Manuscript: bg-2020-20

Title: Oxygen and light determine the pathways of nitrate reduction in sediments of a highly saline lake

RESPONSE TO REFEREE #2 (Oksana Coban)

We would like to thank the reviewer for her valuable comments, which we believe have helped us a lot to improve the manuscript in general, and also some aspects of the discussion. In general, we have restructured the manuscript, rewritten some parts and revised the figures and tables included, moving some of them to supplementary material. In addition, Judith Prommer was added in the co-author list due to her significant contribution in explaining the role of nitrification in the discussion section. Our responses are shown below the reviewer's comments in blue.

Major comments:

Line 26: 'N2O-denitrification' is unclear term. Is it incomplete denitrification to N2O or the last step of denitrification (N2O reduction)? Although you explain it later in the introduction, it should be clear in the abstract itself.

Thank you for the comment. This term is now explained in the abstract by changing "N_2O-denitrification" to "denitrification to N_2O", what we consider adequately explains which step of denitrification is involved without unnecessarily lengthening the abstract. The term N_2O-denitrification is then first mentioned in the introduction and later used for "partial" denitrification.

Lines 26-29: from these two sentences it looks like DNRA was important both under light and in dark. So it is not clear how you make the conclusion in your next sentence about coupling on the anammox and DNRA.

Thanks, we agree that it needed a clarification. So, we have changed to "*DNRA, and especially denitrification to N_2O, were the dominant nitrogen (N) removal pathways when oxygen and/or light were present (up to 82%). In contrast, anoxia and darkness promoted NO_3^- reduction by DNRA (52%) combined to N loss by anammox (28%).*"

Line 47: removing fixed N by producing N2 and N2O gas.

Thanks, of course. We have included N_2O in the description.

Line 60-63: it is not clear as an advance of recent studies at what exactly (supposedly lower than 0.25 mg/L?) O2 concentrations can *nosZ* function; you should provide the concentrations, otherwise this sentence does not make any sense.

We were not able to provide the concentrations such authors used, as the concentrations are not indicated (Wittorf et al., 2016). Therefore, we decided to include the genera of bacteria able to do it by the following sentence (line 45):

… recent studies showed the presence of nosZ gene or nosZ transcripts in potentially non-denitrifying genomes of aerobic genera like Gemmatimonas (Orellana et al., 2014; Yoon et al., 2016; Hallin et al., 2018)…

Lines 75-78: these sentences are not build logically; a previous sentence does not support the following, and the role of light on coupled DNRA-anammox is not well explained

Thank you, we agree with your comment. The “Introduction” section has been reorganized and some changes have been made, including putting coupled DNRA-anammox into context of the different NO_3^- removal pathways described in aquatic sediments.

Line 90-91: your hypothesis is not very clear from the practical point of view. Would these results help to calculate mass balance of a saline lake? Or what is the ultimate goal for the measurements based on this assumption? Elaborate more clear research objectives.

During the reorganization of the introduction, the last paragraph has been rewritten explaining the rationale of the present study (line 85):

As described above, oxygen plays a key role in favoring certain processes over others. In addition, light availability can impact the balance between NO_3^- removal pathways as light will enhance primary production and the production of dissolved oxygen. Here, we tested the hypothesis that oxic and light exposed conditions in the water column promote denitrification over DNRA and anammox. For this purpose, we incubated lacustrine sediments from a eutrophic saline lake (Pétrola Lake, Spain) and applied the revised ^{15}N-IPT to confirm and quantify N-cycling rates. Taken together, these findings not only improve our knowledge of the mass balance of N pollutants in saline lakes, but also of how they contribute to climate change in terms of N_2O release.

Line 135: why were the mesocosms incubated at +25 oC? What conditions is this temperature representative of?

The reason to choose 25ºC was that this value was the mean water temperature of samples collected in Pétrola Lake in the summer months of the 2013 campaign, as shown in Valiente et al. (2018). By 2015, this was the last field campaign with comprehensive summer data, and so we relied on that data, which has been cited in the manuscript (line 134).

Line 148: what is the percentage (atom-%) of 15NO3 in added NO3?

^{15}N-labeled NO_3^- was 98 atom% at ^{15}N. It has been added to the manuscript (line 148).

Line 155: how much water was taken?

We collected 20 mL for inorganic N concentrations and N isotope compositions, and 10 mL for physico-chemical, DOC and DNb. This information has also been added (lines 154-156).

Line 163: why was salinity not measured?

We used Total Dissolved Solids (TDS) as estimator for salinity (Williams, 1966), although we are aware that the use of total dissolved salts is preferable to determine salinity in this type of waters (Boerlage, 2012). However, this determination required equipment which the laboratory where incubations were performed did not possess. Consequently, a sentence has been included in the manuscript to indicate that salinity was estimated from TDS (line 163).

Line 237: this chapter of the results and discussion consists of the results only. Furthermore it is not easy to follow when the information about differences between phases and treatments is given so early at the beginning. In this case a reader has to return constantly from the following subchapters of the discussion to this, first subchapter. I suggest that you incorporate this statistical information into the subchapters 3.2 and 3.3 when discussing results of a specific parameter.

We have restructured the manuscript following your recommendation: “Results and discussion” have been separated in “Results” (Section 3, including all the statistical information) and “Discussion” (Section 4). Former point 3.1 is kept in “Results” section separated in two different sub-sections (“3.1 Differences between treatments in chemical parameters” and “3.3 Measured rates of N-loss processes”). In addition, the relevant information from former point 3.2 (“Hydrogeochemical dynamics during sediment incubations”) has been moved to “Results” (as “3.2 Hydrochemical evolution”), whereas the remaining part is included in the first sub-section of “Discussion” (“4.1 N-removal over time”).

You have too many figures and not all of them provide important enough information to be in the main body of the paper. I suggest you to move Figure 3 to Supplementary information. Also, rethink other ones.

Thanks for the suggestion. Following your recommendations, we have moved former figures 3 (mass balance) and 4 (physico-chemical evolution) to Supplementary Information.

Lines 239-240: you did not provide in the M&M how you measured salinity

As answered above, the use of TDS for estimate salinity has been added to the “Materials and Methods” section.

Line 264: what do you mean by ‘N2-anammox’ here? That suggests like there is another end product of anammox possible?

This term has been explained and the sentence clarified (line 288). The reason to introduce this term was to make a difference between N_2 produced by denitrification and N_2 produced by anammox. From this point, the latter will be referred as N_2-anammox.

Line 314-315: you should explain what are the possible nitrogen converting processes that produce 45N2O and what processes result in 46N2O. Furthermore, it doesn’t look like 45N2O and 46N2O increased at all after time 15 hours. Then your assumption about 15N recirculation by coupled DNRA nitrification does not seem to be supported by the data. Instead you should find an explanation that would fit increase in N2O concentration but not in the 15N in N2O.

Thank you so much for this comment, it gave us the opportunity to better explain the mechanisms occurring during our incubations. As you said, our assumption of ^{15}N recirculation by coupled DNRA and nitrification is not well supported by the data. It could lead to $^{46}N_2O$ (and $^{30}N_2$, discussed below). However, there was a slight increase in $^{45}N_2O$, not observed in $^{46}N_2O$, and especially in $^{44}N_2O$ when accounting for the total N_2O concentration (Figure 1). This imbalance cannot be explained if denitrification was the sole source of both N_2O and N_2.

$^{46}N_2O$ can be produced by denitrification and, as mentioned above, by coupled DNRA-nitrification. Produced $^{15}NH_4^+$ by DNRA may be subsequently nitrified, either by the nitrifier-denitrification mechanism or combined by the hybrid pathway with existing $^{15}NO_2^-$. In both scenarios, it would result in $^{46}N_2O$ due to the merge of two ^{15}N atoms. This trend is not observed in our experiments.

Concerning $^{45}N_2O$, its production could be linked to a direct coupling between externally supplied $^{15}NO_2^-$ (reduced $^{15}NO_3^-$) and internally converted ^{14}N by ammonia-oxidizers. It was noticed that NH_4^+ increased over time in the mesocosms as a result of OM mineralization. This mineralization would supply the $^{14}NH_4^+$ source, which can be combined with $^{15}NO_2^-$ to form $^{45}N_2O$ by the hybrid pathway, as shown by previous studies (Trimmer et al., 2016). The large amount of N_2O at the end of the experiment must be $^{44}N_2O$. It could be formed by $^{14}NH_4^+$ formed by OM mineralization and further processing by the nitrifier-denitrification mechanism, which is preferred under reduced oxygen conditions.

These explanations have been included in the manuscript (lines 368-394) with the following text:

Studies involving the role of N_2O-denitrification in saline aquatic environments are mainly restricted to marine ecosystems. Our high measured rates may be explained by the high biological activity after $^{15}NO_3^-$ addition, in the absence of nutrient limitation and/or low N_2O reductase activity. Nonetheless, the different patterns observed for $^{29}N_2$ and $^{45}N_2O$ (Figure 2) cannot be explained, if denitrification was the sole source of N_2 and N_2O, in which case the proportions of $^{29}N_2$ and $^{30}N_2$ would match the proportions of $^{45}N_2O$ and $^{46}N_2O$ assuming steady state conditions (Trimmer et al., 2006). Differences in $^{29}N_2$ and $^{45}N_2O$ can be attributed to anammox, which can imbalance the proportion of ^{15}N by producing $^{29}N_2$. However, nitrification also produces N_2O during its first step. This step involves the oxidation of ammonia (NH_3) to NO_2^- by either ammonia-oxidizing archaea (AOA) or ammonia-oxidizing bacteria (AOB). AOB contain two distinct N_2O-producing pathways. The first mechanism, referred to as "hybrid formation" involves the combination of one N atom from NO_2^- and one from NH_4^+ or an intermediate of its oxidative metabolism, such as hydroxylamine (NH_2OH) or nitric oxide (NO) (Kozlowski et al., 2016; Frey et al., 2019). The other mechanism is the "nitrifier-denitrification" pathway that sequentially oxidizes NH_4^+ to NO_2^-, which is then reduced to NO and N_2O (Wrage et al., 2001; Frame and Casciotti, 2010).

A possible explanation is the ^{15}N recirculation by coupled DNRA-nitrification (DNRA fueling nitrification to N_2O), which is a process whose importance has recently been highlighted in estuarine sediments (Dunn et al., 2009; Murphy et al., 2016). Although treatments OD and OL meet the conditions for this process to take place, this assumption is not fully supported by $^{45}N_2O$ and $^{46}N_2O$ evolution over time. $^{45}N_2O$ did show an increase over time, but not $^{46}N_2O$ (Figure 2). In addition, the vast majority of N_2O measured during the incubation was $^{44}N_2O$, as the sum of $^{45}N_2O$ + $^{46}N_2O$ did not account for the huge N_2O concentration at the end of the experiments (0.5 mmol/L in OL, above 2.0 mmol/L in OD and AD; Figure 1). DNRA would produce $^{15}NH_4^+$, which would be subsequently either oxidized-reduced by the nitrifier-denitrification mechanism or combined by the hybrid pathway with existing $^{15}NO_2^-$ (from our tracer addition). In both scenarios, it would result in $^{46}N_2O$ due to the merge of two ^{15}N atoms. Alternatively, there could be a direct coupling between externally supplied $^{15}NO_2^-$ and internally ^{14}N by ammonia-oxidizers. As stated in the previous section, NH_4^+ increased as a result of OM mineralization, supplying the $^{14}NH_4^+$ source. This $^{14}NH_4^+$, coupled to $^{15}NO_2^-$, can form $^{45}N_2O$ by the hybrid pathway as shown by previous studies (Trimmer et al., 2016). The large amount of $^{44}N_2O$ formed can be derived by $^{14}NH_4^+$ formed by OM mineralization and further processing by the nitrifier-denitrification mechanism, which is preferred under reduced oxygen conditions (Frame and Casciotti, 2010). To reveal the contribution of N_2O production linked to ammonia oxidation by AOA and AOB, we tried to calculate gross nitrification.

Line 324: the same comment with the previous one, you should assign specific processes to 29N2 and 30N2 production.

The explanation for this question is similar to that described for $^{45}N_2O$ and $^{46}N_2O$. An explanation of the processes leading to $^{30}N_2$ and $^{29}N_2$ production has been added into section 4.1 (line 342).

The production of $^{30}N_2$ can be attributed either to denitrification of $^{15}NO_3^-$, or to coupled DNRA-anammox, by combining the DNRA substrate ($^{15}NO_2^-$) with the DNRA product ($^{15}NH_4^+$) (Holtappels et al., 2011). About $^{29}N_2$, both anammox and denitrification can contribute (Song et al., 2016). For the first one (canonical anammox), existing $^{15}NO_2^-$ can be combined with present $^{14}NH_4^+$, which can be produced by OM mineralization. However, denitrification can play an important role if this formed $^{14}NH_4^+$ by OM mineralization is subsequently nitrified.

Lines 340-341: why would there be an increase in release of CO2 and organic acids after your incubations as compared to natural conditions? Please explain.

Microbial decomposition produces organic acids and CO_2 from the breakdown of larger organic carbon molecules (e.g. Herndon et al., 2015). As a result of the addition of NO_3^- as electron acceptor, and considering that enough organic matter is available to donate electrons, an increase in the microbial metabolism is expected, and as a result, higher release of CO_2 than in natural conditions. The decrease in pH (Table 1) at the end of the incubations we understand was in line with this assumption.

Lines 369-371: I guess you could make a more robust assumption here about the N2O as a product of partial nitrification based on evidence that N2O concentration was increasing over the incubation time but not the 15N in N2O. I suggest you rethink this and probably also make calculations to assume quantitative contribution of other sources (such as nitrification) to N2O production.

Thank you so much for your suggestion. As discussed above, we consider that nitrification had a relative influence on the production of N_2O. For this reason, we calculated gross nitrification and gross NO_3^- consumption rates based on isotope pool dilution (IPD) theory, using ^{15}N at% of NO_3^- on 10 time intervals per mesocosm. Once these rates were calculated, we wanted to cross our values with published values of N_2O production rates by ammonia oxidizers belonging to AOA and AOB, to derive a maximum estimate for nitrifier N_2O emissions and contributions to overall N_2O production. However, gross nitrification rates were below detection limit, therefore obviating the possibility to estimate nitrifier N_2O. For this reason, we consider that unfortunately nitrification was undetectable in this type of mesocosm experiment, probably because it was produced using $^{14}NH_4^+$ as argued above. To summarize our calculations and assumptions, we added (lines 394-395):

Unfortunately, the obtained rates were below LOD, meaning that another type of mesocosm experiments would be needed to measure the contribution of ammonia oxidizers to N_2O production (which was not the main focus of this study).

Table 3: it is not clear what you mean here by ‘canonical anammox’ and ‘N2-anammox’. You also do not explain this in the text.

The term “canonical anammox”, used in Salk et al. (2017), refers to the anammox process which consumes non-DNRA-derived NH_4^+. This explanation has been included in the “Material and Methods” section (line 212).

Minor comments:

Lines 53-54: this sentence does not seem necessary.

Thanks, this sentence has been removed.

Line 59: this sentence seem disconnected from the previous ones. Use a connector like ‘also’ or ‘furthermore’

Thank you for your comment. As listed above, the Introduction sections has been reorganized and the first of these sentences removed from this paragraph.

Line 84: it is questionable if a paper from 2003 can be called ‘recent’

“Recently” has been removed from this sentence.

Line 85: it isn’t clear here why anammox was underestimated. It is more logical to place this sentence at the end of line 88.

Thanks, this sentence has been moved to the end of the paragraph as you suggested.

Lines 253-254: you should state here what kind of differences (i.e., where pH was found to be the highest, and where the lowest).

After the values of the ANOVA analysis, the following sentence has been added (lines 240-241):

At the end of the experiment, the highest mean pH values were found in the oxic treatments, significantly higher than mean pH measured in AD treatment (Table 1).

Lines 337-338: these changes were not statistically significant (Line 248-250), therefore this discussion does not seem necessary.

We agree with your suggestion and part of the sentence has been removed. However, the changes we talk about are related to the temporal evolution of DOC from stage S1 (sharp increase after tracer addition) to the end of the experiment. Therefore, although there were no significant differences between treatments at the end of the experiments, we considered necessary to keep part of the discussion as follows (line 334):

A sharp increase of DOC, probably derived from a bloom collapse, was observed in all the treatments during S1 stage (Figure S3). Afterwards, DOC concentration decreased as a result of heterotrophic metabolism. DON values also support this, as the decreasing percentages of DON:DNb underline the role of OM remineralization throughout the incubation.

Line 345: it is not clear here to what ANOVA results you are referring to.

A reference to section 3.3 has been added. In this section, the second paragraph describes differences in N-processes within each treatment, and the co-dominant role of N_2O-denitrification is shown.

Abbreviations (examples of misuse):

Line 22, 90, 188, 191, 274: ‘nitrate’ should be abbreviated

Line 27: no need to introduce ‘N’ abbreviation here as you don’t use it in the abstract anymore.

Line 50: dinitrogen should be N2

Line 60, 86: nitrous oxide should be ‘N2O’

Line 173, 400: replace ‘nitrogen’ with ’N’

Thank you very much for indicating us those examples. These abbreviations followed in general a confusing and heterogeneous notation throughout the manuscript. So, we have reviewed and corrected them, including all the chemical compounds that were not completely homogeneous in their notation (i.e. NH_4^+, N_2O, N_2).

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