Interactive comment on

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

We like to express our 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 amount of the limitations of the presented data (with very few exceptions where a slight over-reach of data interpretation can be identified). In contrast, Reviewer #2 asked for a more cautious interpretation of the data.

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 on line 392 the limitation of isotope and microbial community data when activity indicators, for example derived from NanoSIMS analyses, are missing (for example: L338-339: … where *AOM* may affect microbial nitrate reduction, although more canonical heterotrophy could also occur). 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 laboratory are not always transferrable to field sites, and hence are a useful complement but not a replacement of field studies. Similar, 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. In addition, we have two native speakers as co-authors who have carefully edited the revised manuscript, 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 build up 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 and must be verified with a numerical model 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, as now stated in the revised manuscript.

Reviewer #2 also commented on the use of Kz from literature data. This issue is addressed in detailed 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 (Oswald, 2015). 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 to 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.

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 our study site. 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 potential of this process to reduce nitrate loading in aquatic environments. These previous publications suggested that this newly discovered process (AOM with nitrate linked with anammox) could have environmental relevance. In this regard, we believe our study and new data provide a valuable contribution to the literature on this topic, while our wording with "has the potential" is very cautious in this context.

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

Comments of Reviewer #2

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

Main points:

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

Response:

While reviewer #2 states that "it does not happen; we know that", no references were provided that would conclusively document that this process is not occurring at our study site. 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 potential of this process to reduce nitrate loading in aquatic environments. These previous publications suggested that this newly discovered process (AOM with nitrate linked with anammox) could have environmental relevance. In this regard, we believe our study and new data provide a valuable contribution to the literature on this topic, while our wording with "has the potential" is very cautious in this context.

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 18: Here, we report vertical concentration profiles and corresponding stable isotope compositions of CH_4, NO_3^-, NO_2^- and NH_4^+ in the water column of Lake Fohnsee, a stratified lake located in southern Germany, which…

L56: Why is nitrate reduction more important in lakes than nitrite reduction, just because there is more nitrate than nitrite? Nitrite is an intermediate and assuming that the most important N loss pathway is complete denitrification, nitrite reduction has to balance nitrate reduction, if nitrite does not accumulate.

Response: In case that there are no alternative electron donors such as DOC or Fe(II) then nitrite will not be formed and one can assume that nitrate reduction with AOM may be more important.

For clarification we change this sentence and added to L 56: If there are no alternative electron donors such as DOC or Fe(II) available microbial nitrate reduction with AOM may be more relevant in aquatic environments than canonical heterotrophy.

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

Model: There is not enough explanation of the model. Obviously, it is not a realreaction-diffusion model, but it also is not just a diffusion model, right? What are thereaction parameters, how are they set? I am not an expert in modelling, but it remainsunclear 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 estimatedbased on fitting of the model to the observational data.

Response: We have re-written this part of the Method section and have already given our view to his 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 literature are not appropriate for this case study.

In the revised text we will use the 1D diffusion model with a degradation term to find some evidence that the observed concentration profile of methane cannot only explained by diffusion. In addition, we have fitted the theoretical methane concentration curve (modelled with diffusion only) to the field data using the rate constant k and compared this value with literature data.

New: Methods

For the 1-D diffusion model, a semi-infinite system was assumed where the lower boundary (at x = 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) that 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:

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

where C [mg/L] is the methane concentration in the water column as a function of distance (depth) x and time, C_0 [mg/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), K_z [m^2 day^{-1}] and 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 Kz = 1.2*10-6 m2/s, corresponding to 0.1 m2/day, was calculated for Lake Fohnsee according to Wenk et al. (2013) and Bless et al. (2014). This value is at the lower range typically applied for methane flux calculations and modeling (0.1-2.1 m2/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 microbial 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):

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

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 Blees et al. (2014) and Roland et al. (2016). 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):

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

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:
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 is 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 in Sebilo et al., 2019 Scientific Reports.
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.

L188: How was complete outgassing of CH4 assured before headspace analysis ?Was brine/NaOH added? Concentrations were calculated based on Henry's Law, but what about the d13C? Is there an isotope shift between CH4 in the headspace and the CH4 dissolved? If so, was that considered?

Response:

- 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 □13C 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 3L of lake water for each depth (water samples for IC, isotopes, DOC and microbiology) and we wanted to exclude mixing between the 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. In addition, it will make sense as suggested by Reviewer #2 to incorporate the isotopes of methane in the modelling part, but this was out of scope for this paper.

Now in section 4.1.

To test the hypothesis if methane diffusion from the lake sediments towards the oxycline can explain the methane concentration profile in the NMTZ, a simple 1D diffusion model with a constant methane input (C_0= 2.6 mg/L) was used (Fig. 4).

Our results provide some evidence that diffusion controlled methane fluxes from the sediment surface to the oxycline are highly insufficient for explaining the non-linear decrease of methane concentrations in the water column (Fig. 4). To test the hypothesis whether AOM controls denitrification, a model run with a 1D diffusion model linked with a first-order degradation rate constant, that was used as fitting parameter (Eq. 3), was performed. Results suggest that a k value of 0.09 [d-1] fits reasonable well the observed depth profile of methane. This k-value is in excellent agreement with the results of Roland et al. (2016) for AOM with nitrate from a temperate lake and may support our hypothesis that AOM with nitrate affects the observed concentration profiles of methane and nitrate. However, micro-aerobic methane oxidation can also play an important role in the water column of this lake.

Can the authors explain why a 90% decrease in ammonium is associated with aï Ad'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 essentiall 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 fractionation in ammonium. We have observed a decrease of ammonium 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 concentration decreases from 0.8 mg to below detection at a water depth of 12 m, probably by diffusion/ and maybe by diffusion controlled aerobic NH4-oxidation etc. as outlined in our manuscript (L 335) and, therefore, no significant isotope fractionation is expected and was observed.

In addition, Wunderlich et al. (GCA 2018) has suggested a transport limitation model, where such small stable isotope fractionation of 4‰ can be explained and also Wenk et al. (2013, Limnol. & Oceagr.) found small stable isotope enrichment of 8 ‰ at Lake Lugano for anammox (here NH_4-concentrations decreased below detection). We will add both references to the revised manuscript and will also give an explanation based on the paper of Wunderlich et al. (2018). To use the expected form of the concentration line when degradation occurs, as suggested by the reviewer is risky because of the low number of data points.

New text § 4.2:

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. 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 1 mg/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 < 0.15 mg/L at 12 m depth. The decrease in ammonium concentration with increasing water depth from around 1 mg/L to 0.8 mg/L is accompanied by an enrichment of ^{15}N in the remaining ammonium shifting the $\delta^{15}N$-NH_4 values from 7.9‰ to 11.6‰ between 22 and 20 m water depth (Fig. 2C), suggesting that ammonium is oxidized anaerobically while enriching the remaining substrate in ^{15}N. Above this water depth there is no isotopic evidence that ammonium is oxidized under anaerobic conditions and the decrease of ammonium concentrations may be only affected by diffusion and by ammonification and nitrification processes that may occur at the oxycline. <u>To explain the moderate isotopic shift of 4 ‰ in $\delta^{15}N$-NH_4 of ammonium Wunderlich et al. (GCA, 2018) has suggested a transport limitation model, where such small isotope fractionation can be explained and also Wenk et al. (2013, Limnol. & Oceagr.) found a small stable isotope enrichment of around 8 ‰ at Lake Lugano for anammox when almost all ammonium was oxidized.</u>

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: We agree and will delete this reference.

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: 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 $d^{18}O$ vs. $d^{15}N$ plot for nitrate a slope lower than 1 is a powerful indicator for the occurrence of *anammox* in an anoxic environment). 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 reoxidize nitrite to nitrate and do not imply that anammox is the main N-loss pathway in all environments. As a result, we developed several lines of evidence to come to the conclusion that anammox may have occurred.

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."

Minor points:

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

References:

KULOYO, O., RUFF, S. E., CAHILL, A., CONNORS, L., ZORZ, J. K., HRABE DE ANGELIS, I., NIGHTINGALE, M., MAYER, B. & STROUS, M. (2020): Methane oxidation and methylotroph population dynamics in groundwater mesocosms. Environmental Microbiology, 22(4): 1222-1237 (early access February 2020; published April 2020). https://doi.org:10.1111/1462-2920.14929

SEBILO, M., ALOISI, G., LAVERMAN, A. M., MAYER, B., PERRIN, E., VAURY, V., MOTHET, A. & LAVERMAN, A. M. (2019): Controls on the isotopic composition of nitrite ($\delta^{15}N$ and $\delta^{18}O$) during denitrification in freshwater sediments. - Scientific Reports, 9: 19206. Doi.org/10.1038/s41598-019-54014-3; published December 16, 2019.

WUNDERLICH, A., HEIPIEPER, H.J., ELSNER, M. AND EINSIEDL, F. (2018) Solvent stress-induced changes in membrane fatty acid composition of denitrifying bacteria reduce the extent of nitrogen stable isotope fractionation during denitrification. Geochimica et Cosmochimica Acta 239, 275-283.