Author Responses:

We deeply appreciate the thorough and helpful comments from the Reviewers. We have revised the manuscript (EGUSPHERE-2026-216) carefully following each comment from the reviewers. Our responses are shown in bold below each comment, and the corresponding changes have been marked in the manuscript for clarity.

RC1:

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 suggestions on 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. Following your recommendation, we have reorganized the Abstract, Discussion, and Conclusions to highlight the central message: estuarine mixing produces a dual response in organic nitrogen dynamics. Specifically, we now emphasize that humic-like organic nitrogen is preferentially transferred into particulate and more refractory pools through adsorption and flocculation processes, while concurrent biological processing alters the remaining dissolved pool toward a composition with greater potential lability. Nitrogen sequestration, eutrophication implications, and broader estuarine relevance are now presented as consequences of this dual response rather than as independent central themes. These revisions have been incorporated into the Abstract, Discussion (sections 4.2 & 4.4), and Conclusions.

The revised text reads:

[Abstract, L16-27] "The results suggest that estuarine mixing drives two co-occurring ON transformation processes with distinct consequences for nitrogen fate. 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 4.2, L312-314] "The illite–quartz-dominated mineral matrix of SPM (Table S1), with its documented affinity for aromatic DOM (Krettek et al., 2023), likely explains the preferential transfer of humic-like components to the particulate phase";

[Section 4.4, L413-417] "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, L442-443] "Beyond the dual transformation mechanism itself, the resulting changes in ON composition have potential implications for downstream nitrogen bioavailability. Different ON forms…";

[Conclusions, L478-479] "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, L486-490] "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, L299-304] "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). Because FI and BIX indicate microbial/autochthonous DOM contribution and recent biological production, respectively, whereas SUVA254 reflects DOM aromaticity, these coordinated optical changes suggest an increased contribution of freshly produced, less aromatic DOM, supporting a shift toward greater potential DON bioavailability during estuarine mixing; direct uptake measurements would further constrain the extent to which this transformed DON pool is biologically utilized";

[Section 4.5, L467-469] "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:

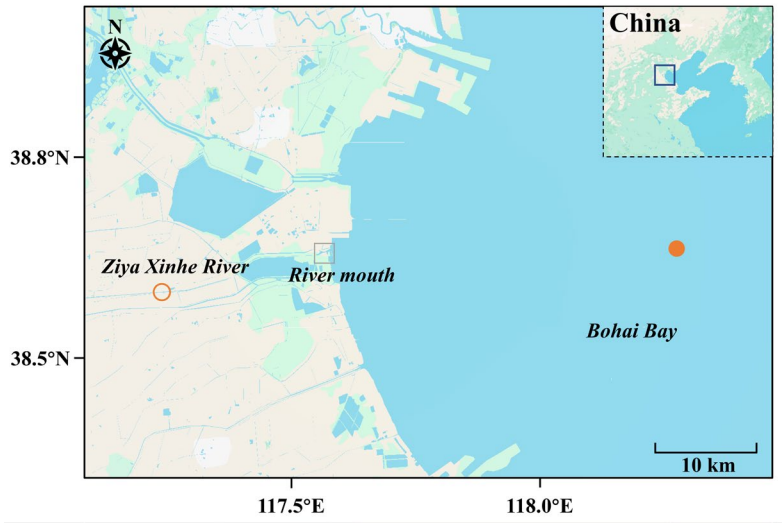


Figure S1. Location of sampling sites in Bohai Bay, China. The riverine endmember (open circle) and marine endmember (solid circle) are approximately 40 km apart.

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, e.g. Sholkovitz, 1976; Schneider et al., 2016.

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:

Thanks for the helpful comments on paper structure. The former Figure 4 has been moved from the Discussion section 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, L200-202] "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, L224-225] "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, L299-300] "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, L333-335] "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, L356-357] "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 suggestion. 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, Deleted] "Our findings thus underscore that DON availability not only fuels productivity but also regulates phytoplankton succession and the potential for harmful algal blooms";

[Section 4.4, L430-433] "Nevertheless, DON remains the dominant fraction of the ON pool (greater than 71%) during DIN-depleted periods or under long residence times, and its modified forms may act as a nitrogen reservoir that could potentially fuel coastal algal blooms. Admittedly, this inference requires validation through in situ monitoring and bioassay experiments conducted in coastal waters";

[Section 4.4, L445-448] "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, L452-455] "Furthermore, this study showed the increase of protein-like components during mixing, underscoring the importance of ON composition in regulating biological utilization. This suggests that both ON speciation and bulk composition could jointly affect its role in supporting productivity. 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, L469-471] "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, L93-94] "Water temperature, salinity, pH, and dissolved oxygen (DO) were measured using a portable multiparameter analyzer (YSI ProPlus, USA) and the values were listed in Table S1".

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

Response:

Corrected.

The revised text reads:

[Section 3.2, L226] "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, L333-335] "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. 3d)".

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,

(1) The concentrations of NaOH and HCl have been added in Text S1;

(2) "Pdl" and "Phl" have been checked and corrected to "PdI" where appropriate;

(3) The typo "doenotes" in the Figure S8 caption has been corrected to "denotes";

(4) "Sampling data" in Table S1 has been corrected to "Sampling date";

(5) The "truly dissolved organic carbon (tDON)" in Table S2 has been

corrected to "truly dissolved organic nitrogen (tDON)."

RC2:

RC2's comments to the Author:

This manuscript investigates organic nitrogen transformation and bioavailability during estuarine mixing using a dual-treatment design (BAM vs. BIM) to separate biological from physicochemical processes. The topic is relevant to BG's scope, and the experimental approach — combining tangential flow filtration, EEM-PARAFAC, stable isotopes, FTIR, and enzyme assays — is comprehensive. The three-pathway conceptual model and the "enhanced nitrogen pump" framework are valuable contributions.

Scientific significance: The BAM/BIM design effectively isolates biological and physicochemical contributions to ON fate, which is difficult to achieve in field studies. The global estuarine ON synthesis (Fig. 5, Table S4) adds breadth.

Scientific quality: The analytical methods are sound, but several issues in data interpretation weaken key arguments — notably an apparent inconsistency between the FTIR description and figure caption (Line 257–258), a questionable zeta potential argument (Line 189–192), and insufficient support for the claimed humic-to-protein transformation pathway (Line 260–275).

Presentation quality: Generally well-organized, but inconsistent abbreviations, ambiguous method descriptions, and a disproportionately long limitations section reduce clarity. The Conclusions section lacks focus.

Response:

We sincerely thank the reviewer for the thorough evaluation and constructive suggestions. We are encouraged that the reviewer recognized both the novelty of the BAM/BIM experimental framework for disentangling biological and physicochemical controls on organic nitrogen fate and the broader value of the global estuarine ON synthesis. At the same time, the reviewer identified several important issues regarding data interpretation, methodological clarity, and presentation quality. We have carefully addressed all comments and summarized the major revisions below.

(1) Scientific quality.

The three data-interpretation issues identified by the reviewer have been fully addressed:

- ***FTIR attribution error (Q4). The mismatch between the Discussion text (which cited "BIM") and the Fig. 4d caption (which showed BAM spectra) resulted from a writing error. We have corrected "BIM" to "BAM," ensuring consistency with the figure and the underlying data.***
- ***Zeta potential argument (Q3). We acknowledge that the original wording incorrectly implied that strongly negative zeta potentials favor adsorption. The revised text (Section 4.1) now distinguishes non-electrostatic adsorption mechanisms from salinity-induced colloidal destabilization and flocculation, and interprets the observed zeta-potential pattern accordingly.***
- ***Humic-to-protein transformation pathway (Q5). We agree that the negative correlation between C2 and (C1+C3) alone does not establish a direct transformation. In the revised Section 4.2, we explicitly acknowledge de novo microbial production of C2 as an alternative, soften the causal language, and emphasize that our inference rests on the convergence of five independent lines of evidence: FTIR spectral shifts, elevated DON-degrading enzyme expression (SDH, CHI), NH_4^+ accumulation, a 6–12% net C2 enrichment above conservative mixing predictions, and published documentation of analogous priming-driven transformations in comparable estuarine systems.***

(2) Presentation quality.

- ***Inconsistent abbreviations and figure labeling. All instances have been corrected: "Phl" → "PdI" (Q10), "phasa" → "phase" in Fig. 2 (Q14), and mislabeled treatment codes ("BEM"/"APM") in the original Fig. S7 (now Fig. S8) have been unified to "BIM"/"BAM" (Q15).***
- ***Ambiguous method descriptions. We now explicitly specify that Zetasizer measurements were performed on 0.45 μm-filtered filtrates (Q1), and provide a clear rationale for the two-step TFF protocol and the pooling of >10 kDa and 1–10 kDa retentates into a single colloidal fraction following standard operational definitions (Q6).***

- ***Limitations section and Conclusions (Q7). Section 4.5 has been condensed from its original length into two focused paragraphs — one on methodological constraints, one on future directions — removing content that restated Discussion findings. The Conclusions have been rewritten to deliver concise, quantitative take-home messages (e.g., 44–71% removal of humic-like DON; 6–12% C2 enrichment above conservative mixing) without repeating the Discussion.***
- ***Grammatical problems. All grammatical errors noted in the Technical Corrections (Q8–Q12) have been corrected in the revised manuscript.***

We believe these revisions have substantially strengthened the scientific rigor, internal consistency, and readability of the manuscript. A point-by-point response to each specific comment follows below.

Specific Comments

Question 1: Lines 119–122: The description of Zetasizer measurements immediately follows FTIR analysis of freeze-dried DON, making it unclear what matrix was actually measured for particle size and zeta potential. The measurement target should be specified.

Response:

Thank you for pointing out this ambiguity. We have revised the Materials and methods section to clearly specify that the Zetasizer measurements were performed on the 0.45 μm-filtered filtrates.

The revised text reads:

[Section 2.3, L142-143] "Particle size distribution, polydispersity index (PdI), and zeta potential of the 0.45 μm-filtered filtrates were measured using a Zetasizer Nano ZS90 (Malvern Instruments, UK) at 25 °C".

Question 2: Lines 142–143: The statement that declining CON and tDON concentrations cause the DON/ON ratio to decrease is logically incomplete — the ratio also depends on concurrent PON changes.

Response:

We thank the reviewer for pointing out this logical incompleteness. In our experiments, all ON fractions (CON, tDON, and PON) decreased in concentration along the salinity gradient; however, DON declined proportionally more than PON, resulting in an increase in the PON proportion and a corresponding decrease in the DON/ON ratio (Fig. S4). The original statement attributed the decline in DON/ON solely to decreasing CON and tDON concentrations without acknowledging the role of the differential decline rates between DON and PON. We have revised the text accordingly.

The revised text reads:

[Section 3.1, L177-179] "Along the increasing salinity gradient, the concentrations of CON, tDON, and PON all declined (Fig. 1; Table S2); however, DON decreased proportionally more than PON, causing the relative contribution of DON to total ON to decline while that of PON increased (Fig. S4)".

Question 3: Lines 189–192: The claim that negative zeta potentials ($\zeta < 0$) promote DON adsorption onto SPM is inconsistent with colloidal chemistry, where strongly negative ζ indicates greater electrostatic repulsion and colloidal stability. The roles of surface adsorption and colloidal destabilization/flocculation need to be distinguished.

Response:

We thank the reviewer for this important correction. The original statement was indeed misleading: strongly negative zeta potentials indicate electrostatic repulsion and colloidal stability, not enhanced adsorption. We have revised the passage (Section 4.1) to clearly distinguish between surface adsorption and colloidal destabilization/flocculation, and to accurately describe how zeta potential governs the latter.

Specifically, in our experiments, zeta potentials remained negative across the entire salinity gradient in both BAM and BIM treatments, but showed a characteristic non-monotonic trend: ζ became least negative (closest to zero) at intermediate salinities, coinciding with maximum mean particle size, and then

became more negative again at higher salinities. This pattern is diagnostic of ionic strength-driven compression of the electrical double layer at mid-salinity, which minimizes electrostatic repulsion, destabilizes colloids, and promotes flocculation and particle aggregation, consistent with the classic estuarine turbidity maximum mechanism. The subsequent return to more negative ζ values at higher salinities likely reflects the depletion of aggregation-susceptible particles and/or a shift in particle population toward marine-origin particles with different surface charge characteristics.

We now clearly attribute DON-to-PON transfer via two separate mechanisms: (1) surface adsorption driven by non-electrostatic interactions (ligand exchange, hydrogen bonding, hydrophobic effects) between humic-like DOM and mineral surfaces, and (2) colloidal destabilization and flocculation driven by double-layer compression at intermediate salinities.

The revised text reads:

[Section 4.1, L248-260] "The non-conservative behavior of ON could be driven by two mechanistically distinct physicochemical processes under abiotic conditions (i.e., biologically inhibited mixing treatments). First, surface adsorption transferred DON onto SPM in the turbidity zone, where high SPM concentrations (Fig. S8b) provided abundant mineral surface area. This adsorption is mediated primarily by non-electrostatic interactions, including ligand exchange, hydrogen bonding, and hydrophobic effects, between humic-like/aromatic DOM and mineral surfaces (Pan et al., 2020; Shimabuku et al., 2017; Yan et al., 2022), and is facilitated by the high proportion (>73%) of illite and quartz in the suspended matter (Table S1), which have a known affinity for aromatic DOM (Krettek et al., 2023). Second, increasing ionic strength along the salinity gradient compressed the electrical double layer of colloidal particles, driving zeta potential values toward neutrality (Fig. 4e). This reduction in electrostatic repulsion destabilized colloids, promoting particle aggregation and flocculation, as evidenced by the concurrent increase in mean particle size (Fig. 4f). The resulting aggregation facilitated the conversion of CON to PON. Additionally, the adsorption of LMW-DOM onto colloids (Kilduff et al., 1996;

Pan et al., 2020) contributed to tDON removal, with efficiency dependent on DOM characteristics (Ateia et al., 2017) and properties of the colloidal material (Lee and Hur, 2016)".

Question 4: Lines 257–258: FTIR evidence is attributed to the BIM treatments, but the Fig. 4d caption states these spectra are from BAM only. This contradiction undermines a key argument in Section 4.2.

Response:

Thank you for carefully identifying this inconsistency. The reviewer is correct that the FTIR spectra were obtained from the BAM treatments only. The attribution to BIM in the original Discussion was a writing error. We have corrected the text to "BAM" and made it consistent with the figure caption and the underlying data.

The revised text reads:

[Section 4.2, L333-335] "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. 3d)".

Question 5: Lines 260–275: The strong negative correlation between C2 and (C1+C3) does not establish that humic-like components are directly transformed into protein-like components. Alternative explanations (e.g., de novo microbial production of C2) could be considered.

Response:

Thank you for this critical comment. We agree that the strong negative correlation between C2 and C1 + C3 does not by itself demonstrate a direct humic-to-protein transformation. In the revised manuscript, we have explicitly acknowledged de novo microbial production of C2 as an alternative or co-occurring mechanism, softened the causal language, and described the transformation pathway as an inference supported by multiple lines of evidence rather than as a directly proven process.

The revised text reads:

[Section 4.2, L320-344] "Strong correlations were observed between C2 and humic-like components (C1 + C3) in the BAM treatments ($n = 21$, $r = -0.95$, $p < 0.001$; Fig. S10c). C2 is a microbially derived protein-like component (Liu et al., 2024), C1 and C3 represent aromatic humic substances (Osburn et al., 2011), with C3 being more susceptible to microbial degradation (Cole et al., 2007; Yao et al., 2024). This inverse relationship is consistent with a pathway in which microbial degradation of humic-like substrates contributes to the production of labile, protein-like DON (Liu et al., 2024; Yao et al., 2024). However, this correlation alone does not prove direct transformation, and de novo microbial production of C2 using ambient DIN, labile DOC, or intracellular metabolites may also contribute to the observed pattern. Several independent lines of evidence support a predominantly transformative mechanism. Following a 3-day incubation, microbes consumed the ambient DOC and nutrients originally present in the riverine and marine waters to stimulate microbial growth and/or respiration, and change ON properties. Previous studies have shown that microbial activity, stimulated by terrestrial inputs, can transform humic-like components into shorter-wavelength humic or protein-like materials via a "priming effect" (Liu et al., 2024; Yao et al., 2024). Elevated expression of DON-degrading enzymes (SDH and CHI) in the BAM treatments (Fig. S9b, c) confirms active enzymatic processing of complex organic N, while concurrent NH_4^+ accumulation (Fig. S8e) is consistent with ammonification of humic-like substrates. 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). Moreover, the strong correlation between microbial and terrestrial DON may imply that enhanced microbial metabolism accelerates DON degradation (Ward et al., 2016), especially in carboxylic acid- and heteroatom-rich fractions, and leads to the formation of labile DON (LDON, C2), which includes amino sugars, proteins, and lipids (Yao et al., 2024). This transformation pathway has previously also been documented in the Yangtze Estuary (Li et al., 2024). Taken

together, the convergence of fluorescence, FTIR, enzymatic, and mass-balance evidence indicates that the observed inverse C2 – (C1 + C3) relationship is best explained by microbial transformation of humic-like DON into bioavailable LDON, potentially supplemented by de novo microbial synthesis. This process resulted in a relative increase in C2 fluorescence intensity of 6% to 12% above the theoretical dilution curve (Fig. 2, Table S2), leading to net tDON enrichment and enhanced overall DON bioavailability in the BAM treatments".

Question 6: Text S1 describes a two-step TFF (10 kDa + 1 kDa) yielding three size fractions, but only two are reported. The rationale for merging the >10 kDa and 1–10 kDa fractions is not explained.

Response:

We thank the reviewer for this question. The two-step TFF (10 kDa followed by 1 kDa) was adopted for operational efficiency: pre-filtering through the 10 kDa membrane first removes high-molecular-weight material, reducing membrane fouling and increasing permeate flux during the subsequent 1 kDa ultrafiltration step. The >10 kDa and 1–10 kDa retentates were then pooled and reported as a single colloidal fraction (CON; 1 kDa–0.45 μm), following the standard operational definition of colloidal substances widely used in water studies (Guo and Santschi, 1997; Yang et al., 2021). This binary partitioning aligns with our research objective, which focuses on the contrasting fates of colloidal vs. truly dissolved ON rather than internal size heterogeneity within the colloidal pool. A brief clarification has been added to Section 2.2 and Text S1 in the revised manuscript.

The revised text reads:

[Section 2.2, L111-114] "TFF was performed in two sequential steps (10 kDa followed by 1 kDa membranes) to reduce membrane fouling and improve permeate flux during the 1 kDa ultrafiltration; the two retentate fractions (>10 kDa and 1–10 kDa) were then pooled as CON, following the conventional operational definition of estuarine colloidal organic matter (Guo and Santschi, 1997; Yang et al., 2021)";

[Text S1] "The two-step TFF protocol (10 kDa followed by 1 kDa) was adopted

as an operational strategy to improve ultrafiltration efficiency: the initial 10 kDa step removes high-molecular-weight macromolecules and particulate residues that would otherwise cause rapid fouling of the 1 kDa membrane, thereby maintaining higher permeate flux and shorter processing times. Although this procedure operationally yields three size fractions (>10 kDa, 1–10 kDa, and <1 kDa), the >10 kDa and 1–10 kDa retentates were pooled and reported as a single colloidal fraction (CON; 1 kDa–0.45 μm). This binary partitioning is consistent with the standard operational definition of colloidal DOM in estuarine and marine biogeochemistry (Guo and Santschi, 1997; Yang et al., 2021) and aligns with the research objective of this study, which addresses the contrasting fates of colloidal versus truly dissolved ON rather than internal size heterogeneity within the colloidal pool".

Guo, L. and Santschi, P. H.: Composition and cycling of colloids in marine environments, Rev. Geophys., 35, 17–40, https://doi.org/10.1029/96RG03195, 1997.

Yang, B., Lin, H., Bartlett, S. L., Houghton, E. M., Robertson, D. M., and Guo, L.: Partitioning and transformation of organic and inorganic phosphorus among dissolved, colloidal and particulate phases in a hypereutrophic freshwater estuary, Water Res., 196, 117025, https://doi.org/10.1016/j.watres.2021.117025, 2021.

Question 7: Section 4.5 (Limitations) is disproportionately long and risks overshadowing the study's strengths. The Conclusions section partly repeats the Discussion and could be more concise and quantitative.

Response:

We thank the reviewer for this constructive suggestion. In the revised manuscript we have condensed Section 4.5 into two focused paragraphs, one on methodological limitations and one on future directions, removing content that restated Discussion findings. We have also rewritten the Conclusions to emphasize concise, quantitative take-home messages without repeating the Discussion.

The revised text reads:

[Section 4.5, L457-476] "Several methodological constraints should be considered when extrapolating these results to natural estuaries. First, freshwater and seawater end-members were collected from a single summer sampling event (July

2024) and incubated under constant temperature and light, which does not capture the influence of multiple water sources (e.g., groundwater, tidal creek return flow, wastewater), tidal dynamics, stratification and resuspension cycles, or seasonal variability. Second, the 3-day incubation is sufficient to capture rapid flocculation, adsorption, and initial microbial transformation, but may underestimate slower processes such as long-term mineralization, sedimentation, and RDON' stabilization. Despite these constraints, the controlled two-end-member design allows us to disentangle rapid salinity-driven partitioning from concurrent biological processing, which are difficult to separate in the field, and provides a mechanistic baseline for interpreting non-conservative ON behavior along estuarine salinity gradients.

Future studies should extend incubation times and incorporate seasonal end-members to assess longer-term ON burial and the generality of these pathways beyond summer conditions. 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. 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. High-resolution molecular tools (e.g., FT-ICR MS, single-cell stable isotope analysis) should also be applied to resolve the full molecular diversity of DON transformations across estuarine gradients (Yan et al., 2024; Arandia-Gorostidi et al., 2024). It would further be valuable to integrate the salinity-dependent partitioning and biological conversion terms identified here into estuarine box or reactive-transport models, coupled with residence time and SPM properties, to generate quantitative predictions of ON export versus retention across estuary types";

[Conclusions, L478-490] "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. The results show that physicochemical processes, specifically salt-induced adsorption and flocculation, act

as a primary pathway for converting humic-like components of DON into refractory particulate forms, removing approximately 44–71% of humic-like DON (C1 + C3) and effectively creating a long-term sink. Crucially, isotopic evidence suggests that biologically modified DON re-adsorbs onto particles, further enhancing their refractoriness. Concurrently, biological activity degrades the remaining labile humic-like fraction, releasing highly bioavailable nitrogen forms such as low-molecular-weight DON and NH_4^+ that fuel microbial production of protein-like substances, as reflected by an approximately 6–12% elevation in C2 fluorescence above conservative mixing predictions. 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".

Technical Corrections

Question 8: Line 30: "where critically mediate" — missing subject.

Response:

Revised.

The revised text reads:

[Introduction, L29] "Estuaries are critical transitional zones between rivers and the sea that critically mediate the flux of nitrogen to the sea".

Question 9: Line 95–96: "to inhibited" — should be "to inhibit."

Response:

Revised.

The revised text reads:

[Section 2.2, L105] "...mixtures amended with 0.1% (v/v) chloroform to inhibit biological activity and isolate physicochemical processes".

Question 10: Line 127 and Fig. S2 caption: "Phl" — should be "PdI."

Response:

Revised.

The revised text reads:

[Section 2.3, L148] "...or PdI following chloroform addition";

*[Figure S2] "... and PdI (d) of the amino sugar (1 mg L^{-1}) + inactivated SPM (50 mg L^{-1}) system was examined, oscillating at 180 rpm for 2 hours. NS means no significance, and '***' denotes $p < 0.001$. Zeta potential analysis showed no significant changes in average particle size, zeta potential, or PdI following chloroform addition (Figure S2, $p > 0.05$), ... ".*

Question 11: Line 188: "are govern by" — should be "are governed by."

Response:

Thank you for noting this grammatical error. The sentence containing "are govern by" has been removed during the revision of Section 4.1 in response to Question 3; therefore, this issue no longer exists in the current text.

Question 12: Line 309: "This study highlight" — should be "highlights."

Response:

Revised.

The revised text reads:

[Section 4.3, L381] "This study highlights that estuarine mixing processes…".

Question 13: Line 351: "component effects" — unclear; perhaps "compound effects"?

Response:

Revised.

The revised text reads:

[Section 4.4, L422] "The interplay of biological and physicochemical processes during mixing can produce compound effects".

Question 14: Fig. 2 legend: "phasa" — should be "phase."

Response:

Corrected.

The modified image is as follows:

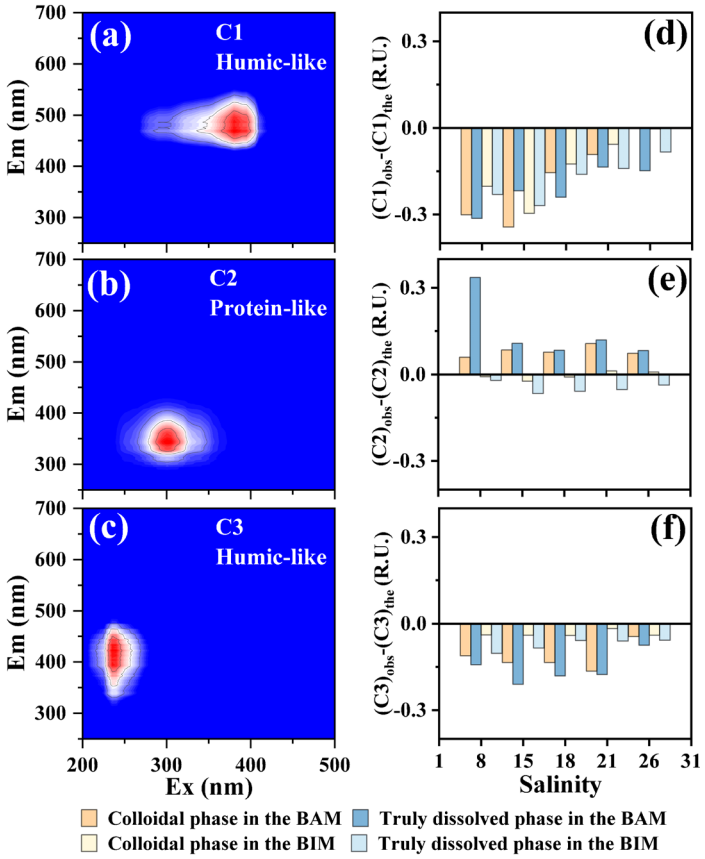


Figure 2. Variations in dissolved organic nitrogen (DON) components in the biologically active mixing (BAM) and biologically inhibited mixing (i.e., physicochemical mixing only, BIM) treatments…

Question 15: Fig. S2a: Treatment labels "BEM"/"APM" are inconsistent with the defined abbreviations "BIM"/"BAM."

Response:

Thank you for carefully checking the figure labels. We re-examined Fig. S2a and confirmed that the treatment labels in this figure are already consistent with the defined abbreviations "BIM" and "BAM." However, this comment prompted us to check all supplementary figures, and we found a similar labeling inconsistency in the original Fig. S7, which has now been renumbered as Fig. S8. The incorrect

labels "BEM" and "APM" have been corrected to "BIM" and "BAM" in the revised supplementary materials.

The modified image is as follows:

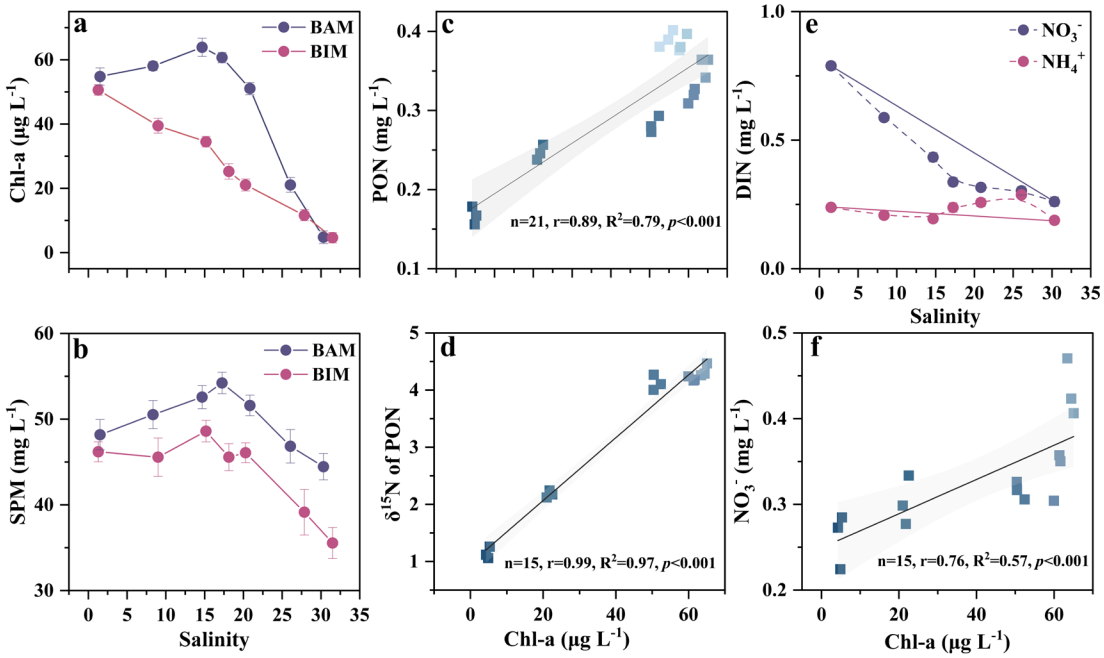


Figure S8. Variations of (a) Chl-a, (b) Suspended Particulate Matter (SPM), (e) nitrate (NO_3^-) and ammonium (NH_4^+) along the salinity gradient in the biologically active mixing (BAM) and biologically inhibited mixing (i.e., physicochemical mixing only, BIM) treatments (a, b). The relationship of (c) Chl-a and PON, (d) Chl-a and $\delta^{15}N$ of PON, (f) Chl-a and NO_3^- in the BAM treatments.

RC3:

RC3's comments to the Author:

Manuscript egusphere-2026-216 employs laboratory incubation experiments to investigate the transformation of dissolved organic nitrogen (DON) and particulate organic nitrogen (PON), as well as nitrogen bioavailability. The study offers valuable insights with clear relevance to future research on nitrogen biogeochemical cycling in coastal waters. Overall, the experimental design is robust and the results are interesting and potentially impactful. However, several aspects require further clarification.

Response:

We sincerely thank the reviewer for the positive evaluation of our experimental design and the significance of this study. We also appreciate the constructive comments, which helped us improve the clarity and rigor of the manuscript. In response, we have added missing references, clarified methodological details, strengthened the explanation of conservative versus non-conservative mixing, added theoretical mixing values and deviations, revised the interpretation of PON isotope trends with statistical support, and defined FI, BIX, and $SUVA_{254}$ in the Materials and methods section. The relevant revised passages are copied below where necessary.

Question 1: Between line 50 and 55: is there a citation for "Humic-like (terrestrial and microbial sources) and protein-like components are considered as the dominant terrestrial DON components entering the sea"?

Response:

We thank the reviewer for noting the missing citation. In the revised manuscript, we have added two supporting references for this statement.

The revised text reads:

[Introduction, L58-60] "Humic-like (terrestrial and microbial sources) and protein-like components are considered as the dominant terrestrial DON components entering the sea (Stedmon and Markager, 2005; Yan et al., 2024)".

Stedmon, C. A. and Markager, S.: Resolving the variability in dissolved organic matter fluorescence in a temperate estuary and its catchment using PARAFAC analysis, Limnol. Oceanogr., 50, 686–697, https://doi.org/10.4319/lo.2005.50.2.0686, 2005.

Yan, Z., Xin, Y., Zhong, X., Yi, Y., Li, P., Wang, Y., Zhou, Y., He, Y., He, C., Shi, Q., Xu, W., and He, D.: Evolution of dissolved organic nitrogen chemistry during transportation to the marginal sea: Insights from nitrogen isotope and molecular composition analyses, Water Res., 249, 120942, https://doi.org/10.1016/j.watres.2023.120942, 2024.

Question 2: Between line 110 and 115: authors mentioned the PN was considered equivalent to PON because PIN is less than 5%. Wonder how much would it affect the isotopic ratios? Especially if the nitrogen isotopic values of PIN vary along the salinity gradient, would it affect the observed trend?

Response:

Thank you for this helpful comment. The variations in δ^{15}N-PIN along the salinity gradient could, in principle, affect the measured bulk PN isotopic composition. To address this issue, we added a sensitivity analysis based on isotope mass balance in the Supplementary Information (Text S2). Because PIN accounted for less than 5% of total PN in all samples, even a large isotopic difference of 20‰ between PIN and PON would shift bulk δ^{15}N-PN by only approximately 1‰, and a conservative difference of 40‰ would result in an offset of approximately 2‰. This potential offset is much smaller than the observed δ^{15}N-PON variation in the BAM treatments, which ranged from 1.1‰ to 8.8‰. Therefore, although salinity-dependent variation in δ^{15}N-PIN cannot be completely excluded, its low contribution to total PN indicates that it would only slightly affect absolute δ^{15}N values and would not change the main isotopic trend observed in this study. Moreover, treating PN as equivalent to PON when PIN is negligible (<5%) is a well-established practice in estuarine and coastal biogeochemistry (Yan et al., 2022), and our approach is consistent with these precedents.

We have added a brief sentence to the revised manuscript to explicitly acknowledge the potential isotopic effect and justify why it is negligible.

The revised text reads:

[Section 2.3, L123-127] "Particulate inorganic nitrogen (PIN) was determined via HCl extraction (Zuo et al., 2016) and accounted for less than 5% of total PN in all samples; therefore, PN was used as a proxy for PON. The potential influence of PIN on δ^{15}N-PON was further evaluated using an isotope mass-balance sensitivity analysis, which indicated that the low PIN contribution would have only a minor effect on bulk δ^{15}N values and would not alter the observed isotopic trends (Text S2)";

[Text S2] "Sensitivity analysis of the potential influence of particulate inorganic nitrogen on δ^{15}N-PON

Particulate inorganic nitrogen (PIN) accounted for less than 5% of total particulate nitrogen (PN) in all samples. Therefore, PN was used as a proxy for particulate organic nitrogen (PON) in this study. To evaluate whether the small PIN fraction could affect the measured nitrogen isotopic composition, we performed a simple isotope mass-balance assessment.

The measured isotopic composition of bulk PN can be expressed as:

$$\delta^{15}N\_PN = (1 - f\_PIN) \times \delta^{15}N\_PON + f\_PIN \times \delta^{15}N\_PIN \quad \text{Equation 4}$$

where f _PIN is the fraction of PIN in total PN. Therefore, the potential offset caused by PIN can be approximated as:

$$\delta^{15}N \approx f\_PIN \times (\delta^{15}N\_PIN - \delta^{15}N\_PON) \quad \text{Equation 5}$$

Because f _PIN was consistently lower than 0.05, the influence of PIN on bulk δ^{15}N-PN would be limited even if δ^{15}N-PIN differed substantially from δ^{15}N-PON. For example, if δ^{15}N-PIN differed from δ^{15}N-PON by 20‰, the resulting offset in bulk δ^{15}N-PN would be approximately 1‰. Even under a more conservative scenario with a 40‰ isotopic difference, the offset would still be approximately 2‰.

In comparison, the observed δ^{15}N-PON values in the BAM treatments ranged from 1.1‰ to 8.8‰, corresponding to a total variation of 7.7‰. To generate such a variation solely through a PIN contribution of 5%, δ^{15}N-PIN would need to differ from δ^{15}N-PON by approximately 154‰, which is unrealistic for this system. Therefore, the observed δ^{15}N variation in the BAM treatments cannot be explained by the minor PIN contribution. In the BIM treatments, δ^{15}N-PON varied within a

narrower range, from 6.2‰ to 7.2‰. Thus, the possible contribution of PIN may introduce minor uncertainty to small within-treatment differences. However, because PIN remained consistently below 5% of total PN, this effect would mainly influence absolute $\delta^{15}N$ values slightly and would not alter the major treatment-level pattern or the interpretation that PN was dominated by PON.

Overall, although salinity-dependent variation in $\delta^{15}N$-PIN cannot be completely excluded, the low contribution of PIN to total PN indicates that its effect on measured $\delta^{15}N$-PN was minor and would not change the main isotopic trends observed in this study".

Yan, X., Yang, J., Xu, M., Wang, H., Dai, M., and Kao, S.: Nitrogen isotope constraint on the zonation of multiple transformations between dissolved and particulate organic nitrogen in the Changjiang plume, Sci. Total Environ., 818, 151678, https://doi.org/10.1016/j.scitotenv.2021.151678, 2022.

Question 3: Between line 115 and 120: does the S in "Additional water quality parameters including S, pH, SPM, and chlorophyll-a (Chl-a) were measured" stand for sulfur? Why is sulfur collected and how would affect nitrogen? I don't think I have seen the relevant discussion in the manuscript.

Response:

We thank the reviewer for pointing out this ambiguity. The "S" refers to salinity, not sulfur. We apologize for the confusion caused by the undefined abbreviation. In the revised manuscript, we have replaced "S" with "salinity" to eliminate any ambiguity.

The revised text reads:

[Section 2.3, L140] "Additional water quality parameters including salinity, pH, ..."

Question 4: In results, the manuscript mentioned the DON indicated a non-conservative mixing and PON indicated a conservative mixing. Do you have data to support it? All I can see from the paragraph and figures is that DON decreases with increasing salinity, which looks like conservative for DON.

Response:

Thank you for this helpful comment. In estuarine mixing studies, conservative behavior is commonly evaluated by comparing observed concentrations with the theoretical two-endmember conservative mixing curve calculated from river-water and seawater endmembers. A decrease in concentration with increasing salinity does not necessarily indicate conservative mixing; it is considered conservative only when the observed values follow the theoretical dilution curve. Recent estuarine studies have applied this approach to DOC, CDOM, nutrients, trace metals, and DON, using deviations from conservative mixing curves to identify net addition or removal (Cheong et al. 2024; Yan et al. 2024).

However, to our knowledge, there is no universal fixed threshold, such as 5% or 10% deviation from the conservative line, that is generally accepted across estuarine mixing studies. The threshold depends on analytical uncertainty, endmember variability, and replicate variability. Therefore, in the revised manuscript, we define non-conservative behavior operationally as a statistically meaningful deviation between the observed concentration and the predicted conservative mixing value, considering the propagated uncertainty from triplicate measurements. We added the calculated theoretical values of nitrogen concentrations and their deviations from conservative mixing to Table S3. To avoid misunderstanding, we have revised the Introduction and Materials and methods section to explicitly define conservative and non-conservative mixing using the two-end-member model, and revised the Results section to clarify the different behaviors of DON and PON in BAM and BIM treatments.

The revised text reads:

[Introduction, L48-49] "Contrary to early models of conservative dilution mixing, in which solute concentrations vary linearly with salinity between the freshwater and seawater end-members (i.e., following the theoretical dilution line; Langmuir et al., 1978; Officer, 1979), ON behavior is often non-conservative, with measured concentrations deviating above (net addition) or below (net removal) the theoretical dilution line";

[Section 2.4, L154-168] "Conservative mixing was evaluated using a two-end-member mixing model based on the river-water and seawater endmembers to distinguish simple physical dilution from internal sources or sinks (Liss, 1976; Langmuir et al., 1978). The theoretical conservative concentration at each salinity was calculated as:

$$X_{mix} = f_r \times X_r + f_s \times X_s \quad (1)$$

$$f_r = (S_s - S_i) / (S_s - S_r),\ f_s = (S_i - S_r) / (S_s - S_r) \quad (2)$$

where X_{mix} was the predicted value under conservative mixing, X_r and X_s were the corresponding values in the river-water and seawater endmembers, respectively, f_r and f_s were the corresponding mixing fractions estimated from salinity, S_i, S_r, and S_s represent the salinity of the mixture, river-water endmember, and seawater endmember, respectively, and X represented nitrogen concentrations and PARAFAC component fluorescence intensities. Deviations between the observed value and the conservative mixing value (X_{mix}) were used to identify non-conservative behavior. The calculated theoretical values and deviations from conservative mixing for nitrogen concentrations and PARAFAC component fluorescence intensities are provided in Tables S3 and S4, respectively. Positive deviations were interpreted as net addition, whereas negative deviations were interpreted as net removal. Thus, a monotonic decrease in concentration with increasing salinity was not necessarily considered conservative unless the observed values followed the theoretical conservative mixing curve";

[Section 3.1, L179-182] "Conservative mixing was evaluated by comparing the observed concentrations with the theoretical two-end-member dilution curves shown as solid lines in Fig. 1. The observed tDON and CON concentrations deviated from the conservative mixing curve (Fig. 1; Table S3), indicating non-conservative behavior";

[Section 3.2, L221-224] "PON showed contrasting behavior between the two treatments. In the BIM treatments, PON concentrations were comparatively closer to the conservative mixing curve, with only slight positive deviation. In contrast, PON in the BAM treatments showed substantial positive deviation from the conservative

mixing curve (Fig. 1e, f). Therefore, PON was not described as conservative under both treatments; rather, it behaved more conservatively in BIM than in BAM.";

[Figure captions, L187-193] "Figure 1. Variations in the concentrations of truly dissolved organic nitrogen (tDON) (a, b), colloidal organic nitrogen (CON) (c, d) and particulate organic nitrogen (PON) (e, f) across different river-seawater mixing ratios in the biologically active mixing (BAM) and biologically inhibited mixing (i.e., physicochemical mixing only, BIM) treatments. Conservative mixing curves (solid lines) were calculated using a two-end-member model based on the river-water and seawater endmembers (Langmuir et al., 1978), while dashed lines represent smoothed fits to the observed data. Deviations of the observed data from the conservative mixing curves indicate non-conservative behavior, with positive deviations representing net addition and negative deviations representing net removal";

[Table S3] "Theoretical conservative mixing concentrations and deviations of observed PON, CON, and tDON concentrations from conservative mixing at each salinity. Observed concentrations used to calculate deviations are provided in Table S2. Theoretical conservative concentrations were calculated using the salinity-based two-end-member mixing model. Positive values of deviations indicate net addition relative to conservative mixing, whereas negative values indicate net removal".

Salinity	Theoretical concentration			Deviation from conservative mixing		
	PON ($mg\ L^{-1}$)	CON ($mg\ L^{-1}$)	tDON ($mg\ L^{-1}$)	PON ($mg\ L^{-1}$)	CON ($mg\ L^{-1}$)	tDON ($mg\ L^{-1}$)
Biologically active mixing (i.e., biological and physicochemical processes occurring concurrently, BAM)						
1.52	0.391	0.829	1.270	0.000	0.000	0.000
8.34	0.338	0.701	1.028	0.049	-0.079	0.183
14.67	0.289	0.582	0.803	0.069	-0.143	0.298
17.24	0.269	0.534	0.711	0.049	-0.148	0.202
20.83	0.241	0.466	0.584	0.041	-0.107	0.125
26.06	0.200	0.368	0.398	0.044	-0.072	0.106
30.31	0.167	0.288	0.247	0.000	0.000	0.000
Biologically inhibited mixing (i.e., physicochemical processes only, BIM)						
1.28	0.381	0.756	1.309	0.000	0.000	0.000
8.98	0.323	0.612	1.026	0.028	-0.108	-0.103
15.18	0.276	0.497	0.798	0.017	-0.114	-0.168
18.13	0.254	0.443	0.691	0.022	-0.102	-0.249

20.27	*0.237*	*0.402*	*0.610*	*0.006*	*-0.108*	*-0.192*
27.85	*0.181*	*0.262*	*0.335*	*0.014*	*-0.082*	*-0.037*
31.5	*0.153*	*0.193*	*0.199*	*0.000*	*0.000*	*0.000*

Cheong, A. Y. L., Annammala, K. V., Yong, E. L., Zhou, Y., Nichols, R. S., and Martin, P.: Distribution of nutrients and dissolved organic matter in a eutrophic equatorial estuary: the Johor River and the East Johor Strait, Biogeosciences, 21, 2955–2971, https://doi.org/10.5194/bg-21-2955-2024, 2024.

Yan, Z., Xin, Y., Zhong, X., Yi, Y., Li, P., Wang, Y., Zhou, Y., He, Y., He, C., Shi, Q., Xu, W., and He, D.: Evolution of dissolved organic nitrogen chemistry during transportation to the marginal sea: Insights from nitrogen isotope and molecular composition analyses, Water Res., 249, 120942, https://doi.org/10.1016/j.watres.2023.120942, 2024.

Question 5: Between line 155 and 160: what are the theoretical fluorescence values?

Response:

Thank you for this comment. The theoretical fluorescence values were calculated using the same two-end-member conservative mixing model as that used for nitrogen concentrations. Specifically, the fluorescence intensities of each PARAFAC component expected under conservative mixing were calculated from the river-water and seawater endmembers, and the deviations were determined by comparing the observed fluorescence intensities with these theoretical values. The calculated theoretical fluorescence values and corresponding deviations for C1, C2, and C3 are now provided in Table S4.

The revised text reads:

[Section 3.1 L196-198] "Compared with the theoretical fluorescence values calculated using the same two-end-member conservative mixing model as for nitrogen concentrations, all three components showed negative deviations in the BIM treatments (Fig. 2; Table S4). In contrast, in the BAM treatments, C1 and C3 showed negative deviations, whereas C2 showed positive deviation (Fig. 2; Table S4)";

[Table S4] "Table S4. Theoretical fluorescence values and deviations from conservative mixing for PARAFAC components in the truly dissolved and colloidal phases. Observed fluorescence values used to calculate deviations are provided in Table S2. Theoretical conservative fluorescence values were calculated using the

salinity-based two-end-member mixing model. Positive values of deviations indicate net addition relative to conservative mixing, whereas negative values indicate net removal".

Salinity	*Theoretical fluorescence values*						*Deviation from conservative mixing*					
	Truly dissolved phase			*Colloidal phase*			*Truly dissolved phase*			*Colloidal phase*		
	C1	*C2*	*C3*	*C1*	*C2*	*C3*	*C1*	*C2*	*C3*	*C1*	*C2*	*C3*
Biologically active mixing (i.e., biological and physicochemical processes occurring concurrently, BAM)												
1.52	*2.098*	*2.140*	*0.396*	*2.614*	*2.004*	*0.398*	*0.000*	*0.000*	*0.000*	*0.000*	*0.000*	*0.000*
8.34	*1.752*	*1.769*	*0.327*	*2.070*	*1.645*	*0.318*	*-0.314*	*0.336*	*-0.143*	*-0.301*	*0.059*	*-0.112*
14.67	*1.431*	*1.425*	*0.263*	*1.566*	*1.311*	*0.243*	*-0.218*	*0.108*	*-0.210*	*-0.344*	*0.085*	*-0.135*
17.24	*1.301*	*1.285*	*0.238*	*1.361*	*1.176*	*0.213*	*-0.241*	*0.083*	*-0.181*	*-0.155*	*0.077*	*-0.135*
20.83	*1.119*	*1.090*	*0.201*	*1.074*	*0.987*	*0.170*	*-0.136*	*0.120*	*-0.176*	*-0.092*	*0.107*	*-0.165*
26.06	*0.854*	*0.806*	*0.148*	*0.657*	*0.711*	*0.109*	*-0.148*	*0.083*	*-0.075*	*-0.002*	*0.073*	*-0.045*
30.31	*0.638*	*0.575*	*0.105*	*0.318*	*0.487*	*0.059*	*0.000*	*0.000*	*0.000*	*0.000*	*0.000*	*0.000*
Biologically inhibited mixing (i.e., physicochemical processes only, BIM)												
1.28	*1.851*	*2.582*	*0.565*	*2.212*	*1.856*	*0.450*	*0.000*	*0.000*	*0.000*	*0.000*	*0.000*	*0.000*
8.98	*1.455*	*2.059*	*0.450*	*1.821*	*1.587*	*0.362*	*-0.236*	*-0.021*	*-0.103*	*-0.203*	*-0.008*	*-0.039*
15.18	*1.135*	*1.638*	*0.358*	*1.506*	*1.370*	*0.292*	*-0.270*	*-0.067*	*-0.081*	*-0.297*	*-0.023*	*-0.040*
18.13	*0.983*	*1.437*	*0.315*	*1.356*	*1.266*	*0.258*	*-0.161*	*-0.058*	*-0.059*	*-0.125*	*-0.009*	*-0.041*
20.27	*0.873*	*1.292*	*0.283*	*1.247*	*1.192*	*0.234*	*-0.141*	*-0.053*	*-0.062*	*-0.057*	*0.012*	*-0.017*
27.85	*0.483*	*0.776*	*0.171*	*0.861*	*0.926*	*0.148*	*-0.084*	*-0.037*	*-0.057*	*-0.004*	*0.009*	*-0.041*
31.5	*0.295*	*0.528*	*0.116*	*0.676*	*0.798*	*0.106*	*0.000*	*0.000*	*0.000*	*0.000*	*0.000*	*0.000*

Question 6: Between line 170 and 175: numbers from −28.3‰ to −26.5‰, 1.1‰ to 8.8‰, −26.1‰ to −25.9‰, and 6.2‰ to 7.2‰, are them statistically different? If not, it would be a stretch to say they decline along the salinity gradient, especially Figure 3 didn't look like these numbers decreased when the salinity increased.

Response:

Thank you for the helpful comment. We agree that the previous statement overgeneralized the trends in PON isotopes along the salinity gradient. To clarify, we analyzed the data using both one-way ANOVA and linear regression.

- ***One-way ANOVA: In both treatments, δ^{13}C-PON and δ^{15}N-PON differed significantly among salinity levels (BAM: δ^{13}C-PON $F(6,14) = 13.10$, $p < 0.001$; δ^{15}N-PON $F(6,14) = 322.43$, $p < 0.001$; BIM: δ^{13}C-PON $F(6,14) = 19.00$, $p < 0.001$; δ^{15}N-PON $F(6,14) = 7.43$, $p = 0.001$). This indicates that isotope values vary across salinity levels in both treatments.***

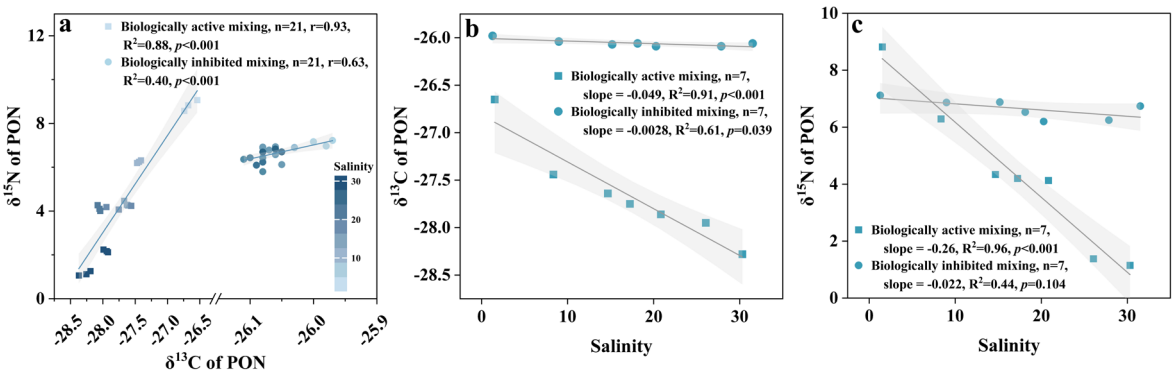
- ***Linear regression: The decrease along the salinity gradient was mainly evident in the BAM treatments (δ^{13}C-PON slope = −0.049‰ per salinity unit, R^2 = 0.91, p = 0.0009; δ^{15}N-PON slope = −0.26‰ per salinity unit, R^2 = 0.96, p = 0.0001). In the BIM treatments, δ^{13}C-PON showed only a weak linear decrease (slope = −0.0028‰ per salinity unit, R^2 = 0.61, p = 0.039), and δ^{15}N-PON showed no significant monotonic trend (slope = −0.022‰ per salinity unit, R^2 = 0.44, p = 0.104).***

Together, these analyses indicate that the significant decrease in PON isotope values along the salinity gradient is mainly observed in BAM treatments, whereas BIM treatments show only minor or non-monotonic variations. Accordingly, we have revised the text to avoid implying that both isotope values declined along the salinity gradient in both treatments.

The revised text reads:

[Section 3.2, L227-232] "The salinity-related decrease in PON isotope values was mainly evident in the BAM treatments, whereas isotope variations in the BIM treatments were relatively small or non-monotonic. Specifically, both δ^{13}C and δ^{15}N values varied significantly among salinity levels as shown by one-way ANOVA ($p < 0.001$). Linear regression analysis based on the mean values at each salinity level further showed significant decreasing trends with increasing salinity for both δ^{13}C-PON and δ^{15}N-PON in the BAM treatments ($p < 0.001$, Fig. S7). In the BIM treatments, the regression analysis indicated only a weak decrease in δ^{13}C-PON with salinity ($p = 0.039$, Fig. S7), while δ^{15}N-PON showed no significant linear trend ($p = 0.104$, Fig. S7)";

[Figure S7]



"Figure S7. Relationships between PON isotopic compositions and salinity under biologically active and inhibited mixing treatments. (a) Relationship between δ¹³C-PON and δ¹⁵N-PON under biologically active mixing and biologically inhibited mixing treatments, with symbol color indicating salinity. (b) Changes in δ¹³C-PON along the salinity gradient. (c) Changes in δ¹⁵N-PON along the salinity gradient. Regression lines are based on the mean values at each salinity level, and shaded areas indicate 95% confidence intervals. δ¹³C-PON and δ¹⁵N-PON showed significant decreasing trends with increasing salinity in the biologically active mixing treatment, whereas isotope variations in the biologically inhibited mixing treatment were relatively small or non-significant".

Question 7: Between line 225 and 230: How does the Fig S5a indicate the decrease in the intensity of C1? I didn't found a decrease trend in the figure and also decrease with what? With time, treatment numbers, or salinity?

Response:

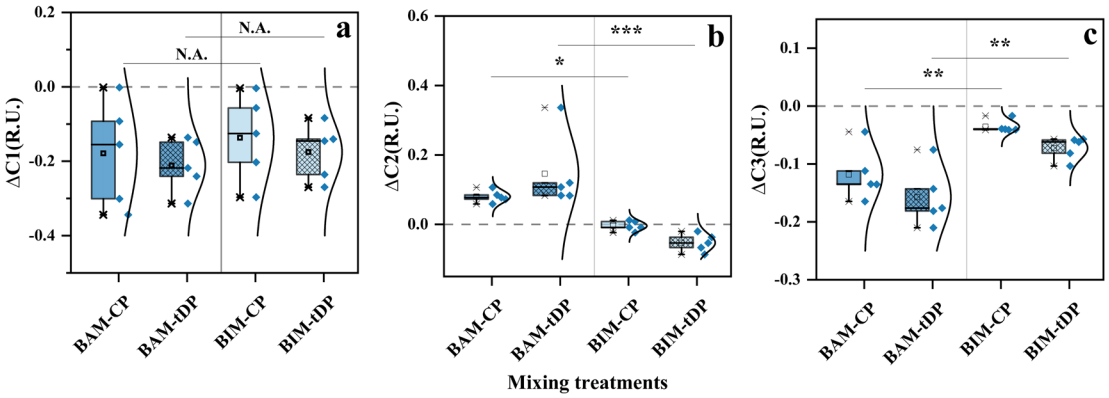
Thank you for pointing this out. We are sorry for the unclear and misleading wording. Fig. S5a does not indicate a decreasing trend with time, treatment number, or salinity. Instead, Fig. S5 shows the deviation between the measured fluorescence intensity and the theoretical conservative mixing/dilution value. The deviation from conservative mixing is illustrated in Fig. 2, where the measured fluorescence intensity of each component is compared with the theoretical conservative mixing curve. Fig. S5 further summarizes these deviations and statistically compares them between BAM and BIM treatments in the truly dissolved phase and colloidal phase.

For C1, the negative ΔC1 values indicate that the measured fluorescence intensity was lower than the theoretical conservative mixing value. The similar ΔC1 values between BAM and BIM treatments, with no significant difference (p > 0.05; Fig. S5a), suggest that the attenuation of C1 relative to conservative mixing was mainly driven by physicochemical processes, with limited biological influence. We have clarified that the "decrease" refers to a negative deviation from conservative mixing rather than a temporal, treatment-number, or salinity-dependent decrease.

The revised text reads:

[Section 4.2, L291-295] "Fig. S6 presents the statistical comparisons of deviations from conservative mixing. For the terrestrial humic-like component C1, ΔC1 values were negative in both treatments, indicating that measured C1 fluorescence intensities were lower than the theoretical conservative mixing values. The absence of a significant difference in ΔC1 between BAM and BIM treatments (p > 0.05; Fig. S6a) suggests that C1 attenuation relative to conservative mixing was mainly controlled by physicochemical processes, with limited additional biological influence (Shutova et al., 2014)";

[Figure S6]



"Figure S6. Deviations of fluorescence component intensities from theoretical conservative mixing values under biologically active and inhibited mixing treatments. Deviations were calculated as ΔC = measured fluorescence intensity − theoretical conservative mixing/dilution value for C1 (a), C2 (b), and C3 (c) in the colloidal phase (CP) and truly dissolved phase (tDP) under biologically active mixing (BAM) and biologically inhibited mixing (BIM) treatments. The dashed horizontal line

*represents conservative mixing (ΔC = 0). Blue diamonds represent individual samples (n = 5), and squares indicate mean values. Statistical significance indicates differences among the corresponding treatments; NS indicates no significant difference, * indicates p < 0.05, ** indicates p < 0.01, and *** indicates p < 0.001".*

Question 8: Between line 230 and 235: what are the fluorescence index and biological index and how are them measured? You would want to address them in the methods section first.

Response:

Thank you for this helpful comment. We agree that the fluorescence index (FI), biological index (BIX), and $SUVA_{254}$ should be defined in the Materials and methods section. FI and BIX are not independently measured parameters, but optical indices calculated from corrected EEM fluorescence spectra, whereas $SUVA_{254}$ is calculated from UV absorbance at 254 nm normalized to DOC concentration. We have added a brief description of these indices in the Materials and methods section and provided their detailed calculation procedures in the Supplementary Information as Text S4. We have also revised the corresponding text to clarify that FI, BIX, and SUVA254 were used as optical proxies for DOM source, recent biological contribution, and aromaticity, respectively.

The revised text reads:

[Section 2.3, L134-139] "Optical indices, including the fluorescence index (FI), biological index (BIX), and specific UV absorbance at 254 nm ($SUVA_{254}$), were calculated from the corrected EEM fluorescence and UV–Vis absorbance spectra. FI and BIX were derived from fluorescence intensity ratios at specific excitation/emission wavelengths, whereas $SUVA_{254}$ was calculated by normalizing UV absorbance at 254 nm to the DOC concentration of the corresponding sample or fraction. Detailed equations and interpretations are provided in Text S4";

[Section 4.2, L299-303] "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). Because FI and BIX indicate microbial/autochthonous DOM contribution and

recent biological production, respectively, whereas SUVA254 reflects DOM aromaticity, these optical changes suggest an increased contribution of freshly produced, less aromatic DOM and thus potentially enhanced DON bioavailability during estuarine mixing";

[Text S4] "Text S4. Calculation of optical indices

Optical indices, including the fluorescence index (FI), biological index (BIX), and specific UV absorbance at 254 nm ($SUVA_{254}$), were calculated to characterize DOM source, recent biological contribution, and aromaticity. Prior to FI and BIX calculation, EEM spectra were blank-subtracted, corrected for inner-filter effects, and Raman-normalized.

Let $F_{Ex,\ Em}$ represent the corrected fluorescence intensity at a given excitation and emission wavelength.

The fluorescence index was calculated as:

$$FI = F_{370,\ 470} / F_{370,\ 520} \qquad \text{Equation 6}$$

where $F_{370,\ 470}$ and $F_{370,\ 520}$ are the fluorescence intensities at emission wavelengths of 470 and 520 nm, respectively, under excitation at 370 nm. FI is commonly used to distinguish terrestrial and microbial/autochthonous DOM sources, with higher FI values generally indicating a greater microbial contribution.

The biological index was calculated as:

$$BIX = F_{310,\ 380} / F_{310,\ 430} \qquad \text{Equation 7}$$

where $F_{310,\ 380}$ and $F_{310,\ 430}$ are the fluorescence intensities at emission wavelengths of 380 and 430 nm, respectively, under excitation at 310 nm. BIX reflects the contribution of recently produced or autochthonous DOM, with higher values indicating stronger biological production.

$SUVA_{254}$ was calculated as:

$$SUVA_{254} = A_{254} \times 100 / DOC \qquad \text{Equation 8}$$

where A_{254} is the UV absorbance at 254 nm measured with a 1-cm quartz cuvette, and DOC is the dissolved organic carbon concentration of the corresponding sample or fraction in mg C L^{-1}. $SUVA_{254}$ is reported in L mg C^{-1} m^{-1} and is commonly used as a

proxy for DOM aromaticity. Higher $SUVA_{254}$ values indicate higher aromaticity, whereas lower values indicate a lower contribution of aromatic DOM".

CC1:

CC1's comments to the Author:

Estuaries act as critical filters for terrestrial nutrients transporting to the ocean. With the increasing anthropogenic nitrogen loading worldwide, understanding the transformation and bioavailability of organic nitrogen (ON) in estuarine mixing zones is becoming crucial for predicting coastal eutrophication. However, most previous studies focused on inorganic nitrogen, while the dynamics of the organic fraction remains less understood. This manuscript investigates the ON transformation and bioavailability dynamics during estuarine mixing, which addresses an important gap. The experimental design is logical, and the data is solid. It is a meaningful paper.

Response:

We sincerely thank the reviewer for the positive evaluation and encouraging comments on the significance, experimental design, and data quality of our study.

Question 1: In Introduction, add quantitative data or recent estimates regarding the global flux of estuarine ON to highlight the significance of estuarine organic nitrogen.

Response:

Thank you for this helpful suggestion. We have added quantitative global estimates of riverine nitrogen export in the Introduction to better emphasize the importance of estuarine organic nitrogen.

The revised text reads:

[Introduction, L30-35] "Globally, rivers deliver an estimated 36–60 Tg N yr^{-1} to coastal waters, including approximately 11.8 Tg N yr^{-1} as dissolved organic nitrogen (DON), highlighting the substantial role of organic nitrogen in land–ocean nitrogen transfer (Mayorga et al., 2010; Tivig et al., 2021). Recent global modeling further suggests that riverine total nitrogen export to the ocean increased from 27.5 Tg N yr^{-1} in 1901–1920 to 40.0 Tg N yr^{-1} in 1995–2014, with DON export increasing by 50.6%, emphasizing the growing importance of estuarine ON transformation under global environmental change (Ma et al., 2025)".

Question 2: The incubation experiments were conducted for 3 days. While this effectively captures rapid mixing processes, the manuscript discusses "long-term" nitrogen sequestration. It would be better to briefly mention the limitations of this short-term incubation in the Discussion section to ensure the extrapolation to long-term sinks is robust.

Response:

Thank you for this helpful suggestion. We agree that the 3-day incubation mainly captures rapid estuarine mixing processes, including flocculation, adsorption, and initial biological transformation, and cannot directly verify long-term nitrogen sequestration. We have therefore revised the Discussion to clarify this limitation and softened the relevant statements.

The revised text reads:

[Section 4.3, L388] "This transformation process likely enhances the potential for ON burial during transport from river to the sea";

[Section 4.4, L425-427] "Collectively, these findings, along with the results from the current study, suggest that coupled biological and physicochemical interactions during mixing not only promote short-term ON transfer into the particulate phase through a top-down PON pump (Fig. 7b, Pathway 1 & 3), but also alter…";

[Section 4.5, L460-462] "Second, the 3-day incubation is sufficient to capture rapid flocculation, adsorption, and initial microbial transformation, but may underestimate slower processes such as long-term mineralization, sedimentation, and RDON' stabilization".

Question 3: Since phytoplankton uptake of DIN involves isotopic fractionation, could this process also contribute to the elevated δ15N values in PON?

Response:

Thank you for this helpful comment. We agree that planktonic assimilation of dissolved inorganic nitrogen (DIN) can contribute to the biological production of PON during the mixing experiments. We have revised the Discussion to explicitly account for this process. However, phytoplankton DIN assimilation generally

involves isotope fractionation that preferentially incorporates ^{14}N, thereby producing isotopically lighter PON and tending to decrease $\delta^{15}N$-PON. This process therefore cannot explain the positive $\delta^{15}N$-PON deviation observed in the BAM treatments.

The observed enrichment instead suggests that a ^{15}N-enriched source was added to or retained within the particulate pool and that this process outweighed the opposing isotopically light contribution from newly produced planktonic PON. We therefore clarified that microbial processing can leave residual DON relatively enriched in ^{15}N, and that the selective re-adsorption of this microbially modified refractory DON (RDON') onto particles most likely increased $\delta^{15}N$-PON. Accordingly, the revised text emphasizes that planktonic DIN assimilation may have contributed to PON production but likely acted as a counteracting process, whereas RDON' re-adsorption dominated the isotopic budget and promoted the accumulation of refractory, isotopically enriched PON.

The revised text reads:

[Section 4.3, L363-380] "The isotopic deviations further constrain this mechanism by indicating that refractory PON accumulation involved the selective re-adsorption of microbially modified, ^{15}N-enriched RDON. In a simple dilution scenario, $\delta^{15}N$-PON would be expected to decrease linearly toward the seawater end-member value. Phytoplankton assimilation of DIN may further lower $\delta^{15}N$-PON through isotopic fractionation (Chen et al., 2024). However, in the BAM treatments, both $\delta^{13}C$ and $\delta^{15}N$ exceeded the theoretical mixing predictions at intermediate salinities, with enrichments of +0.10‰ and +0.45‰, respectively (Fig. 4c, d). This indicates that a process introducing or retaining ^{15}N-enriched material in the PON pool must have operated and outweighed the opposing input of isotopically light phytoplankton-derived PON. ON modified by microbial activity, including microbial products and residues, tends to have a higher C/N ratio, more negative $\delta^{13}C$ values, and elevated $\delta^{15}N$ values. These isotopic shifts are known to result from fractionation during the biosynthesis of cellular components (Abraham and Hesse, 2003; Ogawa et al., 2001).Because phytoplankton DIN assimilation would tend to lower rather than

raise δ^{15}N-PON, the observed positive deviations strongly implicate the selective re-adsorption of microbially modified, ^{15}N-enriched and residual refractory DON (RDON') onto particles as the dominant mechanism. Several complementary observations support this interpretation. The increase in C/N ratios with salinity (Fig. 4f) suggests a shift toward microbially processed organic matter rather than fresh phytoplankton biomass, which typically has lower C/N ratios. In addition, free energy calculations have shown that microbial transformation products, such as amino sugars, are thermodynamically favored for adsorption onto particles (Liu et al., 2024). As no external particles were introduced during the mixing experiments, the net positive δ^{15}N deviation most likely reflects in-situ RDON' adsorption and particle-phase retention, contributing to the accumulation of refractory, isotopically enriched ON in the particulate pool".

Question 4: Figure 7 presents an "Enhanced nitrogen pump model." What exactly is "enhanced"?

Response:

Thank you for raising this important point. We agree that the meaning of "enhanced" was not sufficiently defined in the original version. In our conceptual model, "enhanced" does not simply refer to a quantitatively larger nitrogen flux. Rather, it refers to the mechanistic expansion of the classical nitrogen pump by incorporating microbial transformation processes into the physicochemical framework of estuarine mixing. Therefore, the term "enhanced" refers to the additional microbial transformation pathway and its coupling with adsorption/flocculation processes. This coupling not only strengthens the transfer of ON from the dissolved to particulate phase, but also alters DON composition and bioavailability during estuarine mixing. We have revised the figure caption and related text to clarify this definition.

The revised text reads:

[Figure captions, L435-441] "Figure 7. Conceptual model of nitrogen cycling under estuarine mixing, highlighting the role of organic nitrogen (ON) transformation.

(a) Classical nitrogen pump model dominated by physicochemical processes, including dissolved organic nitrogen (DON) adsorption/flocculation, particulate organic nitrogen (PON) deposition and burial, resuspension, and mineralization. (b) Microbially enhanced nitrogen pump model, in which microbial utilization and transformation of terrestrial DON promote the degradation of humic-like DON, the production of NH_4^+ and labile DON (LDON), and the formation of microbially modified refractory DON (RDON'). The subsequent re-adsorption of RDON' onto particles further promotes refractory PON accumulation and nitrogen retention during estuarine mixing".

Question 5: The authors mention using parametric tests (e.g., t-tests or ANOVA) to compare treatments. It would be rigorous to briefly clarify whether the normality of the data distribution was checked.

Response:

Thank you for this helpful suggestion. We agree that the assumptions underlying the parametric analyses should be clarified. In the revised manuscript, we have added information to the Statistical analysis section stating that data normality was assessed using the Shapiro–Wilk test and homogeneity of variance was evaluated using Levene's test prior to one-way ANOVA. Tukey's post hoc test was applied when the assumptions of normality and equal variance were satisfied.

The revised text reads:

[Section 2.4, L151-154] "Prior to one-way ANOVA, the normality of data distribution was assessed using the Shapiro–Wilk test, and homogeneity of variance was evaluated using Levene's test. Group differences were assessed by one-way ANOVA followed by Tukey's post hoc tests when the assumptions of normality and equal variance were satisfied".

Question 6: I noticed several grammatical and formatting errors that should be corrected:

Line 127: "p" is not italic.

Line 188: "The non-conservative changes... are govern by..." should be "are governed by".

Line 223: "...particle characteristics , typically..." There is an extra space before the comma.

Line 309: "This study highlight that..." should be "highlights".

Line 424: "Crucially, our isotopic evidence that biologically-modified DON re-adsorbs onto particles..." This sentence appears to be a fragment or grammatically incomplete (missing a main verb for "evidence" or "that" should be removed).

Response:

Thank you for your careful corrections. We have revised the manuscript accordingly. Specifically,

(1) "p" has been italicized in Line 149;

(2) The extra space before the comma in Line 290 has been removed;

(3) "This study highlight that" in Line 381 has been corrected to "This study highlights that";

(4) The sentence containing "are govern by" in Line 188 and the incomplete sentence in Line 424 have been removed from the revised manuscript in response to related comments from other reviewers, so these issues no longer occur in the current text.
