Manuscript: bg-2020-20

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

RESPONSE TO REFEREE #1

We greatly appreciate the reviewer's comments, which have helped us to improve the manuscript. In general, we have restructured the manuscript 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 general comments:

As above, please re-consider the structure of how each section is approached and should be proofread. I also suggest it's less confusing to keep Results and discussion as separate sections.

We have restructured the manuscript following your recommendation: "Results and discussion" have been separated in "Results" (Section 3) 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").

Check nitrate/NO3 - nitrite/NO2 - throughout for consistency

Both (NO_3^- and NO_2^-) followed a confusing and heterogeneous notation throughout the manuscript. We have reviewed and corrected it, as well as other chemical compounds that were not completely homogeneous in their notation (i.e. NH_4^+, N_2O, N_2).

Consider the relevance of references you use… some are from water column (e.g. Jensen et al 2011) or sediment with very difference settings.

Thank you for the comment. We acknowledge that Jensen's paper is on water column processes. Nevertheless, we decided to include this reference as it described a very particular interface (the ocean's oxygen minimum zone) where oxygen is limited, similar to the conditions found in the water-sediment interface.

About the references of sediment studies that we provided, we agree that they cover very different settings. However, the literature addressing the three N-removal processes that we studied (denitrification, DNRA and anammox) is very limited in (saline) lake ecosystems. Therefore, we were forced to include references from many different lacustrine and/or saline locations in order to compare our results.

Title: Add in ‘sediment’ somewhere

We added “sediment” in the title, which has been modified so that: Oxygen and light determine the pathways of nitrate reduction in sediments of a highly saline lake

Introduction

The introduction is quite disjointed with no logical direction to draw the reader in. For future potential submissions I would suggest a nicer structure to introduce the reader to your topic – e.g.:

- Shortly introduce N cycle and identify large anthropogenic impacts

- Overview on N cycling processes in sediments, focus on nitrate reducing processes/end products

- Introduce saline lakes and their importance/why are they interesting/understudied

- Potential factors controlling NO3- reducing processes in this lake (light, O2 etc) (I should say that I didn’t realize the study was only on sediments until line 80 as this in not explicitly mentioned before. Additionally, Jensen et al 2011 is a water column study so might not be relevant to sediments (where there should anyway be plenty of NH4+)).

Thank you so much for this valuable comment, not only for this manuscript, but also for future ones. Therefore, the introduction has been rewritten and restructured as:

- First paragraph about N cycle and NO_3^- impacts
- Second and third paragraphs about NO_3^- removal pathways in sediments (stating that the study is in sediments)
- Fourth paragraph about quantification of such processes
- Fifth paragraph about saline lakes
- Sixth paragraph explaining the goal of the study.

Methods

Please re-think the structure and need for the amount of text here

A lot of the text in the Methods section is unnecessary. If you are referencing a method from another paper, it should only be very briefly described in your methods (e.g. a lot of things don’t need to be reiterated from Salk et al).

Thanks. We have removed text from the “Methods” section, especially in sub-sections “2.2 Sediment incubations” and “2.4 Isotope composition of N species”. In addition, the Table showing IPT calculations following Salk et al. has been moved to Supplementary Information.

What is the relevance of the treatments to your study site? Does the lake become stratified/anoxic in some months? What is the phytobenthos? Diatoms? Bacterial mats? This needs to be put into context with better descriptions of your site (in methods and results)

We added such information to section 2.1 (“Study site”) and included in the discussion (“4.1 N-removal over time”) with the following texts:

Section 2.1: *The lake is shallow (maximum depth 2 m), with major water volume oscillations depending on seasonal precipitation, and is not stratified, with a regular mixing throughout the year caused by both wind and cooling. Phytoplankton is present in the water column of Pétrola Lake and includes diatoms (Amphora spp., Nitzschia spp.), cyanobacteria (Oscillatoria spp., Phormidium spp.), and green algae (Chlamydomonas spp., Tetraselmis spp.) (in Spanish: Confederación Hidrográfica del Segura, unpublished data)… Despite that the Pétrola endorheic basin was declared vulnerable to* NO_3^- *pollution by the Regional Government of Castilla-La Mancha in 1998, it still receives a continuous supply of N mainly derived from inorganic synthetic fertilizers (Valiente et al., 2018). As a result, eutrophication of the water layer occurs leading to the dominance of phytoplankton, reducing light levels, and promoting bottom-water oxygen depletion because of bacterial decomposition.*

Section 4.1: *Three different treatments were applied during sediment incubations by modifying oxygen and light conditions in the water column. The darkness treatment mimics the reduction of light derived from enhanced development of planktonic organisms, as commonly observed in shallow eutrophic lakes (Cristofor et al., 1994). In shallow lakes, wind-driven water mixing contributes to avoid anaerobic bottom water (Utsumi et al., 1998). However, shallow eutrophic lakes may exhibit extreme fluctuations in DO concentrations, undergoing anoxia as a result of the collapse of phytoplankton blooms (Robarts et al., 2005) together with high sediment oxygen demand (Mallin et al., 2006). These conditions are found in Pétrola Lake, and therefore, the study of the treatments explained above in this study were: OL (oxygen + light), OD (oxygen + darkness), and AD (anoxia + darkness).*

Section 2.1: What depth were water samples taken?

Surface water samples were collected for the study, what has been added into Section 2.1 (line 115). In addition, a better description of the sampling point has been added (line 112): *The sampling site (control point 2651 in Valiente et al., 2018) was deep enough (approximately 50 cm) to allow us to sample sediment cores and overlying water, but located close to the lake's depocenter without any direct input of polluted freshwater streams or wastewaters. We therefore consider it representative of the natural conditions of the lake.*

Nutrient samples should be filtered (at least 0.2, possibly 0.45um filters) so no nutrients are produced/consumed between sampling/measuring.

To analyze in situ conditions, samples were filtered through 0.45 um. Therefore, such information has been included and the text is as follows (line 115): *To evaluate initial in situ natural conditions (NC), surface water samples were collected, filtered through 0.45 μm pore size nylon filters and stored at 4 °C in darkness prior to further analyses.*

Those samples to refill the mesocosms once arrived to the laboratory were not filtered.

Welti et al use a reservoir to feed the sealed (gas-space-free) mesocosms, I'm very confused about the method description here, was the mesocosm water itself bubbled?

Experiments were adapted from Welti's design. However, in our mesocosms we did not use a reservoir to feed them (we directly bubbled them). So, in line 121, the following sentence has been rewritten: *Mesocosm preparation for core incubations was adapted from previous works (Welti et al., 2012), except for the use of a feeding water reservoir.*

In addition, in Section 2.2 we have added the following sentence (line 129): *In the lab, each mesocosm was filled with lake water and bubbled with either air (oxic treatment) or argon (anoxic treatment).*

Section 2.2:

The first paragraph is a very long way to write ‘the overlying water of each mesocosm was bubbled with either air (oxic treatment) or Argon (anoxic treatment)

Thanks. According to your recommendations, we have reduced the length of the paragraph and such information has been summarized in the sentence included above (line 129).

Why do you seal mescosms with no air space if you’re going to bubble them anyway?

You’re right. The reason to bubble them was to keep the anoxic conditions in the anoxic treatments (bubbled with Argon), but unnecessary in the oxic treatments. However, this sentence has been removed in order to summarize information from Section 2.2.

Line 135: what do you mean by pump? Do you mean a wheel/stirrer to mix the water to avoid stagnation? If not please explain more clearly – and add how the mesocosm water was mixed.

Yes, a small aquarium pump was used to mix the water and avoid stagnation, which has been included in the text (line 132): *a small aquarium pump was installed in the inner wall to prevent stagnation.*

Was light intensity monitored/measured?

Light intensity was not monitored. The reason for not doing so was that the room where the incubations were performed is not directly exposed to sunlight. Natural light entered through a window, in front of which the mesocosms of the light treatment (OL) were uniformly exposed. In addition, the days when the incubations were performed had clear skies (27.07.15 to 01.08.15), so we could assume that the intensity of light received must have been uniform over time.

It’s fine to use Dalsgaard et al 2000 for timing calculations and assuming 1 mm oxygen penetration. However an oxygen penetration depth of 1 mm is not correct for the anoxic treatments. Why was this very high resolution time series chosen? More reasoning should be presented behind this as it seems a bit unnecessary. What is the relevance of 24 h anoxia and darkness in a 2 m deep lake? Diel and seasonal conditions should be better described in terms of biogeochemistry and phytobenthos etc.

The sampling frequency was adjusted on the basis of the oxic treatment, assuming 1 mm of oxygen penetration. Of course, this approach is not valid for an anoxic treatment, where there is no oxygen able to penetrate into the sediment. However, to facilitate comparison between treatments, we decided to adopt the same sampling frequency for all treatments.

We considered that a high resolution of sampling was necessary at the beginning of the experiment, to better know the fate of the tracer added. However, due to the complexity of the processes that can co-occur, finishing our experiment within the first 24 hours would have led us to miss valuable information about the processes that are triggered secondarily (as seen, for example, in the DNRA-anammox coupling). As a practical matter, we decided to decrease the sampling frequency after the first 24 hours.

With regard to the treatment of anoxia and darkness, as noted before, in order to compare the treatments on equal terms, it was necessary to maintain the same sampling frequency. In these shallow, non-stratified and eutrophic lakes, when algae blooms develop, light cannot penetrate (darkness treatment), which together with a high rate of microbial decomposition of organic matter leads to anoxic conditions (anoxic treatment). These conditions of darkness and anoxia can be maintained over times beyond daily cycles.

Was water removed though sampling the mesocosms replaced? Were dilution effects accounted for?

The volume of water removed wasn't replaced in the mesocosms. The option of making an artificial matrix was discarded due to the chemical complexity of the matrix we were working with. Furthermore, we discarded the addition of filtered or otherwise preserved lake water, as it could lead to a change in the nature of the experiment. So, the final concentrations showed in this manuscript were corrected considering the effect of sample removal.

In the anoxic treatment why do you assume all NO3- reduction takes place in the sediment? Why not also the water column?

Thank you for that interesting comment. The role that microorganisms in the water column can play in the AD treatment was not properly explained. Therefore, the paragraph talking about diatom-bacteria aggregates (section 4.1, line 324) has been modified and a new reference has been included (Kamp et al., 2011), which explains how they are able to survive conditions of darkness and anoxia. This paragraph is written as follows:

Based on isotope data, these processes seem to mainly account for the reduction of the added NO_3^-*. However, traceability can sometimes be problematic in* ^{15}*N-IPT studies due to processes such as uptake and intracellular storage (Robertson et al., 2019). Significant inputs of* NO_3^- *may also promote blooms of diatoms (frequent in Pétrola Lake), which are physiologically adapted to grow rapidly under* NO_3^- *rich conditions (Bronk et al., 2007). A phytoplankton bloom was observed after* $^{15}NO_3^-$ *addition in the light treatment (OL), with a subsequent decrease. Even though we cannot prove it, the role of diatom-bacteria aggregates should be considered. These are able to survive anoxia and darkness by reducing* NO_3^- *from the water column to* NH_4^+ *(DNRA), subsequently fueling benthic anaerobic N-cycling (Kamp et al., 2011; 2016), which could alter the time pattern of* ^{15}*N in* NO_3^- *and* NH_4^+*. Therefore, under anoxia and darkness, the reduction of* NO_3^- *may not be limited to the sediments.*

Line 164-165: Is this the name of the site? This has not been introduced/mentioned until now.

Yes, 2651 refers to the control point where samples were collected. This sampling point was already described in a previous study (Valiente et al., 2018), and has been better described as stated previously in this document (line 112).

Section 2.4: A lot of this text is unnecessary and can be streamlined.

Following your recommendation, this section has been considerably shortened.

Line 181: Use original references for the microdiffusion method.

The reference to Brooks et al. (1989) has been included in Section 2.4 (line 183).

Results and Discussion (I suggest it's better to split into two sections)

This section is very confusing to read so please consider re-structuring. All of 3.1 seems to be results and 3.2 onwards is a mixture of results/discussion.

Following your recommendation, and as stated at the beginning of this document, "Results and Discussion" has been separated in "3. Results" and "4. Discussion". Former section 3.2 has been also divided, as part of it was mainly results description.

It's also important to include the in situ conditions you measured and a description of the site. What is the relevance of a longer anoxic incubation to your site?

The in situ conditions (or natural conditions in the text, referred as NC) are included in Table 1. As these samples were collected at time -48 h, they are notated as NC_{-48}. The relevance of long anoxic incubation in this site has been previously explained, as well as the conditions particular conditions of the sampling site.

What are the subscript numbers? E.g. OL72, t(2), F(2,6)

Thanks for the question. Those subscripts for NC, OL, OD and AD refer to the time of sampling. So, OL_{72} means sampling in the OL treatment at time 72 h. This has also been added to the legend of Table 1.

Subscripts following *t* indicate the degrees of freedom for the t-test (per definition, n – 1). In the case of ANOVA, the subscript numbers for F indicate the degrees of freedom between-sample (h – 1) and within-sample (n – h).

Don't forget units (e.g. lines 262-270)

Thanks for the reminder. The values presented in such lines (F-values) correspond to those obtained in the F-test of the ANOVA. These values are obtained by calculating the ratio between the explained variance (or between-group variability) and the unexplained variance (or within-group variability), both in the same units. Therefore, F-values have no units.

In general, a more thorough discussion of data is needed.

We also agree that our manuscript needed some more discussion, especially regarding the dynamics of N_2O (section 4.2). We have made a detailed interpretation of the evolution of $^{45}N_2O$ and $^{46}N_2O$ (Figure 2) to try to explain alternative mechanisms of N_2O production apart from denitrification. As a result, the following two paragraphs have been added (lines 368-394):

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.

The ‘stages’ 1, 2 and 3 have not been defined/introduced until the results, please re-consider how you refer to the experiments.

Thank you for your comment. They have been included in section 2.2 (lines 144-153) when talking about the different timing of the incubations. In addition, we thought that the notation could be misleading, and we've changed by: initial stage or S0 (from sampling to tracer addition); middle stage or S1 (from tracer addition to 24 h); and final stage or S2 (from 24 h to the end of the incubation).

Why is there already so much 15N-N2 at the time when the tracer is added (in Fig 2)? I suggest the data and zero/background correction is checked. There is something wrong here.

According to your suggestion, we have checked the data we presented in Figure 2. For N_2O ($^{45}N_2O$ and $^{46}N_2O$) it is clear that at time 0 the quantity of N_2O is the lowest, and increased to reach more or less a stationary state (but not zero). The explanation for the oscillations are given in the section 4.2, as stated above, linked not only to denitrification but also to other hidden processes (like nitrification). For the N_2 the increase is not visible. Due to the rapid denitrification activity, equilibrium is reached quickly. In fact, concentrations of $^{29}N_2$ and $^{30}N_2$ are close to those of atmospheric air. In spite of that, the value measured at time 0 is not relevant for rate calculations because we worked using the slope value at each specific point for N_2 and N_2O, as explained in section 2.5 (line 208).

Lines 331-312: “…the experiment would only have underestimated N2 production processes…” – surely these are two of the three processes you are investigating?!

This sentence has been removed as it could lead to misunderstanding. The authors wanted to explain that the mass balance was close to 100% (explained in former Figure 3), and any deviation from that could lead to little underestimation in the total N_2 production.

Figs/tables

There seems to be some overlap/repetition between figures, please try and summarize data in fewer figures.

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

Keep colors consistent for N species between figures (i.e. NO3- appears in red, blue, yellow)

Thank you for informing us of this detail. We have solved this inconsistency and kept the same colors for each N species (following those used in Figure 2).

Why is there already so much 15N-N2 at the time when the tracer is added (e.g. Fig 2)? I suggest the data and zero/background correction is checked. There is something wrong here.

We have already made an explanation for this observation in the previous section of the revised document.

Are both fig 3 and 6 necessary as they are quite similar? Comparing them it seems like there is much more N2O-denit and DNRA but hardly any N2-denit in fig 6 than is shown in Fig 3’s 15N recovery. Perhaps you can double check:

29N2 = 1 x 15N,

30N2 = 2 x 15N,

45N2O = 1 x 15N

46N2O = 2 x 15N

Figure 3 has transferred to Supplementary Information. However, Figure 3 and Figure 6 explain different things. On the one hand, Figure 3 represents the mass balance by the recovery of ^{15}N from our measurements. ^{15}N was added as NO_3^- but was measured in 4 different chemical compounds (NO_3^-, NH_4^+, N_2O, N_2). Any measurement of concentration and ^{15}N enrichment comes with an error. The accumulation of steps can inflate the total error on the final values. Therefore, showing values at each time point so close to 100% actually demonstrates how precisely all the measurements were performed.

On the other hand, Figure 6 represents the proportion of each NO_3^- reduction pathway (N_2-denitrification, N_2O-denitrification, DNRA, and anammox) to the total contribution at each time. This calculation is made on the basis of the total NO_3^- reduction.

Is Table 1 necessary as it is just copied from Salk et al?

Table 1 is adapted from Salk et al. (2017), but not just copied from Salk. DNRA parameters included in our table are obtained from the equations provided in the Supplementary Information of Salk’s work. So, you are right about the necessity of including it in the main manuscript and we decided to move it to the Supplementary Information (Table S1).

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