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.*

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 and constructive evaluation. The reviewer recognized the scientific significance of our BAM/BIM experimental design and the value of the three-pathway conceptual model, while raising

substantive concerns regarding data interpretation, methodological clarity, and presentation. We have carefully addressed every point; below we summarize the major revisions organized around the three aspects highlighted in the general comments.

(1) Scientific quality. The three data-interpretation issues identified by the reviewer have been fully resolved:

- ***FTIR attribution error (Q4). The mismatch between the Discussion text (which cited "BIM") and the Fig. 4d caption (which showed BAM spectra) was a writing error. We have corrected the text to read "BAM," ensuring consistency with the figure and the underlying data. No changes to the data, interpretation, or conclusions were necessary.***
- ***Zeta potential argument (Q3). We acknowledge that the original text incorrectly implied that strongly negative zeta potentials promote adsorption. In the revised Section 4.1, we now clearly distinguish two mechanisms: (i) surface adsorption driven by non-electrostatic interactions (ligand exchange, hydrogen bonding, hydrophobic effects), and (ii) colloidal destabilization and flocculation driven by electrical double-layer compression at intermediate salinities, where zeta potentials approach neutrality. The non-monotonic zeta potential trend observed in our experiments is discussed as diagnostic evidence for the latter mechanism.***
- ***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.***

(3) 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 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, L138-139] "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, L169-171] "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, L240-252] "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, 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. 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, L331-355] "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. S9cS10c). C2 is microbially derived protein-like component (Liu et al., 2024), C1 and C3 represents 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 stimulates microbial growth and/or respiration, and changes 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; Xu and Guo, 2017). 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, L475-494] "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, L536-551] "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, L34] "Estuaries are critical transitional zones between rivers and sea that critically mediate the flux of nitrogen to 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, L144] "...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, L397] "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, L442] "The interplay of biological and physicochemical processes during mixing can produce compound effects".

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

Response:

Revised.

The modified image is as follows:

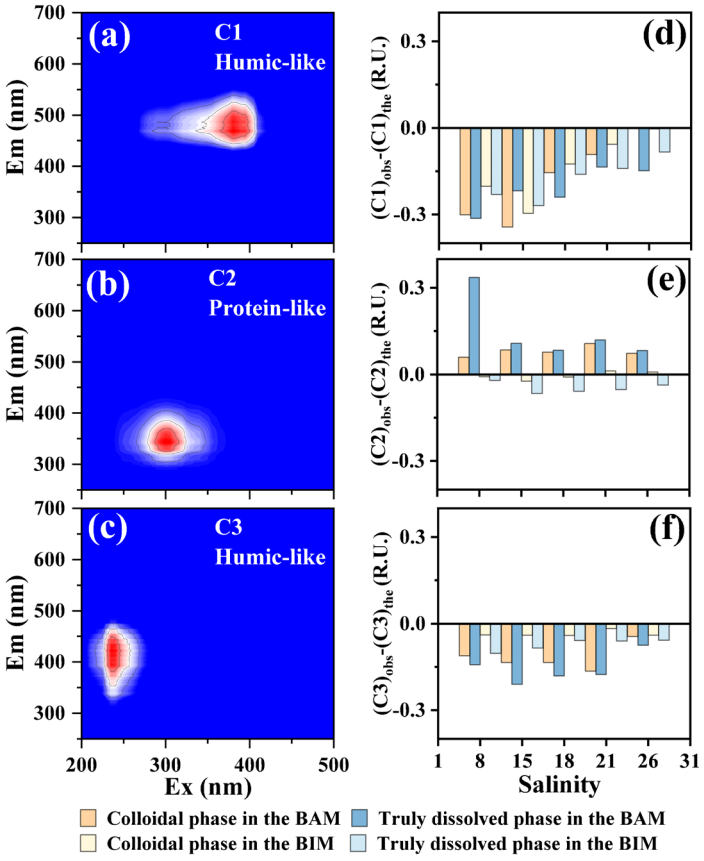

Figure 2. Variations in dissolved organic nitrogen (DON) components in the

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:

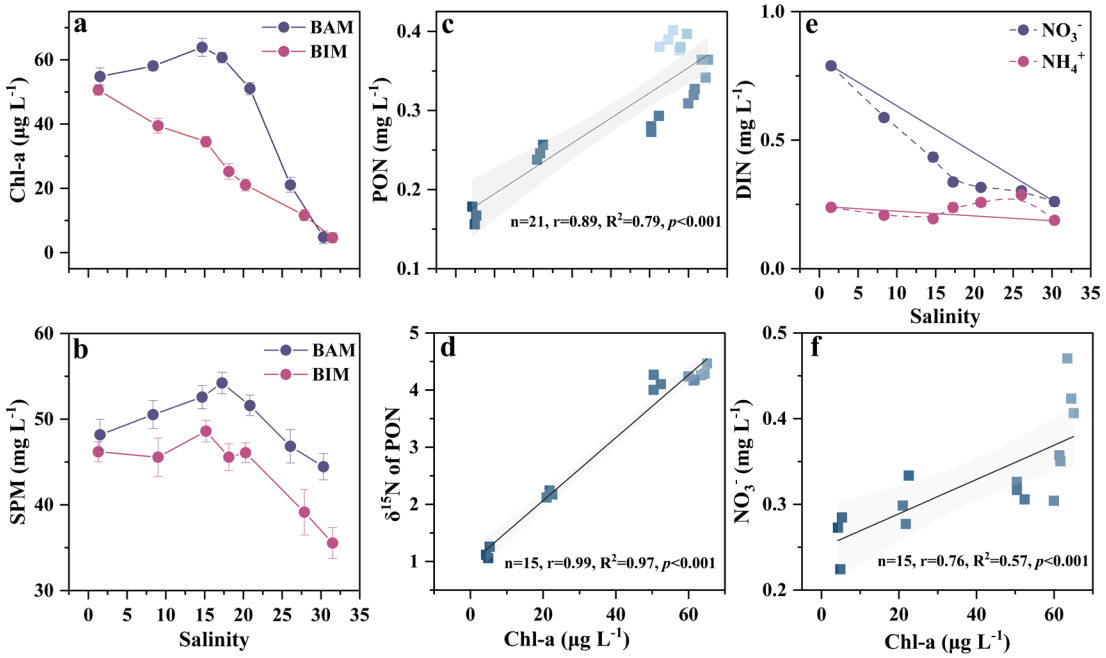

Figure S8. Variations of (a) Chl-a, (b) Suspended Particulate Matter (SPM), (e) nitrate (NO_3^-) and ammonium (NH_4^+) along the salinity gradient in the biologically active mixing (BAM) and biologically inhibited mixing (i.e., physicochemical mixing only, BIM) treatments (a, b). The relationship of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