

# ***Interactive comment on “NO<sub>x</sub> reduction is the main pathway for benthic N<sub>2</sub>O production in a eutrophic, monomictic south-alpine lake” by C. V. Freymond et al.***

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AC: We thank the referees for their valuable comments, which will be very helpful when preparing an amended version of the submitted manuscript. Besides their request for some clarification, which we will address below, both reviewers acknowledge that the presented study covers an understudied ecosystem with regards to N<sub>2</sub>O emission dynamics, and agree in that the paper is well written. The reviewers came to the conclusion that our manuscript will be a valuable contribution to improve the understanding of N<sub>2</sub>O emissions from freshwater environments, and that the quality of our study merits publication in Biogeosciences. The most fundamental criticism was that the paper is

Full Screen / Esc

Printer-friendly Version

Interactive Discussion

Discussion Paper

quite descriptive and possibly biased by the methodological approach. In the revised version of the manuscript, we more critically discuss the potential shortcomings of the labelled-isotope approach used in this study, and we will try to add more quantitative components with regards to the discussion on the potential role of nitrifying organisms. The reviewers asked for clarifications on some specific points and suggested corrections, which may help to further improve the manuscript. Below, we address the editor's and reviewers' concerns point-by-point (AC: author comment; EC: editor comment; RC: reviewer comment).

#### Response to 'Editor review'

1. EC: It will be very useful for the readers if you write the reactions you are studying with the possible outcomes in relation to isotope labeling. For instance, if the substrate is  $^{15}\text{N-NH}_4^+$ , what are the options?, etc.

AC: We thank the editor for this comment and we will add a new figure, which will highlight the studied reactions (nitrification, denitrification, and nitrifier denitrification).

2. EC: Is one sampling site representative of the whole south basin? Is extrapolation of  $\text{N}_2\text{O}$  production to the whole area a sensible calculation? Is there background information on the dynamic of the whole basin?

AC: We agree that it is always problematic to spatially extrapolate from one sampling site only to the whole lake basin. Our focus was on studying the temporal dynamics of the benthic  $\text{N}_2\text{O}$  exchange, and a greater spatial coverage would have exceeded our analytical capacities in the lab (given the significant number of different treatments in replicates). The chosen site represents the deepest spot in the south basin and can likely be considered most representative for the studied system. Previous work on P-accumulation in the sediments of the south basin, however, highlighted the heterogeneity of the catchment geology (PhD Thesis M. Veronesi, 1999), which is partly reflected in the spatial changes of the mineral content (e.g., Fe minerals) of the sediments within the south basin, in turn the P content. With regards to other sediment

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characteristics (porosity, organic N, organic C) the different sites within the South Basin are comparable. In the revised version of the manuscript, we will cite the previous work on sediment heterogeneity, and we will clarify the risks that are associated with extrapolating the average annual N<sub>2</sub>O flux of one sampling site over the area of the entire south basin and over a time span of one year. We will stress that for a more robust quantitative extrapolation, a higher spatial and temporal resolution would be needed. Nevertheless, our estimations show that a considerable amount of N<sub>2</sub>O is produced in the sediments and can be accumulated in the bottom water. The uncertainty of this extrapolation will also be discussed in the conclusions of the manuscript.

3. EC: Since the concentration of added <sup>15</sup>N-NH<sub>4</sub><sup>+</sup> is 10-fold higher than ambient ammonium concentration, it is not straightforward to be claimed as “real rates” since they may be at saturating concentrations (in a Michaelis & Menten-type of kinetics). Are there literature data on half-saturation constants for these reactions? Regarding these fluxes across the treatments, the decrease in fluxes in the amended cores is peculiar. If anything, they should be similar. Could you provide an explanation for that? 4. I do not see the similarity in fluxes in experiments of unamended nitrate and ammonium compared to amended ones (Fig 3). For instance January 2011. Do you have an explanation?

AC: The variation in fluxes between treatments is about the same as for replicate measurements, and we cannot share Dr. Pantoja’s opinion that the NH<sub>4</sub> addition generally leads to a decrease in N<sub>2</sub>O flux. Ammonium concentrations were approximately doubled during anoxic conditions, and 10 μmol <sup>15</sup>N-NH<sub>4</sub><sup>+</sup> L<sup>-1</sup> was added when the ambient NH<sub>4</sub><sup>+</sup> concentration was <1 μmol L<sup>-1</sup>. This was the case in April 2010 and May 2011. In these months, however, the total N<sub>2</sub>O fluxes were quite comparable in <sup>15</sup>N-NH<sub>4</sub><sup>+</sup>-amended and in un-amended cores. From this we conclude, that the additional ammonium did not significantly influence the measured rates. But indeed, during other months the difference between un-amended and amended treatments seems more significant (although at relatively low levels, as in January 2011). We do not really have

[Full Screen / Esc](#)[Printer-friendly Version](#)[Interactive Discussion](#)[Discussion Paper](#)

an explanation for the decrease in N<sub>2</sub>O flux in the labeled experiments. The data suggest that the addition of either DIN substrate stimulates N<sub>2</sub>O reduction by denitrifying bacteria. We will include this aspect in the revised version of the manuscript.

5. EC: Page 9, line 8. Add “Fig. 3” to direct readers

AC: We will add the reference to the figure (it is now Fig. 5c)

6. EC: Page 9, line 17. Show numbers or delete the sentence. We cannot access Wenk in prep.

AC: Page 9, line 22. We now added the rates (‘maximum of >900 nmol L<sup>-1</sup>’).

7. EC: Abstract. It would be informative to see values comparing denitrification and ammonium oxidation in this section

AC: Ammonium oxidation and denitrification rates will be presented in Wenk, C.B. et al., a paper in revision for another journal, and we refer to this paper. Here, we want to focus on benthic N<sub>2</sub>O cycling. Based on our data, we can state that up to 15% of the total NO<sub>3</sub><sup>-</sup> that was reduced was measured as N<sub>2</sub>O in the overlying water. Since we were not able to detect N<sub>2</sub>O production from NH<sub>4</sub><sup>+</sup> oxidation, we are unable show numbers that relativize the denitrification rates to ammonium oxidation.

8. EC: Page 12, lines 22-26. The comparison is not very informative. Your fluxes are from sediment to water, soils fluxes are to the atmosphere. Which fraction of the bottom water N<sub>2</sub>O could reach the atmosphere? On the other hand, the total area of lakes and soil are very different therefore global fluxes of lakes could be small, especially if we consider only the eutrophic lakes. Are they?

AC: We will remove this part.

EC: Page 12, Line 10, 11. Redundant with line 8-9. Delete one of them

AC: We will correct the text as proposed.

Full Screen / Esc

Printer-friendly Version

Interactive Discussion

Discussion Paper



9. EC: No need to abbreviate south basin (SB). It makes reading more difficult, unless you live near Lake Lugano

AC: We will write “south basin” instead.

Response to ‘Anonymous Referee Nr. 1’

RC1: ...evaluate the contribution of the different evaluated rates such as nitrous oxide derived from nitrate labeled experiments with the total fluxes from unamended experiments? What is the actual contribution in percentage and thus the potential role of nitrification?

AC: N<sub>2</sub>O production from 15N-NH<sub>4</sub> remained below detection limit in all experiments. This would theoretically imply that the total N<sub>2</sub>O flux was produced solely by denitrification (or nitrifier denitrification; see below), leaving no space for N<sub>2</sub>O production by ammonium oxidation via the hydroxylamine pool. Obviously, there was always N<sub>2</sub>O mass 44 production from ambient substrates, which could in theory originate from the nitrification of ambient NH<sub>4</sub> from porewaters. Using the isotope pairing technique to differentiate between, and quantify, the different N<sub>2</sub>O production pathways, it is important to know what the ambient substrate concentrations (ammonium and nitrate) are. In the case of ammonium, the f ratio (ratio between ambient 14N-NH<sub>4</sub> and added 15N-NH<sub>4</sub>) is very difficult to determine, since 14N-NH<sub>4</sub> is continuously produced through OM remineralization. However, isotope pairing predicts that even in the case of stark dilution of the labeled ammonium with ambient ammonium from N remineralization within the sediments, a small contribution of NH<sub>4</sub> oxidation to N<sub>2</sub>O production would be indicated by a measurable efflux of 15N-N<sub>2</sub>O in the 15N-NH<sub>4</sub> label experiments. Yet, this was not observed, even during periods when bottom waters were still oxic. We will stress this point in the revised manuscript.

While we did not observe the incorporation of 15N from 15NH<sub>4</sub><sup>+</sup> into N<sub>2</sub>O, the relative yield of N<sub>2</sub>O produced through the ammonia oxidizing bacteria would increase substantially under conditions that stimulate their nitrifier denitrification pathway. As a

Full Screen / Esc

Printer-friendly Version

Interactive Discussion

Discussion Paper



result, as long as there is a source of  $\text{NO}_2^-$  (e.g. denitrification), nitrifier denitrification could be an important source of  $\text{N}_2\text{O}$  even if  $\text{O}_2$ -limitation has slowed the overall rate of ammonia oxidation (and therefore the rate of  $\text{N}_2\text{O}$  production via  $\text{NH}_2\text{OH}$  decomposition). Unfortunately, our experimental set up does not allow us to assess this possibility. But we discuss the potential  $^{15}\text{N}$ - $\text{N}_2\text{O}$  production by nitrifier denitrification in the manuscript.

RC1: The paper also lacks for a statistical support (like multivariable) in order to clearly state the potential relationship with oxygen or other variables such as nutrient concentration variability to the nitrous oxide fluxes observed. I consider that the authors should center the interpretations with the help of statistical approaches. Variable nitrous oxide production reaching significantly high magnitudes in certain periods probably linked also with the response of the microbial community to other factors such as those explored in the main study.

AC: Our main hypothesis, and one of the points we want to make in the paper, was that overall  $\text{N}_2\text{O}$  fluxes are enhanced during periods of anoxia in the bottom waters. The data set is relatively small, and the replicate measurements are not completely independent (two cores with the same inlet water reservoirs and time series). However, albeit the variability among replicate flux measurements during the respective sampling campaigns, the temporal flux variations between the seasons are significant, and we will add some statistical treatment of our data set in the revised manuscript. Temporal differences between rates and sampling time/conditions (anoxic during summer and fall versus oxic) were evaluated using a one-way analysis of variance (ANOVA) and additional variance tests (e.g., Wilcoxon) that may be more appropriate will be considered. The main conclusions we can draw already now from the statistical analysis is: 1.) At a significance level of  $P < 0.05$ , the  $\text{N}_2\text{O}$  fluxes varied significantly between samplings ( $p=0.0005$ ), with highest  $\text{N}_2\text{O}$  fluxes in October 2010. 2.) Similarly, the  $\text{N}_2\text{O}$  fluxes during clearly anerobic conditions were significantly higher than during the samplings when bottom waters were oxygenated ( $p=0.012$ ). January 2011 (when bottom waters

[Full Screen / Esc](#)[Printer-friendly Version](#)[Interactive Discussion](#)[Discussion Paper](#)

were just in transition from anoxia to oxic conditions) appears to be exceptional in this regard, with significantly smaller N<sub>2</sub>O production rates than during all other months. We are still lacking an obvious explanation for this observation, and we will speculate in the revised manuscript about possible mechanisms that keep the N<sub>2</sub>O fluxes out of the sediments at very low levels, or possibly even lead to a returned N<sub>2</sub>O flux (e.g., the stimulation of N<sub>2</sub>O reduction even under unsteady redox conditions). We agree that variations in N<sub>2</sub>O fluxes may be closely linked to microbial community changes (which may be closely coupled to changes in the redox conditions). Unfortunately, we did not study the microbial population dynamics, but we will include this aspect in the revised manuscript.

RC1: At this point the authors can't determine the contribution of nitrification using the experimental settings since many biases are associated with label ammonium determinations. Determine the main pathway which originates nitrous oxide in this environment is a challenge that the authors did not accomplish yet since the technique did not help to decipher the contribution of each pathway in this ecosystem, for example combine the experiments with nitrous oxide isotopomers to decipher the contribution of each pathway. Nitrification could be important in some periods sampled such as January when no significant NO<sub>x</sub> reduction was detected but still unlabeled nitrous oxide is produced in ammonium labeled experiments.

AC: See comments above. Indeed, quantifying N<sub>2</sub>O production by nitrification is difficult, due to the difficulties of determining the concentration/production of ambient ammonium. The reviewer is right that, in this regard, the ammonium-addition approach is problematic. The isotope approach allows us, however, to clearly identify a setting where N<sub>2</sub>O production by ammonium oxidation does not occur at all (i.e., the absence of <sup>15</sup>N<sub>2</sub>O production in <sup>15</sup>N-NH<sub>4</sub> addition experiments). We agree in that nitrification can be important when no significant nitrate flux into the sediments is detected (balancing nitrate consumption by denitrification). But our experiments demonstrate that even if nitrification occurs, it does not necessarily lead to the production and accumu-

[Full Screen / Esc](#)

[Printer-friendly Version](#)

[Interactive Discussion](#)

[Discussion Paper](#)

lation of N<sub>2</sub>O. If unlabeled N<sub>2</sub>O is produced in 15N-NH<sub>4</sub> label experiments, it most likely originates from denitrification or nitrifier denitrification. Unfortunately, N<sub>2</sub>O isotopomers were not measured during flow-through incubations. We measured however the isotopomeric composition in the water column (Wenk, C.B. et al., in prep.). During the sampling campaigns when N<sub>2</sub>O isotopes and isotopomers were analyzed, the redoxcline about 10 m above the sediment was the main site of N<sub>2</sub>O production, with decreasing water column N<sub>2</sub>O concentrations towards the sediments, indicating N<sub>2</sub>O consumption by denitrifying bacteria in the deep suboxic/anoxic water column. Given that several N<sub>2</sub>O producing/consuming processes occur in close vicinity in the deep hypolimnion, a “clean” benthic N<sub>2</sub>O isotope signal could not be discerned. However, the observed N<sub>2</sub>O isotope patterns in the water column are not inconsistent with two different N<sub>2</sub>O sources, in the sediments and at the water column redoxcline, respectively, and N<sub>2</sub>O reduction and diffusive mixing in-between.

RC1: The authors consider dilution effects by remineralization of labeled ammonium as possible explanations of a lack in being able to evaluate nitrification source of nitrous oxide. I think that also a significant assimilation by the microbial community should also be a significant unaccounted sink of labeled ammonium. The authors should explore this possibility since in their experiments they determine higher 44-mass-nitrous oxide fluxes in ammonium labeled experiments compared with nitrate labeled ones in various months.

AC: It is correct that ammonium dilution would have an effect on the assessment of N<sub>2</sub>O production through the ammonium oxidation. It is hard to believe, however, that 15N-ammonium dilution with ambient ammonium would act to completely suppress any 15N-N<sub>2</sub>O production by ammonium oxidation, as this would imply that the 15N-ammonium pool is completely swamped by the ambient ammonium, or that the 15N labeled ammonium added to the inflowing water does not reach the reactive zone. The latter possibility can be excluded, because the diffusive distance to the potential nitrification zone is shorter than it is for 15N-labelled nitrate to the denitrification zone.

[Full Screen / Esc](#)[Printer-friendly Version](#)[Interactive Discussion](#)[Discussion Paper](#)



We will clarify this point in the revised manuscript. As for ammonium uptake, we fully agree with the reviewer. Unfortunately, we did not measure the organic matter after the incubations, to verify whether  $\text{NH}_4$  uptake into the benthic biomass occurred. In any case we thank the reviewer for this valuable comment, and we will consider  $^{15}\text{N}$ - $\text{NH}_4$  assimilation as possible N sink.

Specific comments:

RC1: Title: focus to the main findings. "Nitrous oxide variability in alpine... in a seasonal scale and the contribution of reduction pathways..."

AC: We consider the finding that  $\text{N}_2\text{O}$  is produced through nitrate reduction throughout the year, also under fully anoxic conditions that are thought to be conducive for complete denitrification to  $\text{N}_2$ , as the main finding. At the discretion of the Associate Editor, we could also live with an alternative title like: "Year-round  $\text{N}_2\text{O}$  production by benthic  $\text{NO}_x$  reduction in a monomictic south-alpine lake."

RC1: Sampling and Methods: How the oxygen was determined, detection limit of the technique? Was the oxygen evaluated all through the experiment as well?

AC: Yes, oxygen concentrations in the inlet water reservoirs and the outflow was measured daily with an optical sensor system (PreSens Dipping Probe). The limit of detection, as reported by the company, is  $0.5 \mu\text{M}$ . The analytical error at suboxic oxygen levels is  $0.2 \mu\text{M}$ .

RC1: Results: The rates results should also be shown more clearly like merged in table 1 as average with standard errors for the different cores.

AC: We added a table containing the average  $\text{N}_2\text{O}$  fluxes and corresponding standard errors (Table 2).

RC1: Percentages of contribution of the only pathway evaluated should be determined in order to have an idea of its variability and will allow the authors to discuss other potential contributors such as nitrification.

[Full Screen / Esc](#)[Printer-friendly Version](#)[Interactive Discussion](#)[Discussion Paper](#)

AC: See comment above.

RC1: Statistical analyses (for example canonical analyses or multidimensional) must be run considering the comparison with the other physical-chemical factors that influences the microbial community in this aquatic ecosystem.

AC: See comment above. Temporal differences between rates and sampling time/conditions (anoxic during summer and fall versus oxic) are now evaluated using a one-way analysis of variance (ANOVA). Our statistical efforts focus on the redox conditions, and let us conclude that the N<sub>2</sub>O fluxes change significantly between oxic and anoxic conditions in the water column. Other parameters the reviewer may have in mind are the concentration of DIN in the water column. The latter is not independent of the redox conditions in the water column (e.g., lower nitrate concentrations during anoxic conditions), but may potentially explain (directly or indirectly) observed N<sub>2</sub>O flux variations. We will address this aspect in the revised manuscript.

RC1: Discussion: The discussion will be enriched from the analyses suggested above and should be reformulated accordingly. Paragraph below line 25 under the first subtitle should be rephrased is confusing as it is written, your results vs the discussion.

AC: We will amend the text in the discussion according to the comments we provided above and following the suggestions made by Reviewer 1.

Response to 'Anonymous Referee Nr. 2'

1. RC2: ...although it lacks information on in-situ N<sub>2</sub>O measurements. These measurements could have given a direct estimate of N<sub>2</sub>O fluxes based on surface N<sub>2</sub>O measurements and in-situ N<sub>2</sub>O accumulation below the thermocline, which could have been an independent measure for the N<sub>2</sub>O emissions from the sediments to the water column.

AC: The reviewer is right. An independent assessment of N<sub>2</sub>O production through the investigation of temporal N<sub>2</sub>O concentration dynamics in the bottom waters below the

Full Screen / Esc

Printer-friendly Version

Interactive Discussion

Discussion Paper



thermocline and the analysis of N<sub>2</sub>O concentration profiles would be great and may have helped to verify the observed flux variation presented in this study. Unfortunately, water column N<sub>2</sub>O concentration data for the same sampling dates do not exist, so that a direct comparison was not possible. Also, from water column samplings during different campaigns we know that generally the thermocline is the hot spot of N<sub>2</sub>O production (with one exception in 2009). The water column N<sub>2</sub>O distribution is the result of multiple processes, including N<sub>2</sub>O production and N<sub>2</sub>O reduction in the water column, and N<sub>2</sub>O production within the sediments. Hence, comparing benthic fluxes with turbulent-diffusive fluxes in the water column would not be straight-forward. In this paper we wanted to focus on benthic N<sub>2</sub>O fluxes. In-situ water column N<sub>2</sub>O concentrations (as well as isotope and isotopomer compositions) were measured separately and will be presented elsewhere (Wenk C.B. et al.). As it stands, it seems that water column N<sub>2</sub>O production undergoes similar seasonal variability as the benthic N<sub>2</sub>O fluxes.

2. RC2: I wonder if the authors also measured the oxygen concentrations during their experiments to ensure that the whole sediment core and the overlying water column were anoxic over the time of the experiment.

AC: See comment above. Oxygen concentration in the inlet water reservoirs and the outflow was measured daily with an optical sensor system (PreSens Dipping Probe). Sometimes, we observed suboxic levels O<sub>2</sub> in the samples that were collected. However, we attribute the observed low-level O<sub>2</sub> concentrations to O<sub>2</sub> contamination during the sampling. True oxygen-free conditions in the inlet water reservoir of the anoxic incubations were confirmed by the fact that we detected 15N-NH<sub>4</sub><sup>+</sup> production in the 15N-NO<sub>3</sub><sup>-</sup> labeled inlet water reservoir. This is a strong indicator for active DNRA and thus anoxic conditions.

Specific comments:

RC2: Page 4974, line 11: define abbreviation when used for the first time.

AC: We removed the abbreviation 'SB' and write 'south basin' instead.

[Full Screen / Esc](#)[Printer-friendly Version](#)[Interactive Discussion](#)[Discussion Paper](#)

RC2: Page 4975, line 9: what was the storage temperature during the transport to the laboratory?

AC: The cores were stored upright and in the dark at ambient temperature without active cooling for max. 6 hours during transportation to the home laboratory. Here they were re-conditioned at near in-situ temperature for at least 24 hours.

RC2: Page 4976, lines 20-21: I am curious why N<sub>2</sub>O standards were prepared by reduction of nitrate to N<sub>2</sub>O? The usage of gaseous N<sub>2</sub>O standards is very common, and the preparation of liquid standards from these is quite straightforward. The method presented by Sigman et al. (2001) was developed for measurements of <sup>15</sup>N-Nitrate, not to measure N<sub>2</sub>O concentrations. I would therefore recommend that the authors provide some more information on the precision of the N<sub>2</sub>O standard concentrations, the standard error of the calibration curves and the overall precision of the N<sub>2</sub>O measurements.

AC: We could have used gaseous standards. However, our lab routinely handles dissolved-N standards and we have less routine in the preparation and handling of low-N N<sub>2</sub>O standards. The method presented by Sigman et al. 2001 is a well-established and precise method to determine  $\delta^{15}\text{N}$  and  $\delta^{18}\text{O}$  of NO<sub>3</sub><sup>-</sup> in water samples. In this method, NO<sub>3</sub><sup>-</sup> is converted to N<sub>2</sub>O by denitrifying bacteria lacking N<sub>2</sub>O reductase and N<sub>2</sub>O isotope composition is determined using an isotope ratio mass spectrometer. This method can be used to convert a known amount of NO<sub>3</sub><sup>-</sup> salt in solution to N<sub>2</sub>O, prior to the quantification (sum of masses 44, 45 and 46) in a purge-and-trap-IRMS system. In the revised version of the manuscript we will include an additional figure showing a calibration curve and the used transfer function.

Page 4978, lines 8-11: I would not use the term “increased” in this context as this implies a continuous rise of the N<sub>2</sub>O fluxes, which cannot be verified from two samplings in six months.

AC: We will change ‘increased’ to ‘changed’.

**BGD**

10, C5010–C5022, 2013

Interactive  
Comment

Full Screen / Esc

Printer-friendly Version

Interactive Discussion

Discussion Paper



Page 4978, lines 15-16: “heterogeneity of the sediments”: please elaborate on this term: what differences in the sediment cores could have an influence on N<sub>2</sub>O production?

AC: In the complex matrix of sediments, containing various organic and inorganic particles, micro-environments can form where redox conditions change on very small scale, for example from the surface to the center of a particle. We will add a short explanation in the revised manuscript.

Page 4982, line 15: please cite the original literature (Goreau et al., 1980, Löscher et al., 2012) instead of Bange et al. (2010).

AC: We thank the reviewer for the careful reading. We removed ‘Bange et al., 2010’ and cite the original literature of Goreau et al., 1980.

Page 4983, line 6: “Net N<sub>2</sub>O fluxes varied seasonally”: the presented data from this manuscript do not allow an assessment of a seasonal variability. To do so, a perennial study of the N<sub>2</sub>O fluxes with seasonal coverage would be necessary.

AC: We will write: “Net N<sub>2</sub>O fluxes displayed temporal variations and the proportion of NO<sub>3</sub>-reduced to N<sub>2</sub>O versus N<sub>2</sub> appeared to be enhanced by ongoing anoxia”

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Interactive comment on Biogeosciences Discuss., 10, 4969, 2013.

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10, C5010–C5022, 2013

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