

Global patterns and drivers of coupling between anammox and denitrification processes across inland aquatic ecosystems

Corresponding Author: Dr Yiwen Zhou

This file contains all editorial decision letters in order by version, followed by all author rebuttals in order by version.

Attachments originally included by the reviewers as part of their assessment can be found at the end of this file.

Version 0:

Decision Letter:

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Dear Dr Zhou,

Your manuscript titled "Global patterns and drivers of coupling between anammox and denitrification processes across inland aquatic ecosystems" has now been seen by 2 reviewers, whose comments are appended below. You will see that they find your work of some potential interest. However, they have raised quite substantial concerns that must be addressed. In light of these comments, we cannot accept the manuscript for publication, but would be interested in considering a revised version that fully addresses these serious concerns.

We hope you will find the reviewers' comments useful as you decide how to proceed. Specifically, to be considered for publication in Communications Earth & Environment, we will need you to

* Provide novel and firmly supported mechanistic insights into the coupling of anammox and denitrification in global freshwater systems, in addition to the correlation analysis that is currently presented.

* Present a clear case for the ecological significance of your work.

Should additional work allow you to address these editorial thresholds as well as all the criticisms raised by the reviewers, we would be happy to look at a substantially revised manuscript. If you choose to take up this option, please either highlight all changes in the manuscript text file, or provide a list of the changes to the manuscript with your responses to the reviewers."

When resubmitting, please provide a point-by-point response to the reviewers' comments. Please submit your responses as a separate file, distinct from your cover letter where you can add responses to the Editors' comments that you do not want to be made available to the reviewers. Word files are preferred. We recommend that any figures, tables or graphs that are included in the response to reviewers are also included in the main article or Supplementary Information.

Please bear in mind that we will be reluctant to approach the reviewers again in the absence of substantial revisions.

If the revision process takes significantly longer than three months, we will be happy to reconsider your paper at a later date, as long as nothing similar has been accepted for publication at Communications Earth & Environment or published elsewhere in the meantime.

We are committed to providing a fair and constructive peer-review process. Please do not hesitate to contact us if you wish to discuss the revision in more detail.

Please use the following link to submit your revised manuscript, point-by-point response to the reviewers' comments with a list of your changes to the manuscript text (which should be in a separate document to any cover letter), a tracked-changes version of the manuscript (as a PDF file) and any completed checklist:

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Please do not hesitate to contact us if you have any questions or would like to discuss the required revisions further. Thank you for the opportunity to review your work.

Best regards,

Somaparna Ghosh, PhD
Associate Editor,
Communications Earth & Environment

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REVIEWER COMMENTS:

Reviewer #1 (Remarks to the Author):

This manuscript focuses on the nitrogen loss in global aquatic ecosystems driven by denitrification and anammox. The author compiled a global dataset on anammox and denitrification, encompassing river, lake, wetland, and estuary ecosystems. These results provide a better understanding of the global nitrogen cycle in the aquatic environment and facilitate more accurate assessment of the nitrogen budget of aquatic ecosystems on a global scale. As there are no original data generated in this study, the novelty level is moderate. There are a few problems to be addressed:

1. Please provide a list of all references in the Supplementary Information. It is essential to pay particular attention to differences in sampling methods and analytical approaches. This not only helps ensure the reliability and validity of the research but also aids in explaining potential biases in the results.
2. Be careful with some statements, for example, "we observed a significant positive correlation between the anammox rate and the abundance of denitrifying bacteria (nirS)"- NirS cannot serve as an indicator for denitrifier abundance. "Denitrifying bacteria are more likely to proliferate than anammox bacteria on a global scale"- What does proliferate mean in this context?
3. In most parts of the article, conclusions were drawn based on correlation rather than causation. For example, why anammox bacterial abundance could explain variations in the denitrification rate (line 217)? why increasing denitrification rates with increasing NH₄⁺ concentrations indicate the importance of nitrifier denitrification (line 250)? Please explain these results from a mechanistic perspective, to the best level.
4. In line 324, it states that anammox is strongly dependent on denitrification. If this is true, partial-denitrification likely provides a substantial amount of NO₂⁻ for anammox, by reducing nitrate. Therefore, it is recommended to analyze other functional genes, such as narG and napA as well.
5. What are the geographical distribution patterns of anammox/denitrification rates, as well as relevant genes? A lot of similar analyses could be done to enrich the findings of this study.
6. Is there any implication from this study towards coupled anammox-denitrification in engineered biosystems, e.g., wastewater treatment?

Reviewer #2 (Remarks to the Author):

General comment

This study investigated the global patterns and drivers of coupling between anammox and denitrification processes based on big dataset. The statistical analysis methods used are useful to reveal the possible drivers. However, the key results and findings were not shown in the Abstract section. The factors determining anammox and denitrification rate were not described in detail in the Introduction section. Authors only focused on the impact of global warming. I believe the authors should revise the Introduction to provide a summary of the research status on various factors.

Specific comment

Line 39: Please provide the detailed variations in anammox and denitrification rates.

Abstract: The main drivers of coupling between anammox and denitrification processes were not shown.

Lines 81-82: Why do anammox and denitrifying bacteria compete for organic carbon?

Lines 120-121: This study mainly explored the feedback mechanisms to climate warming, while the title focused on the drivers.

Lines 192-194: Why do authors explore the relationship between denitrification rate with nirS/nirK genes instead of nar genes?

Lines 216-220: A significant relationship between anammox and denitrification rates was observed in this study. Similar relationship was also observed between anammox and denitrifying bacteria. Thus, it is easy to speculate about these positive relationships in lines 216-220. Further, whether these relationships have ecological significance?

Lines 239-241: A low correlation was observed between anammox rate and the nitrite concentration. Why does an increase of nitrite concentration significantly boost the anammox process?

Line 297: This result was calculated by RF model? Similarly in line 303 and figure S6, the total explanation of these factors exceeds 100%. Please provide the reason.

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Version 1:

Decision Letter:

<*** REMEMBER TO ATTACH REVISIONS CHECKLIST (WORD)***>

** Please ensure you delete the link to your author home page in this e-mail if you wish to forward it to your coauthors **

Dear Dr Zhou,

Your manuscript titled "Global patterns and drivers of coupling between anammox and denitrification processes across inland aquatic ecosystems" has now been seen by our reviewers, whose comments appear below. In light of their advice we are delighted to say that we are happy, in principle, to publish a suitably revised version in Communications Earth & Environment, provided you add a more comprehensive discussion of potential mechanisms for the coupling between anammox and denitrification processes.

We therefore invite you to revise your paper one last time to address the remaining concerns of our reviewers. At the same time we ask that you edit your manuscript to comply with our format requirements and to maximise the accessibility and therefore the impact of your work.

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We hope to hear from you within two weeks; please let us know if you need more time.

Best regards,

Somaparna Ghosh, PhD
Associate Editor,
Communications Earth & Environment

REVIEWERS' COMMENTS:

Reviewer #1 (Remarks to the Author):

I'm generally satisfied with the revisions, but I noticed a few minor issues:

1. Some references should be provided in the Introduction, for example, on Lines 87 and 110.
2. Instead of "nitrogen removal" in aquatic ecosystems, "nitrogen loss" is more commonly used.
3. Line 123: Could you clarify what the variable data were?
4. The figure title "Coupling of anammox and denitrification rates" seems inappropriate.
5. The raw data will be made available online after the manuscript is accepted. Did the authors submit the raw data to a public database under a project number that is currently under embargo? If so, this should be specified.

Reviewer #2 (Remarks to the Author):

General comment

This study investigated the global patterns and drivers of coupling between anammox and denitrification processes based on big dataset. The topic is interesting and the statistical analysis methods used provide convincing results to reveal the possible drivers. However, the presentation of the manuscript needs revision. The key results and findings are not shown in the Abstract section. The factors determining anammox and denitrification rate were not clearly explained in the Introduction section. Authors only focused on the impact of global warming. I believe that the authors should revise the Introduction to provide a summary of the research status on various factors.

Specific comment

Line 39: Please provide the detailed variations in anammox and denitrification rates.

Abstract: The main drivers of coupling between anammox and denitrification processes were not shown.

Lines 81-82: Why do anammox and denitrifying bacteria compete for organic carbon?

Lines 120-121: This study mainly explored the feedback mechanisms to climate warming, while the title focused on the drivers.

Lines 192-194: Why do authors explore the relationship between denitrification rate with nirS/nirK genes instead of nar genes?

Lines 216-220: A significant relationship between anammox and denitrification rates was observed in this study. Similar relationship was also observed between anammox and denitrifying bacteria. Thus, it is easy to speculate about these positive relationships in lines 216-220. Further, whether these relationships have ecological significance?

Lines 239-241: A low correlation was observed between anammox rate and the nitrite concentration. Why does an increase of nitrite concentration significantly boost the anammox process?

Line 297: This result was calculated by RF model? Similarly in line 303 and figure S6, the total explanation of these factors exceeds 100%. Please provide the reason.

Reviewer #3 (Remarks to the Author):

A comprehensive data set is created on the global role of denitrification and anammox. The review suggests a significant role of nitrifier denitrification in N loss when ammonium concentrations are higher than nitrate. Although, the authors have comprehensively addressed previous comments. I still have some concerns about their explanation about correlation about anammox and denitrification rates. The authors should also consider the possibility of dissimilatory nitrate reduction to ammonia. This reviewer also disagree with authors response about the role of denitrification and anammox in engineered bioreactors. They are not extensively studied and there is a huge opportunity. In response to second reviewer's comments and authors response about nar gene, I think nar gene is not used as a biomarker for denitrification because this is also present in other organisms.

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Response to reviewers

Dear Editor and Reviewers,

Thank you for your letter and for the reviewers' comments concerning our manuscript entitled "Global patterns and drivers of coupling between anammox and denitrification processes across inland aquatic ecosystems" (COMMSENV-24-1821). These comments are all valuable and very helpful for revising and improving our paper, as well as important for guiding our research. On the basis of the reviewers' comments, we have revised and further improved the manuscript. We hope that our revisions will meet with approval. The revised portion is marked in **blue** in the revised manuscript. The main corrections in the manuscript and the responses to the reviewers' comments are as follows:

Reviewer #1:

This manuscript focuses on the nitrogen loss in global aquatic ecosystems driven by denitrification and anammox. The author compiled a global dataset on anammox and denitrification, encompassing river, lake, wetland, and estuary ecosystems. These results provide a better understanding of the global nitrogen cycle in the aquatic environment and facilitate more accurate assessment of the nitrogen budget of aquatic ecosystems on a global scale. As there are no original data generated in this study, the novelty level is moderate. There are a few problems to be addressed:

Response:

Thank you for the insightful and valuable comments and suggestions to improve the quality of our manuscript. Understanding how anammox drives nitrogen loss from aquatic ecosystems is important given that the anammox process is an environmentally friendly biochemical pathway compared with the denitrification process. Tracing and quantifying anammox variation in various aquatic ecosystems are essential because denitrification is considered a major pathway for nitrogen loss. In particular, a recent study highlighted the overestimation of nitrogen loss by denitrification in natural terrestrial ecosystems (Feng et al., Nature Communications, 2023). In addition, current Earth models do not incorporate anammox. Thus, we constructed a global dataset of anammox and denitrification from aquatic ecosystems. On the basis of this dataset, we employed a mechanistic learning method to explore the interaction between denitrification and anammox. First, we propose a model to predict anammox rates on the basis of denitrification rates in aquatic ecosystems. The

results can be used to assess the contribution of nitrogen loss via both anammox and denitrification processes accurately. Accordingly, we can identify hotspots in the anammox process in aquatic ecosystems on the basis of these results. Overall, there are significant differences that further enhance and expand our understanding of anammox and denitrification in aquatic ecosystems.

We have carefully checked and revised the manuscript according to the reviewer's suggestions and comments, as detailed in our point-by-point response below.

[Reference]

Feng, M., Peng, S., Wang, Y., Ciais, P., Goll, D.S., Chang, J., Fang, Y., Houlton, B.Z., Liu, G., Sun, Y. and Xi, Y. 2023. Overestimated nitrogen loss from denitrification for natural terrestrial ecosystems in CMIP6 Earth System Models. *Nat Commun* 14(1), 3065.

#1. *Please provide a list of all references in the Supplementary Information. It is essential to pay particular attention to differences in sampling methods and analytical approaches. This not only helps ensure the reliability and validity of the research but also aids in explaining potential biases in the results.*

Response:

Thank you for the kind reminder and valuable suggestions. We have already listed all the references in the Supplementary Information. We acknowledge that the sampling methods and analytical approaches introduce potential biases in the field data. Hence, we design several criteria to collect the published data as follows: (1) at least one focal variable (e.g., anammox rates and/or the abundance of anammox bacteria) was reported in the peer-reviewed article; (2) the soil anammox rate was measured via ¹⁵N isotope tracing, and the units for the anammox rate were unambiguous; and (3) the anammox and denitrification rates in the overlying water were excluded from the datasets. Please refer to LL. 397-402 for a detailed description of the strategy. To interpret the data in this study, we employed a mixed-effect model to address the bias in the data (see LL. 430-433). A random forest model is a modeling framework that accounts for nonlinear effects and complex interactions between predictors. To determine the effectiveness of the model, we employed a tenfold cross-validation method to evaluate the performance of the specific model (see LL. 467-476). Thus, we believe that these approaches are available for addressing potential biases.

#2. *Be careful with some statements, for example, “we observed a significant positive correlation between the anammox rate and the abundance of denitrifying bacteria*

(nirS)” - NirS cannot serve as an indicator for denitrifier abundance. “Denitrifying bacteria are more likely to proliferate than anammox bacteria on a global scale” - What does proliferate mean in this context?

Response:

Please accept our apologies for the improper expressions in the manuscript. As you noted, denitrifying bacteria harbor either the *nirK* or *nirS* gene. Hence, we employed *nirK*-type and *nirS*-type denitrifiers to classify the denitrifying bacteria. We have carefully reviewed and corrected the manuscript throughout.

In addition, the term “increase” here refers to growth, and we have modified this term for better understanding on L. 221 of the revised manuscript.

#3. *In most parts of the article, conclusions were drawn based on correlation rather than causation. For example, why anammox bacterial abundance could explain variations in the denitrification rate (line 217)? why increasing denitrification rates with increasing NH_4^+ concentrations indicate the importance of nitrifier denitrification (line 250)? Please explain these results from a mechanistic perspective, to the best level.*

Response:

Our results revealed that abundant denitrifying bacteria consistently promoted anammox bacterial abundance, as evidenced on a global scale (Fig. 3). This phenomenon explains why anammox bacterial abundance can explain variations in the denitrification rate. We have provided additional discussion in LL. 234-237 of the revised manuscript. Additionally, the previous section highlighted the potential mechanism in lines 215-217.

In general, NH_4^+ was the major form of inorganic nitrogen in the sediment, while the NO_3^- and NO_2^- concentrations remained low (Figs. S3 and S4). Thus, NH_4^+ conversion into $\text{NO}_2^-/\text{NO}_3^-$ is the crucial pathway for providing substrates for denitrification. A previous study indicated that the percentage of nitrifier denitrification increased to 66.0% - 77.5% when the soil ammonium content was much higher than the nitrate content (Wrage et al., 2004). Our results also revealed a positive correlation between the denitrification rate and NH_4^+ concentration ($p < 0.001$) (Fig. 4d-2). Therefore, these results suggest the importance of nitrifier denitrification in aquatic ecosystems at a global scale. We have provided more discussion on this matter at LL. 264-270 in the revised manuscript.

[Reference]

Wrage, N., Velthof, G., Laanbroek, H., & Oenema, O. (2004). Nitrous oxide production in grassland soils: Assessing the contribution of nitrifier denitrification. *Soil Biology & Biochemistry*, 36, 229-236.

#4. *In line 324, it states that anammox is strongly dependent on denitrification. If this is true, partial-denitrification likely provides a substantial amount of NO_2^- for anammox, by reducing nitrate. Therefore, it is recommended to analyze other functional genes, such as *narG* and *napA* as well.*

Response:

We appreciate the reviewer's suggestion. We acknowledge that partial denitrification provides a substantial amount of NO_2^- to anammox. Owing to the scarcity of data for quantifying *narG* and *napA* gene abundance from published works, it is difficult to construct a dataset including *narG* and *napA* on a global scale. Nevertheless, our results indicated that NO_3^- concentration is the major driver of anammox. This further reflects that denitrifying bacteria share the substrate NO_2^- with anammox bacteria. In addition, we have added an explanation at LL. 411-414 in the revised manuscript.

#5. *What are the geographical distribution patterns of anammox/denitrification rates, as well as relevant genes? A lot of similar analyses could be done to enrich the findings of this study.*

Response:

According to the reviewer's suggestions, we performed geographical analyses of anammox/denitrification, as well as relevant genes, on the basis of the linear mixed-effect model. We found that there was an insignificant relationship between latitude/longitude and anammox/denitrification. Given these results, we have not incorporated them into our revised manuscript. In fact, we employed the mean annual temperature and mean annual precipitation to explore the variation in anammox and denitrification at a global scale (Fig. 7).

#6. *Is there any implication from this study towards coupled anammox-denitrification in engineered biosystems, e.g., wastewater treatment?*

Response:

The coupling of anaerobic ammonium oxidation–denitrification has been extensively studied in engineering systems, particularly in sewage treatment, through the control of various reaction conditions, such as temperature, organic matter and dissolved oxygen. However, in natural water environments, the abundance of ammonia-oxidizing bacteria and denitrifying bacteria, as well as the coupling of the denitrification process between these groups, remains largely unknown. Additionally,

the contribution of this coupling to the denitrification rate in aquatic ecosystems is still poorly understood. Therefore, our research focuses primarily on natural processes. Similar explorations may have been conducted in biosystems, and the results challenge the prevailing view that nitrogen loss in natural water bodies is predominantly driven by the denitrification process. Our findings reveal the significant role of anaerobic ammonium oxidation in nitrogen budgets. In our future research, we aim to apply insights from the natural environment to engineering applications. We thank the reviewer for these constructive suggestions.

Reviewer #2:

General comment

This study investigated the global patterns and drivers of coupling between anammox and denitrification processes based on big dataset. The statistical analysis methods used are useful to reveal the possible drivers. However, the key results and findings were not shown in the Abstract section. The factors determining anammox and denitrification rate were not described in detail in the Introduction section. Authors only focused on the impact of global warming. I believe the authors should revise the Introduction to provide a summary of the research status on various factors.

Response:

Please accept our apologies for the incomplete expressions of our findings in the abstract section and the summary of various factors in the introduction section. We have incorporated detailed descriptions in the revised manuscript.

Specific comment

#1. *Line 39: Please provide the detailed variations in anammox and denitrification rates.*

Response:

As the reviewer suggested, we have added detailed information on the variations in the anammox and denitrification rates in LL. 39-40 of the revised manuscript.

#2. *Lines 81-82: Why do anammox and denitrifying bacteria compete for organic carbon?*

Response:

Anammox and denitrifying bacteria may also compete for other substrates, such as

organic carbon. Generally, denitrifying bacteria prefer to use organic matter as a carbon source, but anammox bacteria only compete for organic carbon to utilize it as an electron donor (Zhang et al., 2023). Some anammox bacteria can oxidize short-chain fatty acids, which are coupled with the reduction of NO_3^- and/or NO_2^- to NH_4^+ or are disguised as denitrifying bacteria (Kartal et al., 2007). To clarify this matter, we have modified the text in LL. 82—87 in the revised manuscript.

[References]

Zhang, D., Yu, H., Yang, Y., Liu, F., Li, M., Huang, J., Yu, Y., Wang, C., Jiang, F., He, Z. and Yan, Q. 2023. Ecological interactions and the underlying mechanism of anammox and denitrification across the anammox enrichment with eutrophic lake sediments. *Microbiome* 11(1), 82.

Kartal, B., Kuypers, M.M., Lavik, G., Schalk, J., Op den Camp, H.J., Jetten, M.S. and Strous, M. 2007. Anammox bacteria disguised as denitrifiers: nitrate reduction to dinitrogen gas via nitrite and ammonium. *Environmental Microbiology* 9(3), 635-642.

#3. *Lines 120-121: This study mainly explored the feedback mechanisms to climate warming, while the title focused on the drivers.*

Response:

Yes, our primary focus is on the patterns of anammox and denitrification shaped by distinct driving factors in aquatic ecosystems on a global scale. In general, climate change is a crucial force regulating the nitrogen cycle in natural ecosystems. Consequently, climate warming is considered a specific factor affecting both anammox and denitrification. After careful consideration, we have decided to revise the original text in LL. 126-127 (explore the responses of anammox and denitrification to driving factors on a global scale.)

#4. *Lines 192-194: Why do authors explore the relationship between denitrification rate with nirS/nirK genes instead of nar genes?*

Response:

The *nirS* and *nirK* genes encode key enzymes in the denitrification process, representing two distinct types of nitrite reductases. Specifically, *nirS* encodes a copper nitrite oxidase (Cu-NirS), whereas *nirK* encodes a cytochrome cd1 nitrite reductase (Cyd1-NirK). Both enzymes play critical roles in the reduction of nitrite (NO_2^-) to nitric oxide (NO), a pivotal step in the denitrification pathway. The *nar* genes, on the other hand, encode nitrate reductases, which facilitate the initial step of denitrification

by reducing nitrate (NO_3^-) to NO_2^- . Although the *nar* gene is important in the denitrification process, it is not directly involved in the subsequent nitrite reduction. Therefore, the *nirS* and *nirK* genes, which are closely related to the formation of denitrification end products, provide a more direct reflection of the rate of denitrification. Methodologically, the *nirS* and *nirK* genes are relatively easy to detect and quantify in molecular biology studies because of their high degree of conservation and sufficient variability, which allows for differentiation among various microbial populations. This characteristic makes them valuable biomarkers in the investigation of denitrification processes and microbial ecology.

We have added an explanation in the revised text in LL.411-414. Complete denitrification involves multiple steps in the reduction of nitrate to N_2 , and nitrite reductase, encoded by *nirK* and *nirS*, catalyzes the first step in converting dissolved nitrogen into gaseous nitrogen. Therefore, we selected *nirK* and *nirS* as marker genes to represent denitrifying bacteria.

#5. Lines 216-220: *A significant relationship between anammox and denitrification rates was observed in this study. Similar relationship was also observed between anammox and denitrifying bacteria. Thus, it is easy to speculate about these positive relationships in lines 216-220. Further, whether these relationships have ecological significance?*

Response:

Thank you for the valuable suggestions. These relationships indicate that the processes of converting reactive nitrogen (e.g., ammonia and nitrate) into inert nitrogen gas work in concert, thereby contributing to the regulation of nitrogen levels in aquatic environments and mitigating emissions of the greenhouse gas nitrous oxide. This collaboration can increase nitrogen removal efficiency and reduce nitrogen discharge, ultimately improving water quality and preventing eutrophication. Thus, understanding these relationships is crucial for predicting and elucidating the effects of global change on ecosystem functioning. On the basis of the reviewer's suggestions, we have incorporated these implications in Lines 240-247 of the revised manuscript.

#6. Lines 239-241: *A low correlation was observed between anammox rate and the nitrite concentration. Why does an increase of nitrite concentration significantly boost the anammox process?*

Response:

Thank you for your valuable comment. While the correlation between the nitrite

concentration and the rate of anammox may not be particularly strong, nitrite remains an essential substrate for the anammox reaction. Insufficient nitrite levels can hinder the progress of the anammox process. Additionally, in terms of enzymatic reaction kinetics, nitrite reductase may exhibit a limited affinity for nitrite during anaerobic ammonia oxidation, which implies that reaction rates could be constrained at low nitrite concentrations. Conversely, when nitrite concentrations surpass a certain threshold, enzyme activity may reach its maximum, thereby increasing the rates of anaerobic ammonia oxidation.

#7. *Line 297: This result was calculated by RF model? Similarly in line 303 and figure S6, the total explanation of these factors exceeds 100%. Please provide the reason.*

Response:

Yes, the result was calculated via the random forest (RF) model. The total feature importance can exceed 1, especially when it is not normalized. This occurs because the feature importance values are accumulated across multiple trees in the forest, representing the contribution of each feature to the overall model. As a result, the total contribution of the factors can exceed 100%. To improve clarity, we have revised Fig. 7c using %IncMSE (increase in mean squared error). The corresponding supporting information has also been updated accordingly.

Response to reviewers

Dear Editor and Reviewers,

Thank you for your letter and for the reviewers' constructive comments. We are pleased to hear that the reviewers find the manuscript strong overall. The comments are valuable and have been instrumental in revising and improving our manuscript, as well as in guiding our ongoing research. Based on the reviewers' feedback, we have made several revisions to further enhance the manuscript. The revised portions are highlighted in blue in the updated manuscript. Below, we summarize the main corrections and provide our responses to the reviewers' comments:

Reviewer #1:

I'm generally satisfied with the revisions, but I noticed a few minor issues:

Response: We thank the reviewer for the positive assessment and appreciate your careful review. We will address the minor issues promptly to ensure the manuscript meets the highest standards.

#1. *Some references should be provided in the Introduction, for example, on Lines 87 and 110.*

Response: Thank you for pointing this out. We have added relevant references to the Introduction on Lines 87 and 110 to support these statements.

[References]

[17] Kartal, B. *et al.* Anammox bacteria disguised as denitrifiers: nitrate reduction to dinitrogen gas via nitrite and ammonium. *Environmental Microbiology* **9**, 635-642 (2007). <https://doi.org:10.1111/j.1462-2920.2006.01183.x>

11 Deng, D. *et al.* Denitrification dominates dissimilatory nitrate reduction across global natural ecosystems. *Global change biology* **30**, e17256 (2024). <https://doi.org:10.1111/gcb.17256>

23 Zhu, L. *et al.* Algal accumulation decreases sediment nitrogen removal by uncoupling nitrification-denitrification in shallow eutrophic lakes. *Environ Sci Technol* **54**, 6194-6201 (2020). <https://doi.org:10.1021/acs.est.9b05549>

#2. Instead of "nitrogen removal" in aquatic ecosystems, "nitrogen loss" is more commonly used.

Response: Thank you for the suggestion. We have revised the text to use "nitrogen loss" instead of "nitrogen removal" for consistency with common terminology.

#3. Line 123: Could you clarify what the variable data were?

Response: Thank you for your comment. We have clarified the description of the variable data in the revised manuscript. Additionally, we have provided a reference to our dataset for further transparency.

[Reference]

Zhou, Y., Yang, Y., Xu, X., Zeng, Y., & Song, K. (2024). Global patterns and drivers of coupling between anammox and denitrification processes across inland aquatic ecosystems [Data set]. Zenodo. <https://doi.org/10.5281/zenodo.14202839>

Revised text:

“we conducted a systematic review of a constructed dataset that incorporated anammox and denitrification as well as variable data (sediment properties, climate factors, and microbial attributes) across aquatic ecosystems (i.e., rivers, lakes, wetlands, and estuaries)²⁴.”

#4. The figure title "Coupling of anammox and denitrification rates" seems inappropriate.

Response: Thank you for your suggestion. We have revised the figure title to better reflect the content, in accordance with your recommendation.

Revised text:

“Explanation of anammox and denitrification rates across aquatic ecosystems”

#5. The raw data will be made available online after the manuscript is accepted. Did the authors submit the raw data to a public database under a project number that is currently under embargo? If so, this should be specified.

Response: Yes, we have uploaded our dataset to a publicly accessible database. Detailed information about the dataset, including the DOI for reference, has been included in the revised manuscript.

Zhou, Y., Yang, Y., Xu, X., Zeng, Y., & Song, K. (2024). Global patterns and drivers of coupling between anammox and denitrification processes across inland aquatic ecosystems [Data set]. Zenodo. <https://doi.org/10.5281/zenodo.14202839>

Reviewer #2:

General comment

This study investigated the global patterns and drivers of coupling between anammox and denitrification processes based on big dataset. The topic is interesting and the statistical analysis methods used provide convincing results to reveal the possible drivers. However, the presentation of the manuscript needs revision. The key results and findings are not shown in the Abstract section. The factors determining anammox and denitrification rate were not clearly explained in the Introduction section. Authors only focused on the impact of global warming. I believe that the authors should revise the Introduction to provide a summary of the research status on various factors.

Specific comment

Line 39: Please provide the detailed variations in anammox and denitrification rates.

Abstract: The main drivers of coupling between anammox and denitrification

processes were not shown.

Lines 81-82: Why do anammox and denitrifying bacteria compete for organic carbon?

Lines 120-121: This study mainly explored the feedback mechanisms to climate warming, while the title focused on the drivers.

Lines 192-194: Why do authors explore the relationship between denitrification rate with nirS/nirK genes instead of nar genes?

Lines 216-220: A significant relationship between anammox and denitrification rates was observed in this study. Similar relationship was also observed between anammox and denitrifying bacteria. Thus, it is easy to speculate about these positive relationships in lines 216-220. Further, whether these relationships have ecological significance?

Lines 239-241: A low correlation was observed between anammox rate and the nitrite concentration. Why does an increase of nitrite concentration significantly boost the anammox process?

Line 297: This result was calculated by RF model? Similarly in line 303 and figure S6, the total explanation of these factors exceeds 100%. Please provide the reason.

Response: Thank you for your continued feedback. We would like to clarify that these suggestions and comments were addressed in detail in our initial rebuttal letter.

Reviewer #3:

A comprehensive data set is created on the global role of denitrification and anammox. The review suggests a significant role of nitrifier denitrification in N loss when ammonium concentrations are higher than nitrate. Although, the authors have comprehensively addressed previous comments. I still have some concerns about their explanation about correlation about anammox and denitrification rates. The authors should also consider the possibility of dissimilatory nitrate reduction to ammonia. This reviewer also disagree with authors response about the role of denitrification and anammox in engineered bioreactors. They are not extensively studied and there is a huge opportunity. In response to second reviewer's comments and authors response about *Nar* gene, I think *Nar* gene is not used as a biomarker for denitrification because this is also present in other organisms.

Response: Thank you for your detailed and constructive comments. We greatly appreciate your feedback, which has helped us refine our manuscript further. Below are our responses to your concerns:

#1. I still have some concerns about their explanation about correlation about anammox and denitrification rates. The authors should also consider the possibility of dissimilatory nitrate reduction to ammonia.

Response: Thank you for raising concerns regarding the potential role of dissimilatory nitrate reduction to ammonium (DNRA) in influencing the observed correlation between anammox and denitrification rates. We acknowledge that DNRA is a crucial pathway in nitrogen cycling, particularly under conditions such as high organic carbon

availability, low oxygen levels, and abundant nitrate. We are currently preparing a manuscript focusing on the global patterns and interplay between denitrification and DNRA, aiming to address the gaps in understanding these processes. This forthcoming research will explore how environmental factors and microbial communities drive the balance between these nitrogen transformation pathways.

#2. This reviewer also disagree with authors response about the role of denitrification and anammox in engineered bioreactors. They are not extensively studied and there is a huge opportunity.

Response: We appreciate your feedback regarding the role of denitrification and anammox processes in engineered bioreactors. While this manuscript primarily focuses on changes in natural ecosystems, we agree that optimizing process parameters to achieve coupling between anammox and denitrification is a critical challenge for realizing low-carbon, energy-efficient wastewater treatment. This represents an important avenue for future research.

#3. In response to second reviewer's comments and authors response about *Nar* gene, I think *Nar* gene is not used as a biomarker for denitrification because this is also present in other organisms.

Response: We understand and accept your concern that the *Nar* gene is not a specific biomarker for denitrification, as it is also present in other organisms. In the revised manuscript, we have corrected our statements regarding the role of the *Nar* gene and removed potentially misleading content. We have also added a discussion of commonly used marker genes, such as *NirS* and *NirK*, to more accurately reflect mainstream views in denitrification research.

Revised text:

“Importantly, the *nar* gene is not considered a reliable biomarker for denitrification, as it is also present in a variety of non-denitrifying organisms^{44,45}.”

[References]

Zumft, W. G. (1997). Cell biology and molecular basis of denitrification. *Microbiology and Molecular Biology Reviews*, 61(4), 533–616. <https://doi.org/10.1128/mmmbr.61.4.533-616.1997>

Kandeler, E., Deiglmayr, K., Tscherko, D., Bru, D., & Philippot, L. (2006). Abundance of *narG*, *nirS*, *nirK*, and *nosZ* genes of denitrifying bacteria during primary successions of a glacier foreland. *Applied and Environmental Microbiology*, 72(9), 5957–5962. <https://doi.org/10.1128/AEM.00439-06>