

Mechanistic understanding of nitrate reduction as the dominant production pathway of nitrous oxide in marine oxygen minimum zones

Corresponding Author: Dr Xin Sun

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

In this study, authors have investigated the microbial ecology of nitrate reduction to nitrous oxide in oxygen minimum zones using stable isotope incubation experiments and modelling. While nitrous oxide production and two (plus combined and abiotic) pathways contributing to it are well known, this study provides new detailed knowledge on the major nitrous oxide production pathway: nitrate reduction to nitrous oxide, as authors show the process to depend on oxygen and organic matter quantity and quality but not on external nitrite concentrations. Authors updated the existing microbial ecology model to include more relevant pathways and broader oxygen range, which is relevant and allows its wider use when describing microbial dynamics in OMZ.

Methods are appropriate, combining stable isotope incubations to modelling is elegant and allows wider conclusions on N₂O production microbiology/ecology in OMZ. Methods are described in detailed.

Manuscript is well written and constructed. Results are clearly presented and interpreted. The quality and quantity of figures is appropriate.

Overall, I find the study very convincing and interesting. I enjoyed especially conclusions and future directions section. I do not have any major concerns on this study, just some minor remarks.

Authors state that denitrifiers reducing nitrate to N₂O are assumed to use external nitrite (line 62), but that there are some indications on them not doing it, which is why they focus on examining the nitrite-to-N₂O pathway in this study. However, authors do not provide any reference to support the assumption on external nitrite, making the whole assumption rather vague. I would encourage authors to include reference(s) to support this assumption.

While the manuscript is generally well written and constructed, there are parts that need clarifications.

Lines 46-48: Not fully clear what is meant here, maybe some words are missing?

Lines 82-84: Sentence is a bit loose. Potential contributions of ammonia-oxidizing organisms to what?

Lines 101-102: Here, reader gets the impression that all N₂O producing processes were measured in this study, but that was not the case. As this finding is less exciting, being already known, and the key finding is NO₃ reducing denitrifiers not using ambient NO₂, I would rephrase this part to make it clear that only NO₃ reduction was studied in this study.

Line 189: Is this assumption made based on the results presented in Figure S2? Would be good to refer to that here.

Lines 191-193: Where are these results presented?

Lines 217-220: I understand what is meant here, but not an easy sentence to read. Maybe it could be divided into two?

Lines 294-5: Very interesting and I agree with this hypothesis.

There are also some minor spelling mistakes in lines 377 "Vails" and 380 "ODZ"

Line 385: What is meant with natural abundance here?

(Remarks on code availability)

There is a readme-file describing the different files and dataset used. It could be more thorough, but contains key information.

Reviewer #2

(Remarks to the Author)

Review

In this manuscript, the authors the rates of N₂O production in oxygen minimum zones by various pathways including NO₃->N₂O, NO₂->N₂O and NH₃->N₂O. The authors then used an ecological model to explore the niche partitioning of different microbial types involved in N₂O production. The authors found that NO₃->N₂O denitrifiers do not use extracellular NO₂ and that the NO₃-> N₂O pathway is compatible with oxygen and its response to oxygen is heterogeneous. Overall, the topic and the findings are interesting and valuable to better understand the ocean nitrogen cycle. But there's some comments and questions the authors need to address before moving forward.

General comments

I only have comments in the modeling part based on my expertise in modeling.

- It would be really helpful to include a bit more information about the model in the main text. Even just saying that the model describes microbial functional types based on their unique redox chemistry.

- The authors stated in the main text (lines 201-202) that the model does not impose any pre-defined O₂ inhibition term and the microbial O₂ niches emerge from ecological interactions. I think that's the key difference the authors' model and other models. This should be the highlight of the whole modeling part, but there's only two sentences. Please elaborate more on this part and highlight the key features of this model.

- The authors used two ratios (OM:NO₃ and OM:O₂) to explain the ecological niche of different microbial functional types. The related fig.3 is not clear enough to show how the community structure of denitrifiers change along different OM:NO₃ and/or OM:O₂ ratios. I suggest the authors check the resource ratio theory by David Tilman and modify fig.3. Below is a link of the resource ratio theory including example figures.

[https://www.myweb.ttu.edu/nmcintyr/Community Ecology/resource_ratio.pdf](https://www.myweb.ttu.edu/nmcintyr/Community%20Ecology/resource_ratio.pdf)

- The finding that the type of OM matters is really interesting. And the authors found that local in situ OM are more beneficial. But my question is that what if the in situ OM contains compounds that have specific functions rather than just food quality? Cause the denitrifiers and phytoplankton at the same depth may adapted with each other and they may provide some special compounds to each other.

(Remarks on code availability)

The results are reproducible and the code is a usable resource for the community.

The README has enough instructions to run the code but the comments in the code are not enough to fully understand the code.

Version 1:

Reviewer comments:

Reviewer #2

(Remarks to the Author)

Review R1

First of all, the authors did a great job revising this manuscript. And my comments and questions are well replied. But I still have one follow-up comments for Fig.3.

Actually I prefer the new Fig.S5 over Fig.3. I would suggest the authors replace Fig.3 (panels b to j) with Fig.S5 with additional panels (similar style) to show the values of NO₃->N₂O rates, N₂O concentrations, and the proportion of NO₃->N₂O rate in total N₂O production rate etc (which are panels b to g in Fig.3). Or the authors could even try to generate a 3D figure with 3 axis (OM, NO₃, and O₂ supplies). All in all, the current fig.3 is still insufficient to explain the details of the denitrifier community structure.

(Remarks on code availability)

The README and comments in the code is updated and are enough to understand the code.

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Point-by-point responses

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

In this study, authors have investigated the microbial ecology of nitrate reduction to nitrous oxide in oxygen minimum zones using stable isotope incubation experiments and modelling. While nitrous oxide production and two (plus combined and abiotic) pathways contributing to it are well known, this study provides new detailed knowledge on the major nitrous oxide production pathway: nitrate reduction to nitrous oxide, as authors show the process to depend on oxygen and organic matter quantity and quality but not on external nitrite concentrations. Authors updated the existing microbial ecology model to include more relevant pathways and broader oxygen range, which is relevant and allows its wider use when describing microbial dynamics in OMZ.

Methods are appropriate, combining stable isotope incubations to modelling is elegant and allows wider conclusions on N₂O production microbiology/ecology in OMZ. Methods are described in detailed.

Manuscript is well written and constructed. Results are clearly presented and interpreted. The quality and quantity of figures is appropriate.

Overall, I find the study very convincing and interesting. I enjoyed especially conclusions and future directions section. I do not have any major concerns on this study, just some minor remarks.

Response: We are very grateful that you enjoyed our study, and we thank you for providing valuable suggestions to our paper. We carefully incorporate all suggestions below.

Authors state that denitrifiers reducing nitrate to N₂O are assumed to use external nitrite (line 62), but that there are some indications on them not doing it, which is why they focus on examining the nitrite-to-N₂O pathway in this study. However, authors do not provide any reference to support the assumption on external nitrite, making the whole assumption rather vague. I would encourage authors to include reference(s) to support this assumption.

Response: We apologize for the missing citation. In the revised manuscript, we have elaborated on the assumption regarding external nitrite utilization by denitrifiers and provided relevant references.

“This assumption is derived from the widespread presence of dissimilatory nitrite reductases in the periplasm of cultured denitrifiers²¹, where NO₂⁻ can be supplied from the extracellular space. Moreover, NO₂⁻ is often exported from the cytoplasm into the periplasm by transporters due to its toxicity at high intracellular concentrations²², making external exchange a likely scenario.”

New references:

21. Zumft, W. G. *Cell biology and molecular basis of denitrification*. Microbiol. Mol. Biol. Rev. 61, 533–616 (1997).

22. van Spanning, R. J. M., Richardson, D. J. & Ferguson, S. J. *Introduction to the Biochemistry and Molecular Biology of Denitrification*. *Biology of the Nitrogen Cycle* 3–20 (2007).

While the manuscript is generally well written and constructed, there are parts that need clarifications. Lines 46-48: Not fully clear what is meant here, maybe some words are missing?

Response: This sentence has been revised to clarify that even though abiotic N₂O production is recently discovered in the sunlit ocean, it is not relevant to dark OMZs, the focus of this paper.

“Although abiotic photochemical N₂O production has been recently discovered⁶, this pathway is restricted to sunlit environments and is only potentially relevant to the smaller portions of OMZ waters that overlap with the euphotic zone. In the dark, subsurface waters that characterize most of the OMZs, N₂O is primarily produced and consumed by a variety of microbial metabolisms (Fig. 1a).”

Lines 82-84: Sentence is a bit loose. Potential contributions of ammonia-oxidizing organisms to what?

Response: Thanks for point this out! We’ve revised the sentence to “*potential contributions of ammonia-oxidizing organisms to N₂O production*”

Lines 101-102: Here, reader gets the impression that all N₂O producing processes were measured in this study, but that was not the case. As this finding is less exciting, being already known, and the key finding is NO₃ reducing denitrifiers not using ambient NO₂, I would rephrase this part to make it clear that only NO₃ reduction was studied in this study.

Response: Agree! We have revised the paragraph to clarify that we only measured NO₃ reduction to N₂O pathway in this study. The paragraph now starts with the sentence stating that we measured NO₃→N₂O at three stations, followed by the statement that NO₃→N₂O was the largest N₂O producing pathway. We clarified that other N₂O production pathways were measured previously.

Line 189: Is this assumption made based on the results presented in Figure S2? Would be good to refer to that here.

Response: This is a great idea! This assumption is made based on both Fig. S2 and Fig. S3, we’ve cited both figures here.

Lines 191-193: Where are these results presented?

Response: These results are in Fig. 3, and we now have cited Fig. 3 in the sentence.

Lines 217-220: I understand what is meant here, but not an easy sentence to read. Maybe it could be divided into two?

Response: Certainly! We’ve divided the sentence into two to make it easier to read.

“In our model, the NO₃⁻ supply rate is constant, so the change in OM supply rate (y-axes in Fig. 3) drives the change in OM: NO₃⁻ supply. Meanwhile, both the OM supply rate (y-axes in Fig. 3) and the O₂ supply rate (x-axes of Fig. 3) together control the OM:O₂ supply.”

Lines 294-5: Very interesting and I agree with this hypothesis.

Response: Thank you!

There are also some minor spelling mistakes in lines 377 “Vails” and 380 “ODZ”

Response: Thanks! We’ve fixed the typos.

Line 385: What is meant with natural abundance here?

Response: It means unlabeled nitrite with natural abundance isotopic composition. We added “*unlabeled*” to clarify the meaning here.

Reviewer #1 (Remarks on code availability):

There is a readme-file describing the different files and dataset used. It could be more thorough, but contains key information.

Response: Thanks for pointing this out! We have added more details in the readme file to make it more accessible.

Reviewer #2 (Remarks to the Author):

Review

In this manuscript, the authors the rates of N₂O production in oxygen minimum zones by various pathways including NO₃->N₂O, NO₂->N₂O and NH₃->N₂O. The authors then used an ecological model to explore the niche partitioning of different microbial types involved in N₂O production. The authors found that NO₃->N₂O denitrifiers do not use extracellular NO₂ and that the NO₃-> N₂O pathway is compatible with oxygen and its response to oxygen is heterogeneous. Overall, the topic and the findings are interesting and valuable to better understand the ocean nitrogen cycle. But there's some comments and questions the authors need to address before moving forward.

Response: Thank you for your time and efforts in providing us very helpful comments and suggestions. We appreciate that you think our findings are interesting and valuable, and we have carefully revised our manuscript based on your comments.

General comments

I only have comments in the modeling part based on my expertise in modeling.

- It would be really helpful to include a bit more information about the model in the main text. Even just saying that the model describes microbial functional types based on their unique redox chemistry.

Response: This is a great idea! We have added this information in the main text as suggested.

“In this model, the key metabolisms responsible for N and O₂ cycling in OMZs are represented as microbial functional types, parameterized using the redox reactions underlying the metabolisms (Fig. 3a). The denitrifiers in particular are described using the thermodynamics of each denitrifier module as well as a pathway length penalty.”

- The authors stated in the main text (lines 201-202) that the model does not impose any pre-defined O₂ inhibition term and the microbial O₂ niches emerge from ecological interactions. I think that's the key difference the authors' model and other models. This should be the highlight of the whole modeling part, but there's only two sentences. Please elaborate more on this part and highlight the key features of this model.

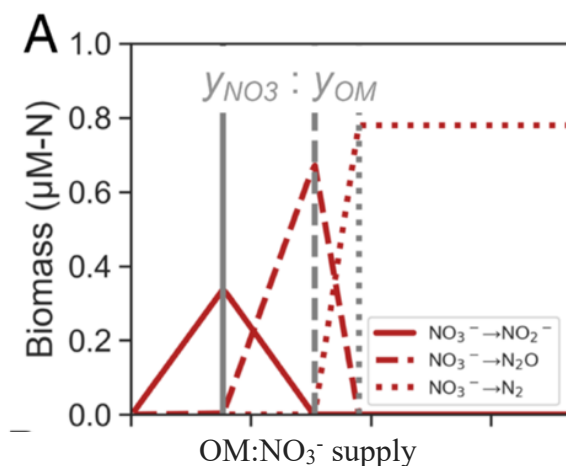
Response: This is indeed an important highlight of our model! In addition to the previous statement, we re-emphasized this point after presenting the results related to the O₂ sensitivity.

“Because our model does not impose fixed O_2 inhibition functions or thresholds for any microbial functional type, these O_2 niches are not pre-specified but arise from outcome of the competitive and cooperative interactions among the functional types.”

- The authors used two ratios (OM:NO₃ and OM:O₂) to explain the ecological niche of different microbial functional types. The related fig.3 is not clear enough to show how the community structure of denitrifiers change along different OM:NO₃ and/or OM:O₂ ratios. I suggest the authors check the resource ratio theory by David Tilman and modify fig.3. Below is a link of the resource ratio theory including example figures.

https://www.myweb.ttu.edu/nmcintyr/Community_Ecology/resource_ratio.pdf

Response: Thank you for this suggestion, and pointing out that Fig. 3 is insufficient to explain the details of the denitrifier community structure. You are spot on with the suggestion: Tilman’s resource ratio theory (RRT) has been central for us in developing a quantitative and mechanistic understanding of the denitrifier community succession along gradients in relative resource supply, but we did not sufficiently make this clear in the manuscript. For example, in our recent study focusing on the modularity of denitrification, we used RRT to theoretically explain the shift in denitrifier community from denitrifiers with single denitrification step to denitrifiers with multiple steps when OM:NO₃ supply increases in a simplified system with constant supply of each resource (Fig. 2A in Sun et al., “Ecological dynamics explain modular denitrification in the ocean,” *PNAS* 2024, attached blow). In this figure, the grey lines are the consumption vectors of each of the three denitrifiers (with line style corresponding to the functional type as in the legend). Each consumption vector is calculated as the ratio of the yields, reflecting the relative demand of OM and NO₃⁻.



However, observations show that OM supply in the ocean is dynamic. When including a time-varying supply of resources, and additionally including all ten of the microbial functional types (our current model in this manuscript) and the dynamic supply of OM, the community shifts cannot be so easily and precisely quantified (i.e., calculated theoretically and then plotted alongside the model simulation using ZNGI plots). This is because during a pulse of OM, the limiting resource for the same microbial functional group changes over time as this pulse of OM decays. However, the general pattern of the community shifts in the model is still consistent with the theory, and we agree that previous version of Fig. 3 alone does not clearly convey the changes in the community structure along the two different resource ratios.

To make it clear, we have added a new figure (Fig. S5) to show the transition from aerobic microbes to anaerobic microbes along OM:O₂, and the composition shift within the denitrifier community along OM:NO₃⁻. We have cited this new Fig. S5 in the main text when discussing the effect of the two resource ratios. Please do let us know if you have additional suggestions, and we would be happy to further incorporate them!

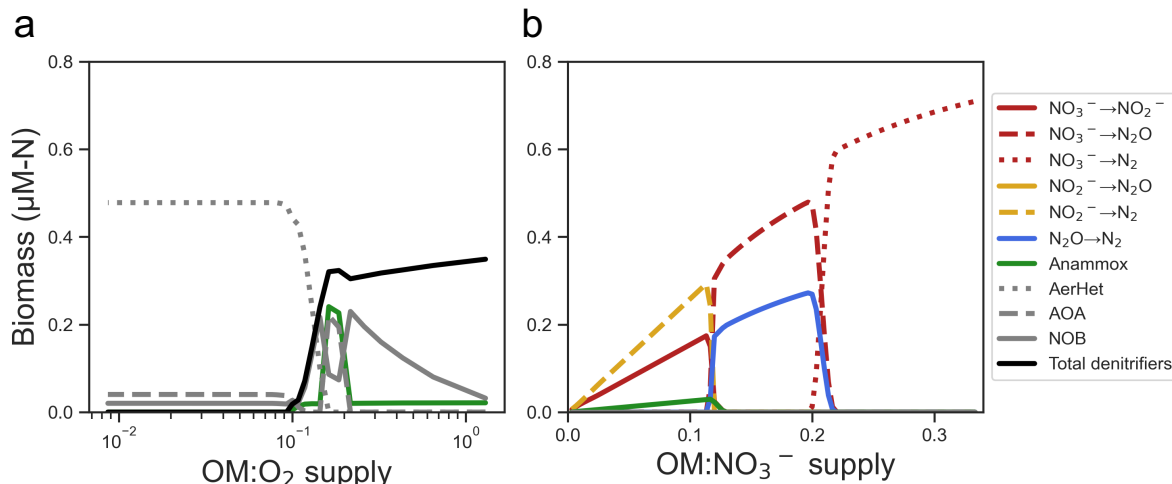


Figure S5 Changes of quasi-equilibrium biomass along (a) an increasing OM to O₂ supply ratio and (b) an increasing OM to NO₃⁻ supply ratio. In (a), OM supply rate is 0.1 μM-N per day, O₂ supply rate varies, and NO₃⁻ supply rate is at 1.2 μM per day. In (b), O₂ supply rate is zero, NO₃⁻ supply rate is at 1.2 μM per day, and OM supply rate varies.

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Response: This is a great point and certainly could be an additional explanation for the observed preference of heterotrophic bacteria for their in situ OM! We have now discussed this point and cited its reference.

“In addition, OM produced by phytoplankton may serve more than just as a carbon source for heterotrophic microbes. OM could also support mutualistic relationships by providing essential micronutrients⁴⁰”

New reference:

40. Cole, J. J. Interactions between bacteria and algae in aquatic ecosystems. *Annu. Rev. Ecol. Syst.* 13, 291–314 (1982).

Reviewer #2 (Remarks on code availability):

The results are reproducible and the code is a usable resource for the community.

The README has enough instructions to run the code but the comments in the code are not enough to fully understand the code.

Response: Thanks for this feedback! We have added more comments in the code to improve clarity.