Author Responses:

*We deeply appreciate the thorough and helpful comments from the Reviewer. We will carefully revise the manuscript (EGUSPHERE-2026-216) carefully following each comment from the reviewer. The response (**in bold**) was made to each comment, and we also marked changes, which is helpful to find how we changed.*

RC1's comments to the Author:

This manuscript addresses an important and timely topic: how estuarine mixing reshapes the partitioning, composition, and potential bioavailability of organic nitrogen. The distinction among PON, CON, and tDON is meaningful, and the comparison between biologically active mixing (BAM) and biologically inhibited mixing (BIM) is a potentially strong feature of the study. The analytical framework is also relatively comprehensive, combining TFF-based fractionation, fluorescence signatures, FTIR, stable isotopes, and ancillary particle and enzyme measurements. Overall, the study has clear potential and is built on a potentially publishable dataset.

Response:

We sincerely appreciate your positive comments and constructive evaluation of our work.

Specific Comments

Question 1: The central message should be sharpened. At present, the manuscript tries to emphasize too many things at once: ON partitioning, DON bioavailability, PON refractoriness, nitrogen sequestration, eutrophication risk, and a broadly generalized estuarine framework. This weakens the paper's main contribution. In my view, the strongest and most defensible message is that estuarine mixing generates a dual response: humic-like ON is preferentially transferred into particulate/refractory pools through adsorption and flocculation, while biological processing simultaneously alters the residual dissolved pool toward a more potentially labile composition. I encourage the authors to reorganize the abstract, discussion, and conclusions around this narrower and clearer core advance.

Response:

Thank you for this insightful and constructive suggestion. We agree that the central message of the manuscript should be more focused. In the revised manuscript, we have reorganized the Abstract, Discussion, and Conclusions to focus on the dual response during estuarine mixing: physicochemical processes preferentially transfer humic-like ON into refractory particulate pools, while biological processing simultaneously shifts the residual dissolved pool toward a more potentially labile composition. We have also reframed nitrogen sequestration, eutrophication risk, and broader estuarine implications as downstream implications rather than co-equal central messages. These revisions have been incorporated into the Abstract, Section 4.4, and Conclusions.

The revised text reads:

[Abstract, L16-29] "Two concurrent but contrasting pathways were identified. First, salt-induced flocculation and adsorption (i.e. physicochemical processes) preferentially transferred a large fraction (63 ± 11%) of humic-like components, mainly terrestrial refractory compounds, into the particulate phase, thereby increasing PON. The isotopic enrichment ($\delta^{13}C$, $\delta^{15}N$) and elevated C/N ratios in PON further suggested the re-adsorption of biologically-modified and $\delta^{15}N$-enriched DON onto particles, enhancing PON refractoriness. Second, biological activity promoted the degradation of residual humic-like components (especially microbial C3), producing labile LMW-DON and ammonium; a strong negative correlation between humic-like and protein-like fluorescence in control treatments evidenced this conversion. Overall, estuarine mixing generates a dual response: physicochemical processes channel humic-like ON into refractory particulate pools, while biological processing simultaneously shifts the residual dissolved pool toward a more labile composition. This mechanistic framework advances our understanding of non-conservative ON behavior across estuarine salinity gradients and has implications for assessing nitrogen bioavailability in receiving coastal waters";

[Section 4.4, L430-434] "This study identifies a dual response during estuarine mixing: physicochemical removal of humic-like ON into refractory particulate pools

and biological enhancement of residual DON bioavailability. This dual mechanism likely operates across estuaries worldwide, with relative intensities modulated by regional hydrodynamics, sediment characteristics, and the strength of coupled biological (microbial degradation, phytoplankton uptake) and physicochemical (adsorption/flocculation) processes";

[Section 4.4, L458-459] "Beyond the dual transformation mechanism itself, the resulting changes in ON composition have potential implications for downstream nitrogen bioavailability. Different ON forms…";

[Conclusions, L536-537] "This study reveals a dual response of organic nitrogen to estuarine mixing, in which physicochemical and biological processes simultaneously but differently reshape ON composition, bioavailability, and fate";

[Conclusions, L547-551] "These results highlight that the dual response of ON during estuarine mixing, characterized by refractory PON accumulation versus enhanced DON bioavailability, cannot be captured by bulk nitrogen metrics alone, underscoring the value of ON speciation in estuarine nitrogen assessments. Future research should test whether this dual mechanism generalizes across seasons and estuary types, and quantify how the balance between physicochemical and biological pathways modulates the bioavailability of ON exported to coastal waters".

Question 2: The manuscript interprets increasing protein-like fluorescence, higher FI and BIX, lower $SUVA_{254}$, and NH_4^+ accumulation as evidence for enhanced DON bioavailability. These are useful and suggestive indicators, but they remain indirect proxies. The study does not include direct DON bioassays, uptake experiments, or response experiments demonstrating that the transformed dissolved nitrogen pool is more readily used by microbes or phytoplankton. Therefore, I suggest that the manuscript consistently distinguish between demonstrated transformation and inferred increases in potential bioavailability.

Response:

Thank you for this important comment. We agree that FI, BIX, $SUVA_{254}$, protein-like fluorescence, NH_4^+ accumulation, and enzyme activities are indirect

indicators rather than direct measurements of DON utilization. In the revised manuscript, we have consistently distinguished between demonstrated compositional transformation and inferred increases in potential bioavailability. We have also softened related statements by using the term "potential bioavailability" where appropriate and added a limitation noting that direct DON bioassays, isotope-labeled uptake experiments, or microbial/phytoplankton growth assays are needed to confirm biological utilization of the transformed DON pool.

The revised text reads:

[Section 4.2, L297-303] "In the BAM treatments, accumulation of tDON was accompanied by increases in FI and BIX and a concurrent decrease in $SUVA_{254}$ (Fig. 3a–c).These optical changes collectively suggest that estuarine mixing processes shifted DON composition toward forms with potentially higher bioavailability. We note, however, that these indicators are indirect proxies; direct uptake experiments would be needed to confirm biological utilization of the transformed DON pool";

[Section 4.5, L485-487] "In particular, direct DON bioassays (e.g., isotope-labeled uptake experiments or microbial/phytoplankton growth bioassays) are needed to validate the inferred increases in DON bioavailability that were based on indirect optical and chemical proxies in this study";

Question 3: L84 provides coordinates for the riverine and marine endmembers and notes that the seawater site was located approximately 40 km downstream of the river mouth, but there is no figure showing the sampling region, the river mouth, and the relative positions of the two endmembers. A map is a basic but important element for a study of this type. It would help readers assess the geographic setting, the representativeness of the chosen endmembers, and the realism of the experimental river–sea mixing framework. I strongly recommend adding a location figure.
Response:

Thank you for this suggestion. We have added a new location map as Figure S1, showing the study area, the Ziya Xinhe River, the river mouth, and the relative positions of the riverine and marine endmembers. The supplementary figures have

been renumbered accordingly..

The added image is as follows:

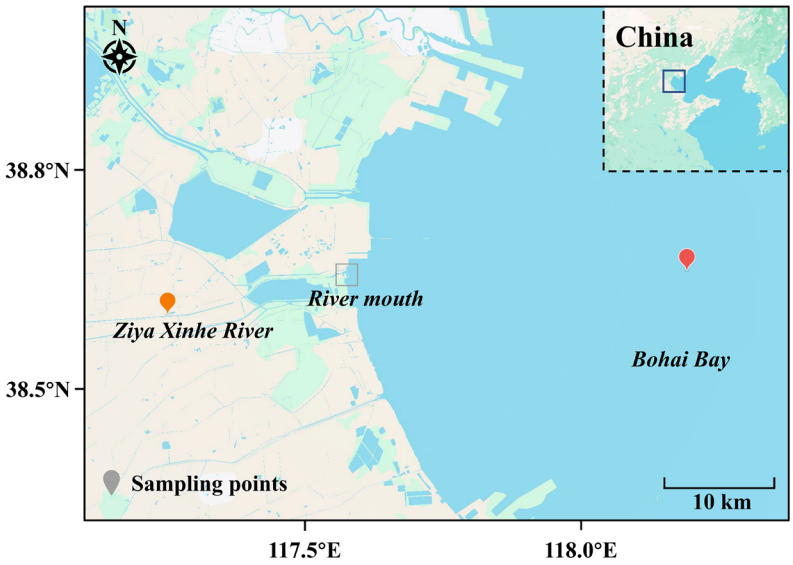

Figure S1. Location of the study area and sampling sites in Bohai Bay, China. The riverine endmember (orange) and marine endmember (red) are approximately 40 km apart.

Question 4: L92-93 states that river water and seawater were mixed at 10:0, 8:2, 6:4, 5:5, 4:6, 2:8, and 0:10, but the manuscript does not explain why these particular ratios were selected. Were they chosen to represent specific salinity zones in the estuary, to match field observations, or simply to generate a broad mixing gradient? This matters because the interpretation of non-conservative behavior may depend on how well the critical salinity range is resolved. The authors should provide a clear rationale for this design choice.

Response:

Thank you for raising this point. We have added a clear rationale for the selected mixing ratios in the Materials and Methods section. These ratios were chosen to span the full river–sea salinity gradient, with relatively finer resolution in the low-to-mid salinity range where non-conservative processes such as flocculation and adsorption–desorption are often most pronounced (Sholkovitz, 1976; Canuel and Hardison, 2016; Osburn et al., 2016). This design is also consistent with mixing schemes used in previous estuarine simulation studies, and relevant references (e.g.,

Sholkovitz, 1976; Schneider et al., 2016) have been added.

The revised text reads:

[Section 2.2, L100-103] "These ratios were selected to span the full salinity gradient with finer resolution in the low-to-mid salinity range, where non-conservative behavior is most pronounced in estuarine systems, and are consistent with mixing designs adopted in previous estuarine simulation studies (Sholkovitz, 1976; Schneider et al., 2016)".

Canuel, E. A. and Hardison, A. K.: Sources, Ages, and Alteration of Organic Matter in Estuaries, Annu. Rev. Mar. Sci., 8, 409–434, https://doi.org/10.1146/annurev-marine-122414-034058, 2016.

Osburn, C. L., Handsel, L. T., Peierls, B. L., and Paerl, H. W.: Predicting Sources of Dissolved Organic Nitrogen to an Estuary from an Agro-Urban Coastal Watershed, Environ. Sci. Technol., 50, 8473–8484, https://doi.org/10.1021/acs.est.6b00053, 2016.

Schneider, A. B., Koschinsky, A., Kiprotich, J., Poehle, S., and Do Nascimento, P. C.: An experimental study on the mixing behavior of Ti, Zr, V and Mo in the Elbe, Rhine and Weser estuaries, Estuar. Coast. Shelf Sci., 170, 34–44, https://doi.org/10.1016/j.ecss.2015.12.002, 2016.

Sholkovitz, E. R.: Flocculation of dissolved organic and inorganic matter during the mixing of river water and seawater, Geochim. Cosmochim. Acta, 40, 831–845, https://doi.org/10.1016/0016-7037(76)90035-1, 1976.

Question 5: Figure 4 presents direct experimental observations, including FI, SUVA254, BIX, FTIR, POC, and C/N changes along the salinity gradient. They are primary results. Moving Figure 4 to the Results would also improve the structure of the paper.

Response:

We agree with the reviewer. In the revised manuscript, the former Figure 4 has been moved from the Discussion to the Results section and renumbered as Figure 3. The descriptive results for FI, $SUVA_{254}$, BIX, and FTIR spectra have been added to Section 3.1, while the POC and C/N results have been added to Section 3.2. The Discussion now focuses on the mechanistic interpretation of these observations.

The revised text reads:

[Section 3.1, L193-197] "Optical indices of DON also varied along the salinity gradient (Fig. 3a–d). In all treatments, the fluorescence index (FI) and biological index (BIX) increased with rising salinity (Fig. 3a, c), while the specific UV absorbance at 254 nm ($SUVA_{254}$) decreased (Fig. 3b). FTIR spectra of the BAM treatments revealed an increase in amide-related absorption peaks (N–H and C=O stretching) and a decrease in humic-associated nitro group ($–NO_2$) signals with increasing salinity (Fig. 3d) ";

[Section 3.2, L201-202] "Moreover, the proportion of PON and C/N ratio increased with rising salinity in both BAM and BIM treatments (Fig. 3e, f) ";

[Section 4.2, L297-298] "In the BAM treatments, accumulation of tDON was accompanied by increases in FI and BIX and a concurrent decrease in $SUVA_{254}$ (Fig. 3a–c) ";

[Section 4.2, L344-346] "These transformations were further supported by FTIR spectroscopy, in which the increase in amide-related peaks and decrease in humic-associated $–NO_2$ signals reflect partial removal of humic-like components and concurrent enrichment of protein-like material in the BAM treatments (Fig. 3d) ";

[Section 4.3, L376-377] "As discussed, PON content exceeded conservative mixing predictions in both treatments, with concurrent increases in C/N ratios (Fig. 3e, f) ".

Question 6: The manuscript ends by linking its findings to coastal eutrophication risk and bloom development. This is a reasonable broader implication, but it extends beyond what is directly shown in a short-term controlled mixing experiment based on one summer sampling event. The transformed ON pool may indeed have ecological implications, but the paper does not directly demonstrate downstream biological responses in the receiving coastal system. I recommend framing this as a likely implication or testable hypothesis, not as an outcome already established by the present study.

Response:

Thank you for this constructive comment. We agree that the present short-term

controlled mixing experiment does not directly demonstrate downstream biological responses in receiving coastal waters. In the revised manuscript, we have softened the related statements and reframed coastal eutrophication risk and bloom development as potential implications or testable hypotheses rather than established outcomes. We have also added a limitation stating that seasonal field observations, bioassays, mesocosm experiments, and coupled biogeochemical modeling are needed to verify these ecological consequences.

The revised text reads:

[Section 4.1, L274-276] "Our findings thus suggest that the enhanced bioavailability of DON during estuarine mixing could potentially influence downstream primary productivity and phytoplankton community structure";

[Section 4.4, L450-454] "Nevertheless, DON remains the dominant fraction of the ON pool (greater than 71%) during DIN-depleted periods or long residence time, and its modified forms may act as a nitrogen reservoir that could potentially fuels coastal algal blooms. Of course, this inference requires validation through in situ monitoring and bioassay experiments conducted in coastal waters";

[Section 4.4, L461-465] "For example, the increased availability of ON may lead to its transport into coastal waters, where it could serve as a supplementary nitrogen source, potentially elevating the risk of algal blooms. Such effects, if sustained at ecosystem scales, could cascade through food webs and alter the ecological stability and function of estuarine and coastal ecosystems";

[Section 4.4, L471-473] "The potential role of mixing-transformed ON in the increased frequency and distribution of algal blooms in coastal regions therefore deserves further investigation in future field studies";

[Section 4.5, L487-489] "More broadly, establishing direct evidence linking mixing-induced ON transformations to downstream biological responses such as bloom initiation and trophic cascades would require seasonal field observations, mesocosm experiments, and coupled biogeochemical modeling".

Technical Corrections

Question 7: L87 “dissolved oxygen (DO) were measured in Table S1”

Response:

Revised.

The revised text reads:

[Section 2.1, L94] "dissolved oxygen (DO) were measured (shown in Table S1)".

Question 8: L171-172 “While in the BAM treatments” it should almost certainly refer to BIM?

Response:

Revised.

The revised text reads:

[Section 3.2, L223] “While in the BIM treatments, …”.

Question 9: There is an apparent inconsistency regarding Figure 4d: the figure caption says FTIR is shown for BAM only, whereas the Discussion interprets the FTIR trends as occurring in the BIM.

Response:

Thank you for pointing out this inconsistency. We have corrected the text to make it consistent with the figure caption. The FTIR results are now described as being from the BAM treatments only.

The revised text reads:

[Section 4.1, L344-346] “These transformations were further supported by FTIR spectroscopy, which showed an increase in amide-related peaks (N–H and C=O stretching) and a decrease in humic-associated nitro group (–NO_2) signals in the BAM treatments (Fig. 4d)”.

Question 10: Supplement

(1) Text S1the TFF cleaning protocol appears to omit the concentrations for NaOH and HCl (“mol L^{-1} NaOH” and “mol L^{-1} HCl”)

(2) “Pdl” should presumably be “PdI,” and “Phl” also appears to be a typo or mislabeled parameter. Please check both the text and the figure labeling.

(3) The supplement still contains typographical errors such as “doenotes” in the Figure S8 caption.

(4) Table S1 “Sampling data” should be “Sampling date.”

(5) Table S2 in the supplement incorrectly refers to “truly dissolved organic carbon (tDON)” and should be corrected to nitrogen.

Response:

Thank you for your careful corrections. We have checked and revised the Supplementary Information accordingly. Specifically, the concentrations of NaOH and HCl have been added in Text S1; “Pdl” and “Phl” have been checked and corrected to “PdI” where appropriate; the typo “doenotes” in the Figure S8 caption has been corrected to “denotes”; “Sampling data” in Table S1 has been corrected to “Sampling date”; and “truly dissolved organic carbon (tDON)” in Table S2 has been corrected to “truly dissolved organic nitrogen (tDON).”
