

Response to reviewers' comments

Reviewer #1:

The manuscript entitled "Macro constraints and local eutrophication shape sediment nitrogen removal in the Eastern China Plain lakes" is well-structured, deeply considered, and generally well-written. The study encompasses a substantial amount of methodology and data, reflecting a significant workload. By utilizing multiple biotic and abiotic indicators to explore how spatial scale and environmental effects shape nitrogen removal processes in lakes, this work merits publication. My specific comments are as follows:

1. 132–142. The language in this section requires polishing; for instance, there are issues with incomplete sentences. Additionally, the relevant software packages mentioned here can be uniformly referred to as "QIIME2".

Response: We thank the reviewer for this constructive suggestion. We have carefully polished the language in lines 132–142 to ensure complete sentences and smooth flow. In addition, our analytical workflow involved both metagenomic sequencing and 16S amplicon sequencing, so we have provided detailed descriptions of the software packages to allow readers to clearly distinguish between them. We have also verified that the software package mentions are consistent and standardized throughout the manuscript. We have attached the revised relevant content below for your review:

Metagenomic libraries were constructed using the ALFA-SEQ DNA Library Prep Kit and sequenced on the Illumina NovaSeq 6000 platform (PE150 mode; Guangdong Magigene Biotechnology Co., Ltd., Guangzhou, China). Raw reads were quality-filtered and adapter-trimmed using *fastp* and *Trimmomatic*, yielding approximately 12 Gbp of high-quality clean data per sample. Subsequent bioinformatic analyses encompassed *de novo* assembly, Open Reading Frame (ORF) prediction, the construction of a non-redundant gene catalog (unigenes), and gene abundance quantification (Steinegger and Söding, 2018). Nitrogen-cycling genes and their taxonomic affiliations were annotated using Diamond (v0.9.32) by aligning sequences

against the NCycDB (Tu et al., 2019) and the NCBI non-redundant (NR) protein database, respectively. Taxonomic classification was subsequently determined using the Lowest Common Ancestor (LCA) algorithm to generate comprehensive gene abundance profiles. Finally, functional annotation was filtered based on an alignment coverage threshold of > 40%.

2. 255. Is the expression "NO₂-N" accurate? Similar formatting issues appear elsewhere with terms like "NO₃-N", "PO₄-P", etc. Please ensure proper subscript and superscript formatting for all chemical notations (e.g., NO₂⁻-N, PO₄³⁻-P).

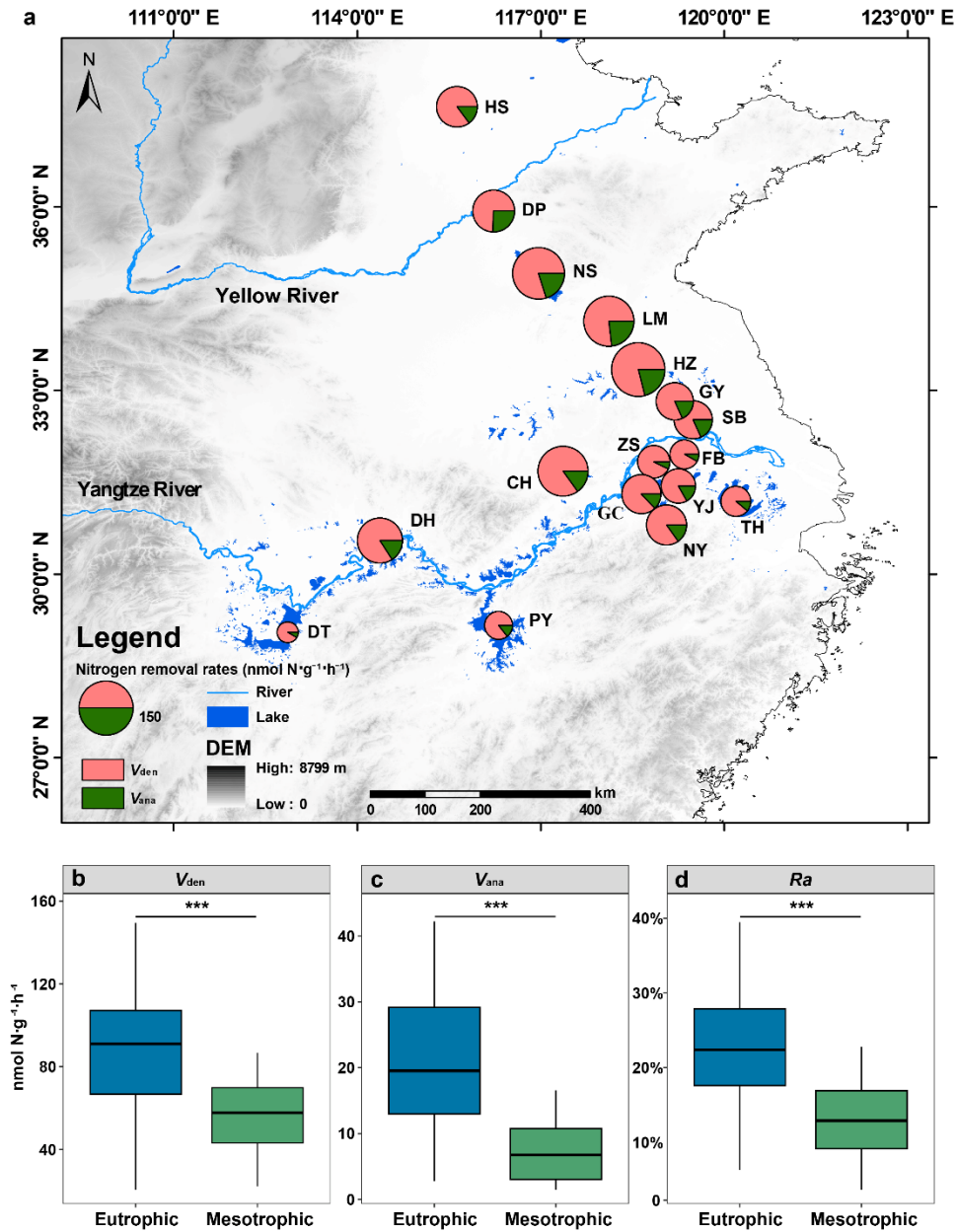
Response: Thank you for the reminder. We greatly appreciate your valuable suggestion and would like to further discuss it with you. As we understand, the reviewer's suggestion refers to the inorganic anions nitrite (NO₂⁻-N), nitrate (NO₃⁻-N), and orthophosphate (PO₄³⁻-P). These species are conventionally expressed in molar units (mol/L), whereas our manuscript uses mass concentrations (mg/L). After further consideration and reviewing other papers (e.g., DOI: 10.1016/j.watres.2023.120047, mentioned in "2.2. Measurement of environmental factors"), as well as consulting Chinese environmental protection industry standards for water quality determination (e.g., HJ/T 197-2005, HJ/T 346-2007). Nevertheless, we remain uncertain whether our proposed modifications fully align with common practices in the field. We kindly seek your understanding and welcome any further suggestions you may have on this matter.

3. 574 and L.588. There are some formatting errors in the bibliography. Please thoroughly cross-check and correct the reference formats one by one according to the journal's official citation guidelines.

Response: We thank the reviewer for pointing this out. We have carefully cross-checked the entire reference list against the journal's official citation guidelines. All references are now uniformly formatted in accordance with the journal's requirements. These changes will be uniformly incorporated into the revised manuscript.

57 4. Figure 2. This figure appears to be incomplete. Please check and correct this.

58 **Response:** Thank you for the reminder. We have carefully checked Figure 2 and
 59 completed the missing parts. The changes here are as follows:



60

61 **Figure 2** Spatial patterns of nitrogen removal rates in the Eastern China Plain lakes (a)
 62 and their variations across sites with different trophic states (b–d). Note: (1) The size
 63 of the pie charts is proportional to the total nitrogen removal potential (the sum of
 64 mean denitrification and anammox rates). (2) Statistical significance was determined
 65 using the Mann-Whitney U test with Benjamini-Hochberg correction, indicated by

asterisks (***: $p < 0.001$). (3) SB, Lake Shaobo; GY, Lake Gaoyou; HZ, Lake Hongze; LM, Lake Luoma; NS, Lake Nansi; DP, Lake Dongping; HS, Lake Hengshui; TH, Lake Taihu; FB, Fangbian reservoir; YJ, Yaojia reservoir; ZS, Zhongshan reservoir; NY, Lake Nanyi; GC, Lake Gucheng; CH, Lake Chaohu; PY, Lake Poyang; DH, Lake Donghu; DT, Lake Dongting.

5. Figure 4c. You need to explicitly clarify what the indices in the figure represent. Generally, "Observed species" refers to the number of species or the ASV (Amplicon Sequence Variant) count. Stating this directly would significantly improve clarity and ease of understanding for the reader.

Response: We thank the reviewer for this constructive suggestion, which greatly improves the clarity of our figures. We believe that the "Observed species" you referred to is likely Figure 4b rather than Figure 4c, is that correct? In the caption of Figure 4b, we have explicitly clarified that "Observed species" denotes the number of observed Amplicon Sequence Variants (ASVs). The revised caption is provided below:

Figure 4 Sediment microbial community characteristics in the Eastern China Plain lakes. (a) Taxonomic composition of the top 20 phyla. (b) Alpha-diversity indices, where "Observed species" denotes the number of observed amplicon sequence variants (ASVs). (c) Microbial beta-diversity based on PCoA. (d) LEfSe analysis identifying discriminative taxa at the phylum level between groups. Note: SB, Lake Shaobo; GY, Lake Gaoyou; HZ, Lake Hongze; LM, Lake Luoma; NS, Lake Nansi; DP, Lake Dongping; HS, Lake Hengshui; TH, Lake Taihu; FB, Fangbian reservoir; YJ, Yaojia reservoir; ZS, Zhongshan reservoir; NY, Lake Nanyi; GC, Lake Gucheng; CH, Lake Chaohu; PY, Lake Poyang; DH, Lake Donghu; DT, Lake Dongting.

6. The supplementary materials contain a wealth of detailed information, which is commendable. However, there is some noticeable overlap between these materials and the data already deposited in public repositories. To avoid creating an

unnecessary reading burden for the audience, please consider streamlining and condensing the volume of the supplementary materials.

Response: We sincerely thank the reviewer for this constructive suggestion to streamline the supplementary materials. We completely agree that reducing redundancy will improve readability for our audience. In response to your comment, we have carefully reviewed the supplementary materials and removed sections containing redundant data that are already fully accessible in public repositories. Meanwhile, we have retained the tables presenting regional mean values, as these summaries are essential for facilitating readers' direct understanding and interpretation without requiring them to parse raw repository files. Thank you again for your suggestion.

7. Specifically, where are the spatial constraints manifested, and how exactly do they exert their limitations? Please strengthen the discussion and elaboration regarding these aspects in the manuscript.

Response: We sincerely thank the reviewer for this insightful question, which has helped us significantly improve the depth and clarity of our discussion. While Section 4.3 of our original manuscript touched upon the specific manifestations of spatial constraints and their limitations, the descriptions were not sufficiently explicit. In response to your constructive suggestion, we have thoroughly revised and polished Section 4.3 to provide a clearer and more rigorous explanation. The changes here are as follows:

Macro-ecological studies consistently demonstrate that nitrogen cycling is significantly correlated with geographic factors such as latitude, longitude, and altitude (Fuhrman et al., 2008; Tu et al., 2019). Our findings reveal that latitudinal gradients and spatial structural variables (e.g., PCNM2) constitute the primary macro-scale background governing nitrogen removal processes across the Eastern China Plain lakes (Figs. 5, S6). Specifically, latitude acts as a robust proxy for climatic

drivers—including temperature, precipitation, and seasonal irradiance (Ding et al., 2025)—consistent with the subtropical to temperate monsoon climate characteristics of this region (Method S1, Tab. S3). Such climatic variations reshape microbial habitat quality by modulating hydraulic residence times, organic matter mineralization pathways, and the thermal, dissolved oxygen, and nutrient stratification regimes (Manzoni et al., 2014; Hu et al., 2020). These shifts subsequently exert profound effects on functional gene abundance, highlighting the critical roles of dispersal limitation and spatial heterogeneity in shaping microbial biogeography (Du et al., 2023). Notably, the response of V_{ana} to latitude was significantly stronger than that of V_{den} (Fig. 5c, g). This discrepancy is likely attributable to the broader metabolic versatility and phylogenetic distribution of denitrifying communities. Conversely, anammox communities exhibit narrower ecological niche specialization and heightened sensitivity to macro-environmental fluctuations, rendering their spatial distribution subject to stricter geographic constraints (Gao et al., 2018).

Despite the study area being situated in Eastern China—a region characterized by intense anthropogenic disturbance (Fig. S7)—the Human Activity Intensity (HAI) across various buffer zone scales showed no direct, significant effects on nitrogen removal rates (Fig. 5A, B). This decoupling suggests that anthropogenic influences undergo a sequential process of environmental filtering followed by micro-environmental redistribution (Keck et al., 2025). Specifically, while human activities drive external loadings of nitrogen, phosphorus, and organic matter, thereby altering lake trophic states and sediment characteristics (Wei et al., 2024), it is the local water-sediment physicochemical properties that constitute the immediate habitat for microbial metabolism. These local factors directly regulate functional genes and nitrogen removal rates through substrate availability and redox conditions, acting as the primary proximate drivers responsible for the spatial heterogeneity observed in this study (Fig. 6a, c).

Furthermore, we have expanded the discussion in Section 4.1 to further elucidate how these spatial constraints operate and influence the ecological processes. We believe

these revisions now offer readers a much more comprehensive understanding of the mechanisms behind these spatial limitations. The changes here are as follows:

However, the contribution of anammox was non-negligible in specific systems (Fig. 2), underscoring its distinct environmental dependency (Zhu et al., 2013). Furthermore, variations in latitudinal gradients among lakes introduce climatic differences such as regional temperature and precipitation. These differences, coupled with variations in geological backgrounds and watershed runoff generation and concentration processes, collectively highlight the critical regulatory role of spatial constraints—exerted by local environments—in shaping nitrogen removal processes.

Thank you again for your valuable comments. These comments will significantly enhance the clarity and quality of the paper. We have made the necessary revisions to address your comments and look forward to your further review.

Reviewer #2:

The manuscript investigates the strong effects of varying lake trophic statuses on nitrogen removal processes (denitrification and anammox) within the large-scale environmental context of the Eastern China Plain lakes. Overall, this is a well-conducted study—the methodology is comprehensive and sound, and the data are compelling. Furthermore, the conclusions offer valuable complements and insights for related research in this field. However, I have a few comments below to be addressed within the text. Additionally, there are some issues (mostly minor) with a few figures. The language of the manuscript is generally good, with only a few instances requiring proofreading and editing. Specific comments:

1. The manuscript draws an important conclusion regarding the decoupling between functional gene abundance and actual nitrogen removal rates. While this is a significant finding, I have a query: could dissimilatory nitrate reduction to ammonium (DNRA) potentially influence this conclusion? This potential impact should be discussed. Please discuss the uncertainties introduced by DNRA into the two nitrogen removal processes.

Response: We sincerely thank the reviewer for this insightful and valuable question. We agree that dissimilatory nitrate reduction to ammonium (DNRA) can introduce uncertainties regarding the fate of nitrate and nitrogen substrate competition. Specifically, while DNRA retains nitrogen within the system as ammonium rather than removing it as gas, its competition for $\text{NO}_3\text{-N}$ substrate can potentially influence both denitrification and anammox pathways. In our revised manuscript, we have expanded the Discussion section to address this point by incorporating a thorough qualitative evaluation of how DNRA might introduce uncertainties into nitrogen removal processes and its potential competition for nitrate fates. The changes are as follows:

Nitrogen and dissolved organic carbon (DOC) serve as critical metabolic substrates for nitrogen-removing microorganisms and act as key environmental factors of these

processes (Liu et al., 2018; Pascal et al., 2025). Our results demonstrated that both V_{den} and V_{ana} were significantly elevated in eutrophic sites compared to mesotrophic ones (Fig. 2b, c). Subjected to prolonged exogenous nutrient loading, the Eastern China Plain lakes generally sustain high substrate availability (Fig. S1) (Zhou et al., 2022). This enrichment likely enhances microbial substrate utilization efficiency, thereby stimulating and accelerating the corresponding nitrogen cycling processes (Zhou et al., 2021). Notably, previous studies have demonstrated potential substrate competition between DNRA and both denitrification and anammox processes. However, the reaction flux of DNRA is typically one to two orders of magnitude lower than that of denitrification. In typical eutrophic lakes, where $\text{NO}_3\text{-N}$ is generally abundant, the impact of this substrate competition on overall nitrogen removal rates remains relatively limited (Jiang et al., 2023). We recommend that future studies incorporate multi-isotope tracing techniques to achieve comprehensive, multi-pathway monitoring of the nitrogen cycle. Furthermore, organic carbon and ionic conditions act as critical electron donors for denitrification (Yoon et al., 2015). The effective supply of electrons (Fig. S1c–f) directly fueled elevated V_{den} in lakes such as Nansi and Luoma (Fig. 2a, Tab. S5). However, high concentrations of SO_4^{2-} may exert an inhibitory effect on denitrification through competitive inhibition for electron donors or sulfide toxicity (Jiang et al., 2020), which likely accounts for the relatively lower V_{den} observed in Lake Hengshui (Fig. S1f).

2. The elucidation of the driving mechanisms behind the nitrogen removal processes relies heavily on correlation analysis and structural equation modeling (SEM), resulting in a relative lack of causal verification. While it may be challenging, please supplement with relevant causal evidence if possible. Alternatively, you should add a prospective discussion on future research directions to address this limitation.

Response: We thank the reviewer for this constructive suggestion. As we currently lack direct experimental data for causal validation, we have addressed this limitation

by adding a prospective suggestion in the revised manuscript. Specifically, we suggest that future studies should further validate the driving mechanisms of nitrogen removal using key enzyme activity assays and targeted controlled experiments. We believe this addition provides a constructive direction for future research in this field. The changes are as follows:

Elucidating the driving mechanisms of sediment nitrogen removal is crucial for understanding the resilience of lacustrine ecosystems to eutrophication. This study reveals that while denitrification dominates nitrogen removal in the Eastern China Plain lakes, anammox serves as a critical, highly environmentally dependent complementary process. Notably, eutrophication significantly amplified both the overall nitrogen removal capacity and the relative contribution of anammox, with local physicochemical properties and microbial communities demonstrating a pronounced joint driving effect on these processes. The observed decoupling among microbial community, functional gene abundance, and nitrogen removal rates highlights the intricate complexity of microbial responses to environmental perturbations. This complexity is further evidenced by the stark contrast between the functional redundancy of denitrifying bacteria and the ecological niche specificity of anammox bacteria. Furthermore, we demonstrate that macro-scale factors (spatial, climatic, and anthropogenic) indirectly drive nitrogen removal by reshaping local water-sediment physicochemical properties, rather than acting as direct determinants of process rates. These findings enrich our fundamental understanding of nitrogen removal, particularly the anammox process, and its multidimensional drivers in the Eastern China Plain lakes. Given the sensitivity of nitrogen removal processes to habitat conditions, we suggest validating driving mechanisms using key enzyme activity assays and targeted controlled experiments. Furthermore, we advocate that future lake management strategies must rigorously account for the overarching constraints jointly imposed by trophic status, alongside spatial and climatic gradients, to ensure the sustainability of nitrogen purification ecosystem services.

3. Fig.1: The approximate locations of the different lakes must be clearly marked on the map. Although they are mentioned in subsequent figures, omitting them in the study area map does not conform to standard publication practices.

Response: We thank the reviewer for pointing this out. We have updated Figure 1 to clearly mark the approximate locations of the different study lakes in accordance with standard publication practices. The changes are as follows:

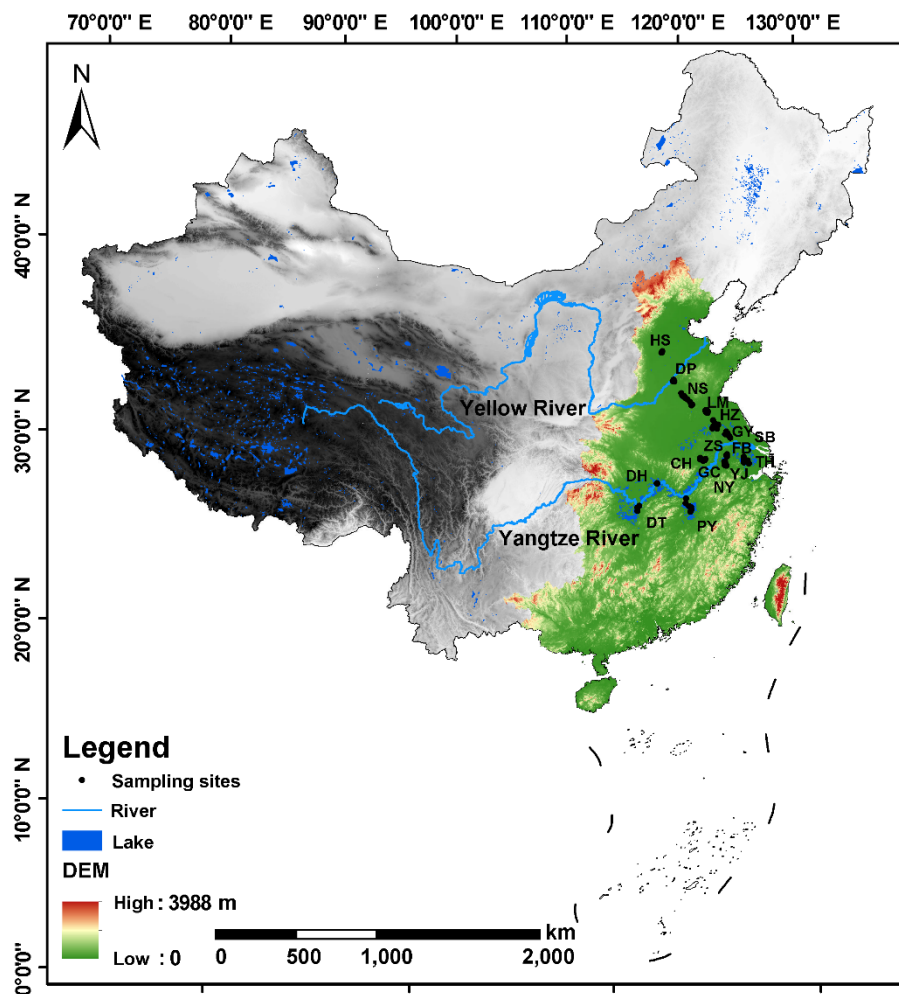


Figure 1 Overview of the Eastern China Plain lakes region and distribution of sampling sites.

Note: SB, Lake Shaobo; GY, Lake Gaoyou; HZ, Lake Hongze; LM, Lake Luoma; NS, Lake Nansi; DP, Lake Dongping; HS, Lake Hengshui; TH, Lake Taihu; FB, Fangbian reservoir; YJ, Yaojia reservoir; ZS, Zhongshan reservoir; NY, Lake Nanyi; GC, Lake Gucheng; CH, Lake Chaohu; PY, Lake Poyang; DH, Lake Donghu; DT, Lake Dongting.

4. Fig.2 (and all other figures): All abbreviations used in the figures must be explicitly defined; please provide their full terms in the figure captions. Additionally, the English phrasing in the captions could be further polished.

Response: We thank the reviewer for this helpful suggestion. We have thoroughly checked all figures throughout the manuscript, explicitly defined all abbreviations in their respective figure captions, and further polished the English phrasing to ensure maximum clarity and professionalism. These changes will be uniformly incorporated into the revised manuscript.

5. Fig.5: The terms " V_{ana} " and " V_{den} " in the axis titles are not properly formatted as subscripts. Please correct them to standard variable notation.

Response: We thank the reviewer for this constructive suggestion. We have carefully checked and revised Figure 5 to ensure that " V_{ana} " and " V_{den} " in the axis titles are properly formatted as standard subscripts. The updated figure has been integrated into the revised manuscript.

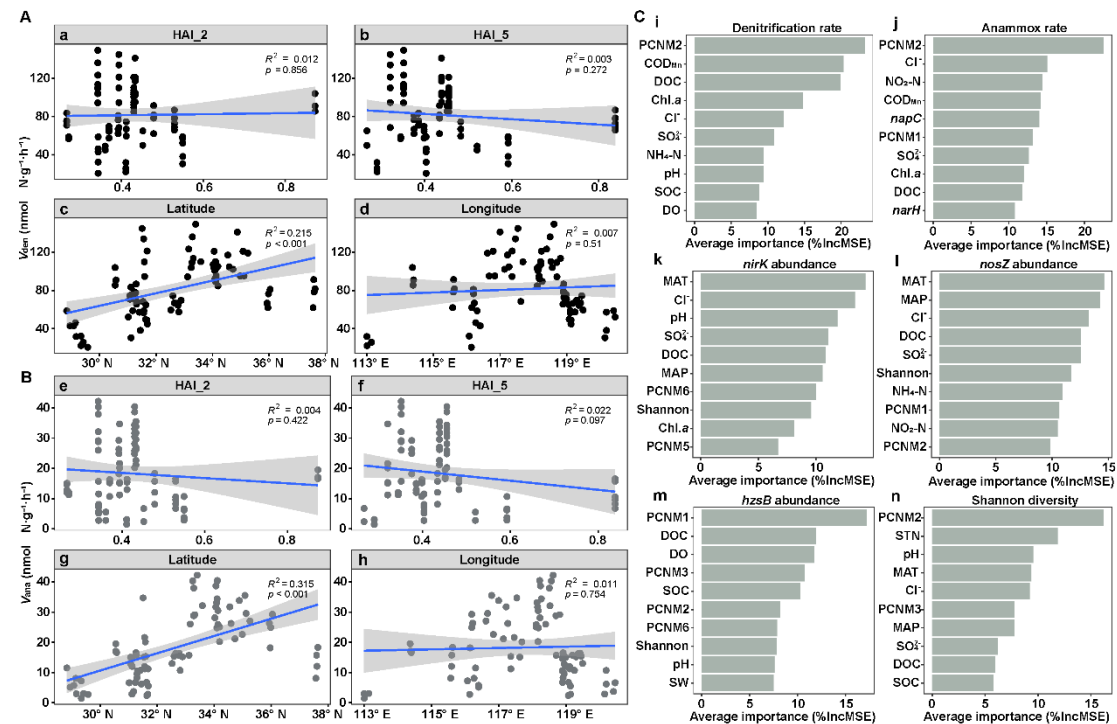


Figure 5 Drivers of nitrogen removal processes in the Eastern China Plain lakes.

Linear regressions of denitrification (A) and anammox (B) rates against Human Activity Intensity (HAI) and geographic location. The solid lines represent ordinary least squares (OLS) linear regressions, with statistical significance evaluated using two-tailed Student's t-tests. (C) Key predictors of nitrogen removal rates, functional gene abundance, and microbial Shannon diversity identified via Random Forest regression models. Note: PCNM, Principal Coordinates of Neighbor Matrices; DOC, dissolved organic carbon; SOC, sediment organic carbon; DO, dissolved oxygen; MAT, mean annual temperature; MAP, mean annual precipitation; SW, sediment water content; STN, sediment total nitrogen.

6. Fig.5A: Please specify the statistical testing methods used for the regression analysis. And, Fig.5C: The resolution of this panel needs to be improved.

Response: We thank the reviewer for pointing this out. Based on our data analysis script, we have explicitly specified the statistical testing methods used for the regression analysis in the figure caption. For Figure 5C, we have re-exported the plots at a high resolution (300 dpi, TIFF format) to ensure optimal visual clarity in the revised manuscript. The changes are as follows:

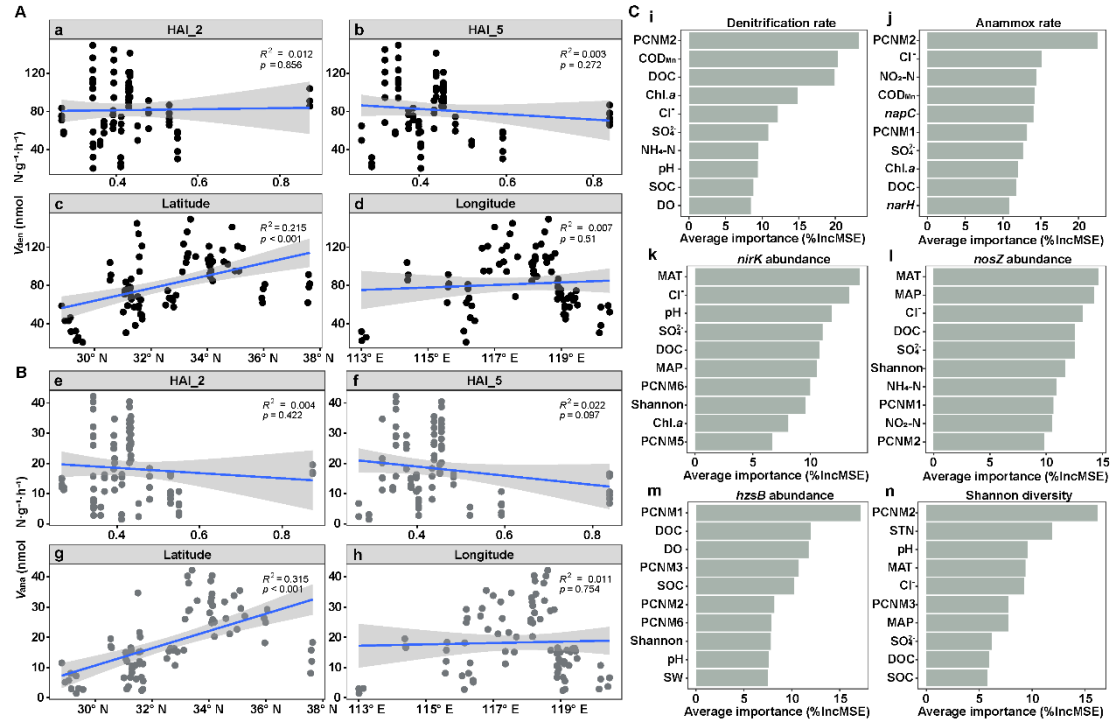


Figure 5 Drivers of nitrogen removal processes in the Eastern China Plain lakes. Linear regressions of denitrification (A) and anammox (B) rates against Human Activity Intensity (HAI) and geographic location. The solid lines represent ordinary least squares (OLS) linear regressions, with statistical significance evaluated using two-tailed Student's t-tests. (C) Key predictors of nitrogen removal rates, functional gene abundance, and microbial Shannon diversity identified via Random Forest regression models. Note: PCNM, Principal Coordinates of Neighbor Matrices; DOC, dissolved organic carbon; SOC, sediment organic carbon; DO, dissolved oxygen; MAT, mean annual temperature; MAP, mean annual precipitation; SW, sediment water content; STN, sediment total nitrogen.

7. Fig.6: Please include the definitions of the path coefficients for the model in the figure caption to ensure full clarity and interpretability for the readers.

Response: We sincerely thank the reviewer for these valuable suggestions to improve our manuscript. For Figure 6, we have expanded the figure caption to explicitly define the path coefficients, indicating that they represent standardized effects that quantify the strength and direction of hypothesized causal relationships between latent

variables. The changes are as follows:

Figure 6 Causal mechanisms driving nitrogen removal in the Eastern China Plain lakes. Partial Least Squares Path Modeling (PLS-PM) disentangling the direct and indirect effects of multiple drivers on denitrification (a) and anammox (c) rates, alongside their standardized total effects (b, d). Note: (1) Arrows represent significant unidirectional causal relationships. Red and blue arrows denote positive and negative effects, respectively. Numbers adjacent to arrows indicate standardized path coefficients (*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$), represent standardized regression coefficients that quantify the relative strength and direct/indirect effects of hypothesized causal relationships. (2) R^2 values represent the proportion of variance explained for each dependent variable. (3) Goodness of Fit (GoF) is displayed at the top of each model. (4) To ensure model robustness, indicator variables for each latent construct were selected based on ecological relevance and preliminary collinearity screening, with measurement validity confirmed through outer model loadings and cross-loadings (retaining indicators with loadings > 0.70 where applicable). (5) PCNM, Principal Coordinates of Neighbor Matrices; DOC, dissolved organic carbon; DO, dissolved oxygen; MAT, mean annual temperature; MAP, mean annual precipitation; HAI, Human Activity Intensity.

8. Lines 105–110: The reliability of field survey data (such as water transparency and parameters measured by portable water quality instruments) is highly dependent on the number of measurement replicates. Therefore, please provide specific details regarding the number of replicates and related methodological info.

Response: We thank the reviewer for this helpful suggestion. To ensure data reliability and minimize random errors, we have updated the Methodology section to explicitly state that triplicate measurements were performed for all field survey data—including water transparency and portable water-quality parameters—with their average values

taken for subsequent analyses. The changes are as follows:

Water temperature (WT), electrical conductivity (EC), dissolved oxygen (DO), and pH were measured on-site (YSI Professional Plus, USA). Water depth (WD) and secchi depth (SD) were determined using a Speedtech SM-5 depth sounder and Secchi disk, respectively. To minimize random errors, triplicate measurements were performed, and their average was taken to ensure data reliability. Detailed analytical parameters and methods for water and sediment samples are provided in Tab. S2. Trophic status of the lake water was evaluated using the Comprehensive Trophic Level Index (TLI) (Supplementary Method S2).

9. Lines 156–159: Please briefly explain how variable selection or dimensionality reduction was performed in the Partial Least Squares Path Modeling (PLS-PM) to verify and ensure the statistical robustness of the model.

Response: We sincerely thank the reviewer for these valuable suggestions to improve our manuscript. For Lines 156–159, based on our PLS-PM workflow and statistical evaluation, we have updated the text to clarify how variable selection and dimensionality reduction were implemented. The changes are as follows:

Figure 6 Causal mechanisms driving nitrogen removal in the Eastern China Plain lakes. Partial Least Squares Path Modeling (PLS-PM) disentangling the direct and indirect effects of multiple drivers on denitrification (a) and anammox (c) rates, alongside their standardized total effects (b, d). Note: (1) Arrows represent significant unidirectional causal relationships. Red and blue arrows denote positive and negative effects, respectively. Numbers adjacent to arrows indicate standardized path coefficients (*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$), represent standardized regression coefficients that quantify the relative strength and direct/indirect effects of hypothesized causal relationships. (2) R^2 values represent the proportion of variance explained for each dependent variable. (3) Goodness of Fit (GoF) is displayed at the top of each model. (4) To ensure model robustness, indicator variables for each latent

construct were selected based on ecological relevance and preliminary collinearity screening, with measurement validity confirmed through outer model loadings and cross-loadings (retaining indicators with loadings > 0.70 where applicable). (5) PCNM, Principal Coordinates of Neighbor Matrices; DOC, dissolved organic carbon; DO, dissolved oxygen; MAT, mean annual temperature; MAP, mean annual precipitation; HAI, Human Activity Intensity.

Thank you again for your valuable comments. These comments will significantly enhance the clarity and quality of the paper. We have made the necessary revisions to address your comments and look forward to your further review.