/L, the analytical error was 0.5 % of the measured value for oxygen. In addition to the in-situ measurements, samples for the laboratory-based measurement of major anion and cation concentrations, and water isotopes (δ^2H, $\delta^{18}O$) were field-filtered with 0.2µm PES filters and stored in airtight 1.5 ml glass vials. Samples for isotope analysis of nitrite ($\delta^{15}N$, $\delta^{18}O$), nitrate ($\delta^{15}N$, $\delta^{18}O$) and ammonium ($\delta^{15}N$) were field-filtered with 0.2µm PES filters and stored in PE vials. Isotope samples were frozen at -23°C until processing. Samples for analysis of DOC (Dissolved Organic Carbon) concentrations were collected in 50 ml glass bottles, filtered with 0.45 µm PVDF filters and measured immediately in the laboratory. Samples for the concentrations and isotope analysis of methane ($\delta^{13}C$) were transferred into 200 ml glass vials without headspace and sealed with crimped butyl stoppers. Samples for molecular-biological investigations were collected in 2 L sterile glass bottles. Subsequently the 2L water samples were divided in two 1L samples for replicate measurements and each sample was filtered in the laboratory using a 0.2 µm sterile filter. The filter including the microbial biomass was kept frozen at -23°C prior to analysis.

2.3 Determination of water chemistry and DOC

The samples were analyzed with ion chromatography for concentrations of nitrate, nitrite, ammonium, and sulfate. The analyses were performed in triplicate using two parallel Thermo Scientific ICS1100 instruments with CS12A (cations) and AS9-HC (anions) columns, respectively. Values are reported as mean values (n=3) with an uncertainty of less than 10%. The detection limits are 0.008 mmol/L for nitrate and 0.007 mmol/L for nitrite and 0.005 mmol/L for ammonium.

DOC concentrations were determined by lowering the pH of the samples to remove inorganic carbon and subsequent spectral analysis of CO_2 after combustion (Analytic Jena Multi N/C 3100) with a measurement uncertainty of ±5% and a detection limit of 0.5 mg/L.

2.4 Analytical model to evaluate methane diffusion and the potential of micro-aerobic oxidation of methane in the water column

For the 1-D diffusion model, a semi-infinite system was assumed where the lower boundary (at z = 0) is kept at a constant input concentration C_0, and the initial concentration throughout the system is zero. The following formula (Eq. 1) from Crank (1975) represents an analytical solution, which was used to determine the methane concentration as a function of depth (resolved in 0.1 m intervals) along the 10 m long water column below the oxycline at time t:

$$C\,(z, t) = C_0 \operatorname{erfc} \frac{z}{2\sqrt{(K_z t)}} \qquad \text{(Eq. 1)}$$

where C [µmol/L] is the methane concentration in the water column as a function of distance (depth) z and time, C_0 [µmol/L] is the constant concentration of methane at the lower boundary, located at a depth of 22 m below the lake surface (bottom of the water column), and K_z [m^2 day^{-1}] represents the turbulent diffusion coefficient for methane in water. For modeling, time t was set to 90 days. This corresponds to the period where stagnant conditions for lake water are assumed to prevail (no mixing and advection) so that methane is transported within the water column by diffusion only. For methane a turbulent diffusion coefficient of $K_z = 1.2*10^{-6}$ m^2/s, corresponding to 0.1 m^2/day, was calculated for Lake Fohnsee according to Wenk et al. (2013) and Bless et al. (2014) with the system specific parameter a_0 of 0.000343 cm^2s^{-2}. The K_z value is at the lower range typically applied for methane flux calculations and modeling (0.1-2.1 m^2/day) at stratified lakes such as at Lake Rotsee and Lake Lugano (Oswald et al., 2015; Wenk et al., 2014).

If the diffusing substance is microbially degraded or immobilized, the differential equation for diffusion needs to be extended by additional reaction terms. If first-order degradation is considered, an analytical solution is also available from Crank (1975), which was used for 1-D modelling of methane diffusion and degradation (Eq. 2):

$$C(z,t) = \frac{C_0}{2} \exp\left(-z\sqrt{k/K_z}\right) \operatorname{erfc}\left(\frac{z}{2\sqrt{K_z t}} - \sqrt{kt}\right) + \frac{C_0}{2} \exp\left(z\sqrt{k/K_z}\right) \operatorname{erfc}\left(\frac{z}{2\sqrt{K_z t}} + \sqrt{kt}\right) \qquad \text{(Eq. 2)}$$

where, k is the first-order degradation rate constant [day^{-1}]. Here we used the k-value as fitting parameter and compared it to literature data from Roland et al. (2017). If the argument kt in Eq. (2) is large enough so that erfc is approaching 2 at the left hand side and 0 at the right hand side, Eq. (3) simplifies as follows (Crank, 1975):

$$C = C_0 \exp\left(-x\sqrt{k/K_z}\right) \qquad \text{(Eq. 3)}$$

2.5 Measurement of stable isotope ratios

The natural abundance stable isotope ratios of nitrogen ($^{15}N/^{14}N$) in NH_4^+, NO_3^-, NO_2^- and oxygen ($^{18}O/^{16}O$) in NO_3^- and NO_2^- as well as carbon isotope ratios ($^{13}C/^{12}C$) of methane constitute a powerful tool to identify biogeochemical transformation processes involving these compounds. During AOM and denitrification the lighter isotopes (^{12}C, ^{14}N, ^{16}O) react preferentially leading to an enrichment of the heavier isotopes (^{13}C, ^{15}N, ^{18}O) in the residual substrate pool (CH_4, NO_3^-, NH_4^+) and an enrichment of the lighter isotopes in the newly formed products CO_2, NO_2^-, and N_2. Stable isotope ratios of C, N and O are reported using the conventional delta (δ) notation expressed as $\boldsymbol{\delta} = \left(\frac{R_{sample}}{R_{standard}} - \mathbf{1}\right)$ [‰] where R_{sample} and $R_{standard}$ are the ratios of heavy versus light isotopes in the sample and an international standard, respectively.

2.5.1 Water isotope composition

Hydrogen and oxygen isotope ratios of water ($^{18}O/^{16}O$ and $^{2}H/^{1}H$) were analyzed by off-axis laser spectroscopy using a water analyzer (Los Gatos Instruments IWA-45EP) with a precision of 0.1‰ for $\delta^{18}O$ and 0.5‰ for $\delta^{2}H$ and are reported with respect to Vienna Standard Mean Ocean Water (V-SMOW).

2.5.2 Isotope compositions of nitrate, nitrite and ammonium

$\delta^{15}N$ and $\delta^{18}O$ values of nitrate and nitrite as well as $\delta^{15}N$ values of ammonium were obtained by the production of N_2O following modified protocols of procedures reported by McIlvin and Altabet (2005), Semaoune et al. (2012) and Zhang et al. (2007), respectively. Nitrite was converted to N_2O using acetic acid buffer sodium azide, 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, $\delta^{15}N$ = -63‰ and Lb2, $\delta^{15}N$ = +2.7‰). Corrections of the raw $\delta^{15}N$ values were made based on the known values of the nitrate and nitrite standards. In a second aliquot of the sample, nitrate was first reduced to nitrite in an activated column of cadmium and the mixture of both nitrate and nitrite was reduced to N_2O via azide. The yield of conversion was better than 95%. 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. 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. Ammonium was oxidized to nitrite using hypobromite (BrO^-). The nitrite produced from ammonium oxidation was then transformed into dissolved N_2O by buffered azide solution for subsequent analysis. The isotope compositions of all N_2O samples were measured with an isotope ratio mass

spectrometer (IRMS, Delta Vplus, Thermo Scientific, Bremen, Germany) in continuous-flow mode with a purge-and-trap system coupled with a Finnigan GasBench II system (Thermo Scientific, Bremen, Germany). Results are reported in the internationally accepted delta notation in ‰ with respect to the standards AIR for $\delta^{15}N$ and Vienna Standard Mean Ocean Water (V-SMOW) for $\delta^{18}O$. Ammonium, nitrate and nitrite reference materials subject to the same analytical procedures were used to calibrate the isotopic composition of N_2O. The standards USGS25, $\delta^{15}N$ = -30.4‰, IAEA-N1, $\delta^{15}N$ = 0.4‰, IAEA-N2, $\delta^{15}N$ = 20.3‰, IAEA-305, $\delta^{15}N$ = 39.8‰ were used for ammonium reference materials and USGS34, $\delta^{15}N$ = -1.8‰, $\delta^{18}O$ = -27.9‰, USGS35, $\delta^{15}N$ = +2.7‰, $\delta^{18}O$ = +57.5‰ and USGS32, $\delta^{15}N$ = +180‰, $\delta^{18}O$ = +25.7‰ were used to calibrate nitrate measurements; Laboratory nitrite standards Lb1, $\delta^{15}N$ = -63‰ and Lb2, $\delta^{15}N$ = +2.7‰ were used to calibrate nitrite isotope analyses). The precision for $\delta^{15}N$ values of ammonium was ± 0.3‰. The precision for $\delta^{15}N$ values was ± 0.5‰ and for $\delta^{18}O$ ± 0.8‰ of nitrite and nitrate.

2.5.3 Concentrations and carbon isotope ratios of dissolved methane

The concentrations and carbon isotope ratios of dissolved methane in the lake water samples were determined from the same bottle 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. Subsequently, the concentration of methane in the headspace was determined by manual injection of >2 ml of gas into a gas chromatograph (Bruker 450) with a measurement uncertainty of < ±5%. The concentration of dissolved methane in the water samples (in mg/L) was subsequently determined using Henrys Law (EPA, 2002).

The carbon isotope ratios of methane in the headspace of the same samples were analyzed on a ThermoFisher MAT 253 isotope ratio mass spectrometer (IRMS) coupled to Trace GC Ultra + GC Isolink (ThermoFisher) after manual injection of <1 ml of gas. We assumed negligible C isotope fractionation between dissolved methane and methane in the headspace (e.g. Feux, 1980) and therefore report the measured $\delta^{13}C$ values for headspace methane. Carbon isotope ratios of methane are reported in the standard delta notation in ‰ relative to the VPDB standard. Instrument stability and linearity was ensured by daily measurements of an in-house methane mix of 5% CH_4 (balance helium). Carbon isotope analyses of methane were standardized by measurements of Isometric Instruments (Victoria, BC, Canada) gases containing methane with known $\delta^{13}C$ values including the following: B-iso1 ($\delta^{13}C$ = -54.5‰, $\delta^{2}H$ = -266‰), L-iso1 ($\delta^{13}C$ = -66.5‰, $\delta^{2}H$ = -171‰), and H-iso1 ($\delta^{13}C$ = -23.9‰, $\delta^{2}H$ = -156‰). The precision for carbon isotope analyses on dissolved methane was better than ±0.5‰.

2.6 DNA extraction

Microbial biomass was collected on 0.22 µm cellulose acetate filters (Corning Inc., 1 NY, USA) in the laboratory after sampling and stored frozen on dry ice and later at -23°C until DNA extraction. Total DNA for groundwater microbial community analysis was extracted from frozen filters as previously described (Brielmann et al., 2009).

2.7 Quantitative gene sequencing

Quantitative PCR (qPCR) was performed using the custom primer dual indexed approach that is commonly applied in microbial ecology community analyses (Kozich et al., 2013), and targets the V4 hypervariable region of the 16S rRNA gene using updated 16S rRNA gene primers 515F/806R (515F: 5′ – GTGYCAGCMGCCGCGGTAA– 3′, 806R: GGACTACNVGGGTWTCTAAT) as described previously (Coskun et al., 2018). These 'universal' primers cover all major groups of Bacteria and Archaea, and have the 'Y' ambiguity code insertion into the 515F forward primer to increase the coverage of Archaea (Parada et al., 2016). qPCR reactions were prepared using an automated liquid handler (pipetting robot), the EpMotion 5070 (Eppendorf), was used to set up all qPCR reactions and standard curves. The efficiency values of the qPCR were <90% and R^2 values >0.95% qPCR was performed using white 96-well plates. The technical variability of 16S rRNA gene qPCR measurements was determined to be consistently <5% under the EpMotion 5070.

Barcoded V4 hypervariable regions of the amplified 16S rRNA genes from the qPCR were sequenced on an Illumina MiniSeq following an established protocol (Pichler et al., 2018). This yielded a total of >2 000 000 raw sequencing reads that were then subjected to quality control. In order to quality control the OTU picking algorithm for the data, we also sequenced a "mock community" alongside our environmental samples. The mock communities contained a defined number of species (n=18) all containing 16S rRNA genes >3% difference. Pichler et al. (2018) USEARCH version 10.0.240 was used for quality control and OTU picking,(Edgar, 2013) OTUs were clustered at 97% sequence identity. The taxonomic relationship of OTU representative sequences were identified by BLASTn searches against SILVA database (www.arb-silva.de) version 132. To identify contaminants, 16S rRNA genes from extraction blanks and dust samples from the lab were also sequenced. These 16S rRNA gene sequences from contaminants were used to identify any contaminating bacteria in our samples. All OTUs containing sequences from these 'contaminant' samples (<5% of total) were removed prior to downstream analysis.

The qPCR and sequencing data were then used to quantify the abundance of individual 16S rRNA genes per OTU across the sampled water column, in the different biogeochemical zones. The fractional abundance (percent total sequences per sample) of each 16S OTU was multiplied by the total number of 16S rRNA genes per sample. This provided quantitative gene abundance per OTU, converting the relative abundance in the 16S rRNA gene libraries into quantitative values.

3 Results

3.1 Temperature, sulfate and DOC depth-profiles

DOC concentrations were highest at the lake surface with concentrations of nearly 5 mg/L and decreased to values of around 3 mg/L at the lake bottom (Fig. 1A). The lake water surface temperature was 18°C and decreased to 5°C at a water depth of around 12m (Fig.1A). As a result, summer warming resulted in a stratification of lake Fohnsee with the development of an anoxic hypolimnion between 12 m and 22 m from around May to September with a constant temperature of 5°C.

Sulfate concentrations were 0.1 mmol/L in the epilimnion and remained unchanged within the analytical uncertainty in the anoxic hypolimnion (Fig. 1A). Sulfate concentrations only decreased from a mean value of around 0.1 mmol/L to 0.07 mmol/L very close to the water/ lake- sediment interface.

3.2 Depth-profiles of O_2, NO_x^-, NH_4^+, and stable water and nitrogen isotopes

Aerobic conditions were prevalent within the epilimnion with a steep oxygen concentration gradient from > 0.28 mmol/L at the surface towards < 0.625 µmol/L below 12 m (Fig. 1B). The average concentration of nitrate in the epilimnion was 0.12 mmol/L (Fig. 1B). Below 12 m, in the nitrate-methane transition zone (NMTZ), dissolved oxygen concentrations decreased below detection (< 0.625 µmol/L) and at a water depth of 21 m nitrate concentrations decreased to < 0.008 mmol/L, while nitrite concentrations peaked at 0.02 mmol/L at a water depth of 20 m. Ammonium concentrations decreased from around 0.06 mmol/L at the lake bottom to the oxycline and were below detection (< 0.005 mmol/l) above a water depth of 12 m (Fig. 1B). **Figure 1A and 1 B**

$\delta^{15}N$ and $\delta^{18}O$ values of dissolved nitrate increased in the anoxic water column (O_2 concentration < 0. 0.625 µmol/L at a water depth below 12 m) of the lake from 6.7‰ to 45.4‰ for $\delta^{15}N$ and from around 1.7‰ to 21.5‰ for $\delta^{18}O$ (Fig. 2C). Simultaneously, $\delta^{15}N$ of nitrite increased from 0.1‰ to 25.9‰ concurrently with increasing $\delta^{15}N$ values of nitrate, while $\delta^{18}O$ values of nitrite remained quite constant over the water depth (Fig. 2C). The $\delta^{15}N$ values of ammonium increased from 7.9‰ at the lake bottom to 11.6‰ near the NMTZ, while simultaneously decreasing ammonium concentrations from 0.060 mmol/L to 0.045 mmol/L were observed (Fig. 2). The oxygen isotope ratios of lake water varied between -10.4 and -9.5‰ for $\delta^{18}O$. $\delta^{2}H$ values were -73.0‰. In the aquifer the $\delta^{18}O$ value was very close to -10‰ (Fig. 1B) supporting earlier findings that lake water is mainly derived from groundwater (Braig et al., 2010). **Figure 2A to C**

3.3 Depth-profile of methane concentrations and C isotope ratios

Concentrations of dissolved methane were highest in the methane zone (from 22 m to 20 m) near the lake bottom with concentrations of 0.16 mmol/L but decreased to concentrations below the detection limit towards the NMTZ (from 20 m to 12 m). With decreasing methane concentrations, $\delta^{13}C_{CH4}$ values increased from -72 ‰ at the lake bottom to -39 ‰ at a water depth of 18 m in the NMTZ (Fig.2B). Above a water depth of 18 m, methane concentrations were too low for stable isotope analyses. The steepest counter-gradients of nitrate and methane concentrations were observed at a water depth between 18 and 21 m (Figs. 2A and 2B), exactly, where nitrite concentrations peaked (Fig. 2A).

3.4 Microbial community distribution in the water column of Lake Fohnsee

To identify the microbial taxa potentially responsible for mediating the the N and C cycling processes identified in the chemical and stable isotope profiles, we performed high-throughput Illumina sequencing of the V4 hypervariable region of the 16S rRNA genes together with quantitative PCR (qPCR) at selected depths throughout the water column corresponding to the distinct geochemical zones identified in the vertical chemical profiles. Analysis of similarity (ANOSIM) performed on the data revealed that significantly (R: 0.57, $P = 0.002$) different microbial communities inhabited four geochemical zones in the water column, the oxic lake water (6 m), the upper NMTZ (12-14 m), the lower NMTZ (16-18 m), and a methane rich zone near the lake bottom, where nitrate and nitrite concentrations decreased towards to the detection limit (20-22 m) (Figs. 2A and 3). The differences in the communities are attributed to a decrease in the *Verrucomicrobia* and *Actinobacteria* with depth, and a large increase in the relative abundance of *Gammaproteobacteria* at a water depth of 22 m (Fig. 3B). While present at a lower relative abundance, *Epsilonproteobacteria*, *Deltaproteobacteria*, and *Bacteroidetes* also increased with increasing depth below the oxycline (Fig 3B).

The relative abundance of populations (operational taxonomic units sharing 97% sequence identity) was converted into quantitative terms by multiplying the fractional (relative) abundance of the populations against the total number of 16S rRNA gene copies per sample determine by qPCR. This revealed a peak in microbial abundance just below the oxic-anoxic transition zone between 12 and 14 m, as well as the presence of known operational taxonomic units (OTUs) affiliated with anaerobic methane oxidizers (*Crenothrix*, NC10) and an OTU affiliated with the anammox bacterium *Candidatus* 'Anammoximicrobium' (Fig. 3C). The methane oxidizing *Crenothrix* and NC10 OTUs showed peak abundance below the oxic – anoxic transition zone at 12-14 m, whereas the anammox bacteria *Candidatus* 'Anammoximicrobium' showed peak abundance in this zone and in the deeper water zone between. 20 and 21m (Fig. 3C).

Figure 3

4 Discussion

4.1 Evidence of AOM coupled with denitrification in the nitrate-methane transition zone (NMTZ)

To test the hypothesis whether methane diffusion from the lake sediments towards the oxycline (as opposed to microbially mediated AOM with nitrate reduction) can describe the observed depth profiles of methane in the water column, a simple 1D diffusion model with a constant methane input (C_0= 0.16 mmol/L) was applied (Fig. 4). The results indicated that diffusion processes alone are insufficient for explaining the non-linear decrease of methane concentrations in the water column. Therefore, a model run that considers methane diffusion combined with degradation was performed. Results showed that a k-value of 0.03 [day^{-1}] for methane oxidation in the hypolimnion represents a good fit between observed and modelled methane concentrations (Fig. 4). Interesting, the results are in agreement with the results of Roland et al. (2017) for microbially mediated AOM with nitrate (k~ 0.07 [day^{-1}]) from a temperate lake during the summer period, whereas aerobic methane oxidation rate

constants where generally about a factor of 10 higher. 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).

Figure 4

The vertical distribution of electron acceptors in the water column of lake Fohnsee was in agreement with the expected order of decreasing free-energy yields (Appelo and Postma, 2005). Nitrate concentrations decreased in the water column at a depth below 12 m, where model results suggest that dissolved O_2 was available at most in trace amounts (Fig. 1B). Sulfate concentrations of around 0.1 mmol/L remained unchanged throughout the water column in the presence of nitrate. Near the water – sediment interface sulfate concentrations decreased slightly (Fig.1A). Decreasing sulfate concentrations at the bottom of the lake and nitrate concentrations at the same water depth of less than 0.015 mmol/L can be thermodynamically explained by partial bacterial sulfate reduction at low sulfate concentrations in lake sediments (Vuillemin et al., 2018), in micro-environments of particles near the lake sediment surface (Bianchi et al. 2028), or by mixing effects between sulfate-free water from the sediments, where methanogenesis may occur, and sulfate-containing lake water.

Decreasing nitrate concentrations in the water column indicates microbial nitrate reduction in the anoxic water column of the lake coupled with the oxidation of DOC (heterotrophic denitrification) or methane (n-damo) that are both present in Lake Fohnsee water (Figs. 1A and 2B). Stable isotope data were used to test the hypothesis whether denitrification occurred in zones where methane concentrations decreased. Methane is formed by methanogenesis in the sediments (Conrad et al., 2007; Norði et al., 2013) and diffuses upwards toward the oxycline. The $\delta^{13}C$ value of -71.6‰ for dissolved methane at the bottom of lake Fohnsee (Fig. 2B) indicates a biogenic source (Norði et al., 2013; Rudd and Hamilton, 1978). In absence of dissolved oxygen (< 0.625 µmol/L), methane concentrations decreased and $\delta^{13}C$ values of methane increased to values of -38.6‰ toward the oxycline (Fig. 2B), providing evidence for AOM. At this depth interval, nitrate concentrations also decreased and $\delta^{15}N$ and $\delta^{18}O$ values of nitrate increased from around 5‰ to 45‰ and from around 1‰ to 22‰ (Fig. 2C), respectively, while nitrite concentrations peaked (Fig. 2A). This provides clear evidence that denitrification was occurring in the water column, and the chemical and isotopic data demonstrate that some of the denitrification was coupled with microbial AOM (n-damo) in the NMTZ between a water depth of 16 and 20 m. However, on the basis of our isotope data we cannot exclude that denitrification is coupled to the common anaerobic hetertorophic nitrate reduction, and methane oxidation is also affected by trace amounts of oxygen in suboxic waters, as shown by Blees et al. (2014) for lake Lugano.

4.2 Evidence of anammox at the bottom of the NMTZ

Several lines of qualitative and quantitative evidence indicate the co-occurrence of anammox, denitrification, and AOM towards the bottom of the NMTZ. As expected the nitrite concentration at a water depth of 20 m was highest where nitrate reduction occurred (Fig. 2A). Between this depth and the lake bottom, our data strongly suggest that *anammox* is the main sink of NH_4^+. Ammonium occurs in concentrations of up to 0.06 mmol/L at the bottom of the water column at 22m, likely stemming from the heterotrophic degradation of organic nitrogen (e.g., proteins and amino acids) close to the sediment – water interface, and is subsequently transported from the methane zone near the lake sediments into the overlying water column (Norði et al., 2013; Wenk et al., 2014), where the NH_4^+ concentration decreases continually towards < 0.005 mmol/L at 12 m depth. The decrease in ammonium concentration with decreasing water depth is accompanied by an enrichment of ^{15}N in the remaining ammonium shifting the $\delta^{15}N_{NH4}$ 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.

To explain the moderate isotopic shift of 4 ‰ in $\delta^{15}N$ of ammonium, Wunderlich et al. (2018) suggested a transport limitation model, where small nitrogen isotope fractionation during denitrification can be explained. Briefly, a partial transport limitation of nitrate into the cell in relation to nitrate reduction would shift the apparent kinetic isotope effect during denitrification towards a value of unity. Similar processes could be assumed for ammonium oxidation during anammox at lake Fohnsee. As dissolved ammonium concentrations are very low and probably diffusion controlled, ammonium uptake may represent the rate limiting step and nitrogen isotope fractionation may be low compared to values observed by Brunner et al. (2013) in laboratory studies where equilibrium conditions can be assumed. Wenk et al. (2013) also found a small isotopic shift in nitrogen of around 8 ‰ for anammox at Lake Lugano when almost all ammonium was oxidized and suggested a similar isotope model for the observed low nitrogen isotope fractionation during anammox.

The $\delta^{18}O$ 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 $\delta^{18}O$ values of nitrite was estableshed in equilibrium with lake water with a $\delta^{18}O$ of -10‰. However, Sebilio et al. (2019) found that the $\delta^{18}O$ values of nitrite are lower (< +4‰), 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.

Above a water depth of 20 m there is no isotopic evidence that ammonium is oxidized under anaerobic conditions and the decrease of ammonium concentrations may be affected by diffusion and by ammonification and nitrification processes that may occur at the oxycline. We observed a difference of $\delta^{15}N$ values ($\Delta\ \delta^{15}N$) 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 in the water column, as the numerically dominant bacteria found throughout the water column were the Gammaproteobacteria many of which are facultative anaerobes that perform heterotrophic nitrate reduction. When new nitrate is formed as metabolic product by nitrite oxidation during anammox, the $\delta^{15}N$ value of the newly formed nitrate is affected by an inverse isotope effect (preferential removal of ^{15}N from the nitrite pool during oxidation to nitrate) resulting in nitrate that is strongly enriched in

^{15}N (Brunner et al., 2013). In this study the difference between $\delta^{15}N$ values of nitrate and nitrite ($\Delta\delta^{15}N$) increased from 11‰ in NMTZ to > 26‰ at the water depth of 20 m, where $\delta^{15}N$ values of ammonium increased while NH_4^+ concentrations decreased (Figs. 2A and C). 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 ($\Delta\delta^{15}N$ of +31‰). The reason for the observed small isotopic differences between nitrite and nitrate ($\Delta\delta^{15}N$) during the *anammox* process within in this study ($\Delta\delta^{15}N$ of +26‰) compared to the results ($\Delta\delta^{15}N$ of +31‰) found in a laboratory experiment (Brunner et al. 2013) could be the result of different anammox strains in lake water and the microcosm-experiment, limiting environmental concentrations of nitrite, or that the suggested inverse isotope effect by anammox was superimposed on "normal" isotope effects during denitrification in the lake water at a water depth of 20 m.

Furthermore, the deviation of the slope of $\delta^{18}O$ versus $\delta^{15}N$ values on a dual isotope plot (2D plot) for nitrate from the expected value of 1 for microbial denitrification (Knöller et al., 2011; Wunderlich et al., 2012) can be used to identify anammox. Granger and Wankel (2016) used a modelling approach linked with pH-dependent isotope exchange reactions between water-oxygen and nitrite-oxygen (Buchwald and Casciotti, 2010; Casciotti et al., 2007; Casciotti et al., 2010) 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. During anammox, when nitrite is reduced with ammonium as electron donor and nitrate is produced, one oxygen atom from water having a $\delta^{18}O$ 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 $\delta^{18}O$ values of nitrate remaining low, while $\delta^{15}N$ of the remaining nitrate is affected by an inverse nitrogen isotope effect and values continue to increase. The $\delta^{18}O$ vs. $\delta^{15}N$ plot for nitrate samples from depths between 20 and 22 m in our study displays a slope of 0.5, while the slope was 0.8 in the NMTZ between 20 and 12 m, much closer to the typical trajectory for denitrification of ~1 obtained under laboratory experiments (Fig. 5). The much slower slope of 0.5 on the $\delta^{18}O$ vs. $\delta^{15}N$ plot for nitrate is an additional line of evidence that strongly suggests that anammox occurred at the bottom of the NMTZ between 20 m and 21 m.

4.3 Crenothrix, NC10, annamox, and heterotrophic bacteria in the water column of lake Fohnsee

We identified gamma-proteobacterial methane oxidizing bacteria related to *Crenothrix* that reach their peak abundance particularly in the NMTZ of the water column of the lake (between 12-20m). The abundance of *Crenothrix* rRNA gene copies reaches up to 10^5 (Fig. 3B), which is 2-3 orders of magnitude higher biomass reported for *Crenotrhix* in the Swiss alpine Lake Rotsee and Lake Zug (Kits et al., 2015; Oswald et al., 2017), where they may act as denitrifying methanotrophs that also have the capability for aerobic metabolism. The facultative metabolism of *Crenothrix* likely allows them to adapt to changing environmental conditions, supporting any nitrate reducing ANME-2d (with lower doubling times) in the

denitrification zone of stratified lakes (Deutzmann et al., 2014). We did not detect any representatives of the ANME-2d in our 16S dataset – despite relatively deep sequencing depth (>150 000 reads per sample), indicating that if they were in the lake water, they were at abundances below our detection limit. ANME-2d may, therefore, be major contributors to AOM in bioreactor studies (Haroon et al., 2013; Shen and Hu, 2012) and sediments, but not in the water column of this lake.

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 linked with denitrification exists, may suggest that this organism was also partially contributing to the anaerobic oxidation of methane with nitrite (*n-damo*). However, 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 a portion of the water column. 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.

Within the anoxic regions of the water column (NMTZ and methane zone), the OTU affiliated with '*Candidatus* Anammoximicrobium' is ubiquitous (Fig. 3B) and its lack of detection in the oxic zone indicates that it is a strict anaerobe. '*Candidatus* Anammoximicrobium' is an aggregate forming bacterium corresponding to a new genus within the Planctomycetes that is capable of anaerobically oxidizing ammonium with nitrite, and has been previously found to carry out anammox in a wastewater bioreactor (Khramenkov et al., 2013). The '*Candidatus* Anammoximicrobium' and NC10 bacteria both utilize nitrite as a terminal electron acceptor, and they co-occur at depth of 20 m, where highest nitrite concentrations were observed (Fig. 2B). While activity indicators such as transcriptomes or NanoSIMS are needed to prove the anammox activity of *Candidatus* Anammoximicrobium' in our samples, the stable isotope and geochemical profiles indicate that this OTU is present in a geochemical setting where anammox may take place. This, together with its affiliation to '*Candidatus Anammoximicrobium*', indicates that this OTU has the potential to perform anammox in the aquatic environment of Lake Fohnsee at a depth of 20 m. In the water depth where nitrite was available due to denitrification via anaerobic methane oxidation, both anammox and NC10 bacteria could compete for the same available nitrite as speculated for *Crenothrix* and NC10 bacteria in the NMTZ as shown in Fig. 6.

As heterotrophic denitrification is a common process in freshwater ecosystems that have abundant organic matter, it is likely that heterotrophy was also responsible for some of the observed consumption of nitrate. 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 heterotrophic nitrate and nitrite reducers. However, microbes belonging to the Gammaproteobacteria class are very abundant in our samples, and in addition to the methane-oxidizing genus *Crenothrix*, is well known to consist of many species that are capable of heterotrophic nitrate and nitrite reduction, a trait that is widespread throughout the Gammaproteobacteria class. The relative abundance of Gammaproteobacteria increases with depth into the anoxic zone (Fig. 3b), and in addition to methane-oxidizing *Crenothrix*, consisted of many heterotrophic

Gammaproteobacteria groups including the genera *Pseudomonas*, *Acidovorax*, *Alteromonas*. Thus, some of the other Gammaproteobacteria that gradually accumulated in deeper waters of the lake (Fig. 3b) are heterotrophs that, in addition to *Crenothrix*, may have performing nitrate and nitrite reduction, and denitrification.

The detected microorganisms at Lake Fohnsee were found to be ecologically important in driving the C and N cycles of other stratified lakes and freshwater reservoirs (Deutzmann et al., 2014; Naqvi et al., 2018; Oswald et al., 2017). This makes it highly likely that these microbial groups are also potentially responsible for the removal of nitrogen and methane at Lake Fohnsee.

Figure 6

5 Conclusion

While aerobic methane oxidation in lake water has been known to occur for over a century, knowledge on anammox and AOM coupled with denitrification in natural anoxic environments within stratified lakes is scarce. Our field study results show that AOM, denitrification and anammox may co-occur in the anoxic water column of stratified Lake Fohnsee. This provides a natural environment context from a seasonally stratified lake, that supports previous bioreactor studies that showed a coupling of *n-damo* and anammox under more controlled conditions (Haroon et al., 2013). The linkage of the N and C cycles that we have observed in the stratified waters of Lake Fohnsee could be an important process in stratified lakes contributing to the removal of nitrogen and methane from freshwater ecosystems.

Data availability

Illumina sequencing data for community analyses are deposited at NCBI BioSample (www.ncbi.nlm.nih.gov/biosample) under accession number PRJNA541816.

Author contribution

FE has designed the study, AW has performed the field work and the measurements of stable water isotopes. Instrumentation and methodology were provided by MS for N isotopes and BM for CH_4, FE has performed the modelling study, ÖC and WO have performed the qPCR, whereas WO has interpreted the data, FE, AW, BM, MS and WO have discussed the results, and FE wrote the original manuscript supported by BM and WO, whereas AW has visualized the isotope and water chemistry data.

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Conflict of Interest

The authors declare no conflict of interest.

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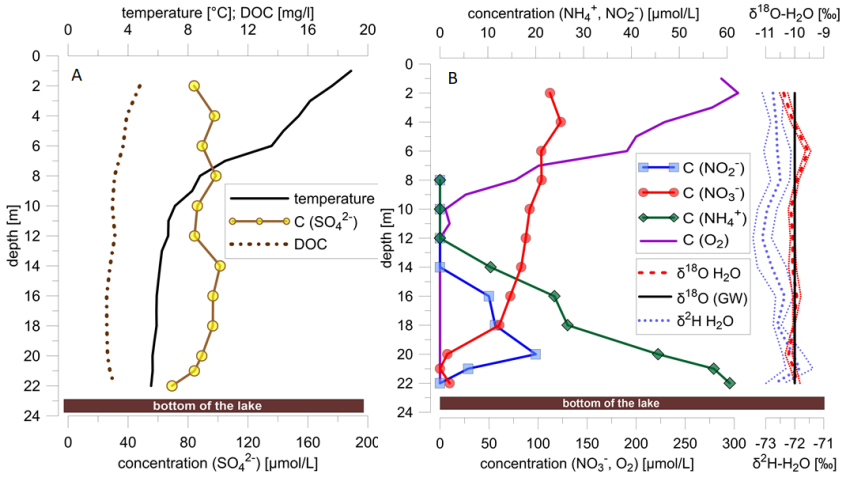

Figure 1A and B. Temperature profile and vertical distribution of concentrations of DOC, sulfate (left) and (B) dissolved nitrate, nitrite, ammonium, and dissolved oxygen, δ^2H and $\delta^{18}O$ values of lake water and $\delta^{18}O$ value of groundwater (GW) (right).

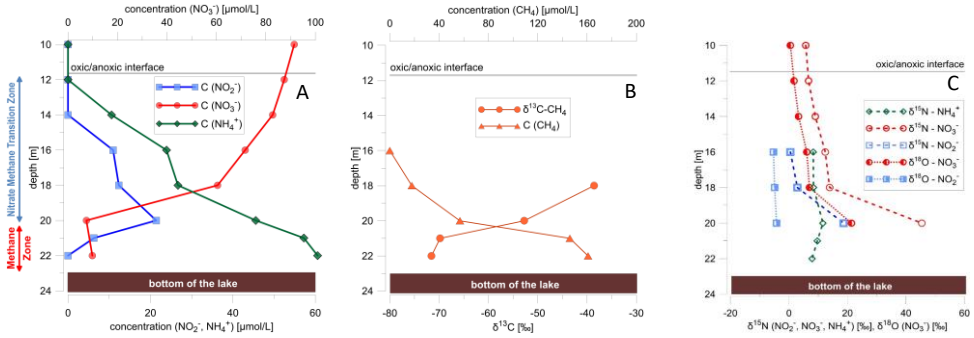

Figure 2A-C. Water column profiles below the oxycline (10 to 22 m) for methane, nitrate, nitrite and ammonium concentrations and (B) stable isotope data and concentration profile of methane ($\delta^{13}C$), and (C) nitrate ($\delta^{15}N$, $\delta^{18}O$), nitrite ($\delta^{15}N$, $\delta^{18}O$), and ammonium ($\delta^{15}N_{NH4}$) isotopes.

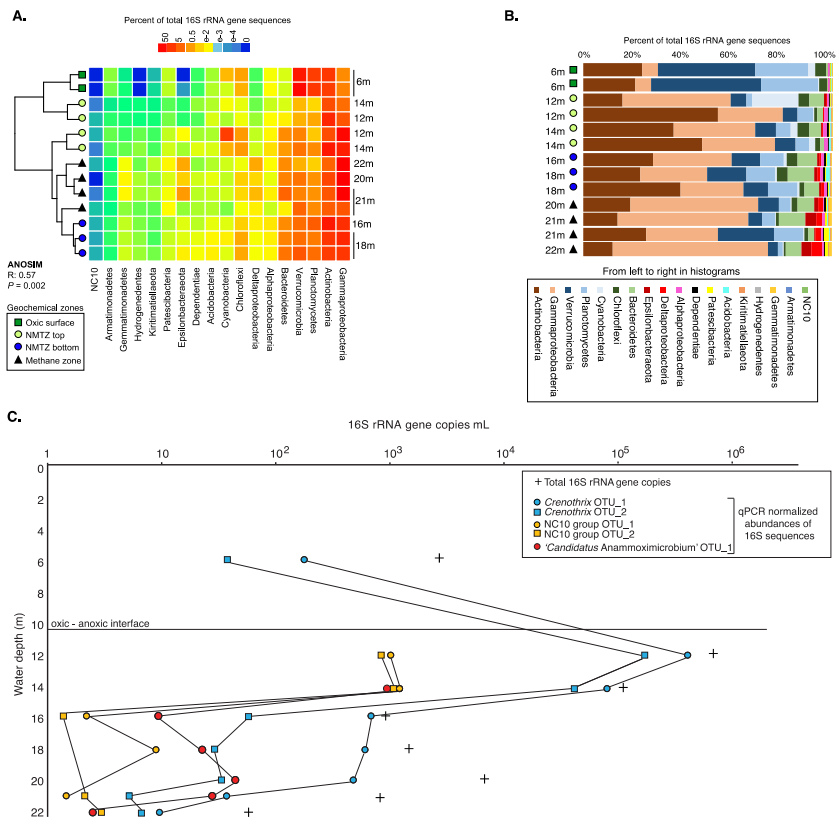

Figure 3A-C. Analysis of 16S rRNA gene data from microbial communities in the stratified lake. (A) Heatmap showing the relative abundance of specific groups in the 16S rRNA gene sequencing data, and corresponding heirarchical clustering analysis (analysis of similarity (ANOSIM) P value = 0.002) of four geochemically defined zones. For those depths where replicates were obtained, the data for both replicates are shown. (B) The relative abundance of 16S rRNA gene sequences affiliated with the major groups across the stratified water column. (C) Abundance of 16S rRNA gene copies determined via qPCR, and the qPCR normalized absolute abundances of 16S rRNA gene sequence relative abundances from key populations (OTUs) potentially involved in AOM and anammox, specifically those affiliated with Crenothrix, NC10, and potential anammox bacteria.

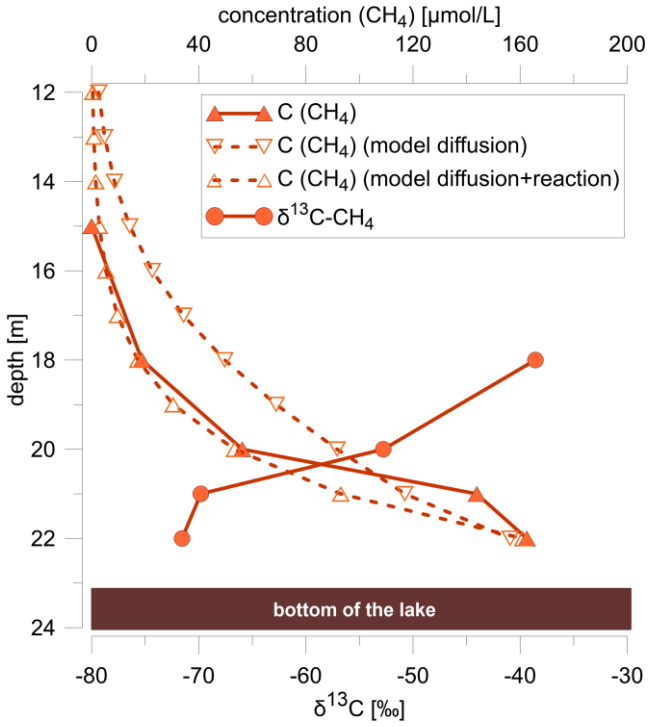

Figure 4. Depth-profiles of methane concentration (filled triangles) and its isotopic composition (filled circles) within the water column, modelled methane concentrations (open triangles) using a 1D-diffusion model with a turbulent diffusion coefficient for K_{zCH4} of 0.1 m^2 day^{-1} (model diffusion), and a 1D diffusion model additionally linked with a degradation term (first order rate constant k= 0.03 d^{-1} (model diffusion and reaction).

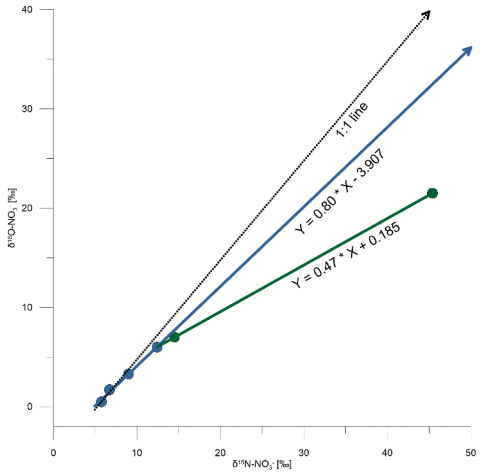

Figure 5. $\delta^{18}O$ versus $\delta^{15}N$ plot of nitrate with the typical trajectory of 1 for denitrification obtained under laboratory conditions (black line), calculated trajectory of 0.8 for the *n-damo* zone (20m and above, blue line) and around 0.5 for the *anammox* zone (20-22m, green line).

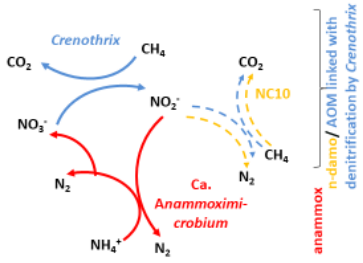

Figure 6. Conceptual model of the coupled N and C cycles in the anoxic water column of Lake Fohnsee