https://doi.org/10.5194/egusphere-2026-216
Preprint. Discussion started: 9 February 2026

Estuarine Mixing Drives Organic Nitrogen Transformation and Bioavailability Dynamics

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Abstract. Estuaries act as critical transition zones for nitrogen transport, where the dynamics of inorganic nitrogen has been extensively studied. In contrast, organic nitrogen (ON), encompassing particulate organic nitrogen (PON) and dissolved organic nitrogen (DON), is strongly influenced by estuarine mixing of freshwater and seawater. However, the mechanisms driving ON transformation and their implications for bioavailability remain poorly understood. Here, estuarine mixing experiments are conducted across salinity gradients to explore ON transformation and changes in nitrogen bioavailability driven by physicochemical and biological processes. Using tangential flow filtration, optical signatures, and stable isotopes ($\delta^{13}C$, $\delta^{15}N$), we quantified ON composition and molecular characteristics. DON dominated the ON pool (> 71%) throughout the mixing process, with the low-molecular-weight (LMW) fraction accounting for 49 ± 7.8%. 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. Meanwhile, biological activity promoted the degradation of residual humic-like components (especially microbial C3), producing labile LMW-DON and ammonium. This conversion was evidenced by a strong negative correlation between humic-like and protein-like components in control treatments. 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. Overall, estuarine mixing drives conversion of humic-like ON into refractory particulate forms, while simultaneously enhancing the bioavailability of the residual dissolved nitrogen pool, thereby influencing nitrogen cycling and eutrophication risks at the river–sea interface.

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1 Introduction

Estuaries are critical transitional zones between rivers and sea, where critically mediate the flux of nitrogen to sea (Kellerman et al., 2014; Wan et al., 2023; Wu et al., 2023). While dissolved inorganic nitrogen (DIN) has been extensively studied (Cai et al., 2000; Zhou et al., 2023; Tang et al., 2024, 2025), organic nitrogen (ON) remains less understood, despite often constituting the majority (greater than 50%) of the total nitrogen pool (Berman & Bronk, 2003; Jani & Toor, 2018; Yan et al., 2024). ON is operationally fractionated into particulate (PON) and dissolved (DON) forms (Bronk et al., 2007; Yan et al., 2021). Recent advancements, such as tangential flow filtration (TFF), further separate DON into high-molecular-weight colloidal (CON) and low-molecular-weight truly dissolved (tDON) fractions (Edokpa et al., 2018; Rostad et al., 1997; Xu et al., 2018). These techniques enable detailed investigations into the physicochemical behavior and microbial bioavailability of different ON pools (Pan et al., 2020; Xu et al., 2018; Fagerberg et al., 2009; Li et al., 2020; Seitzinger et al., 2002). Given that excessive export of terrestrial ON can drive coastal eutrophication and hypoxia, understanding the transformation and fate of these distinct ON fractions in estuaries is a scientific priority.

In estuaries, salinity gradients drive complex biological and physicochemical processes that govern the fate of ON (Jani & Toor, 2018; Zhang et al., 2022; Wu et al., 2023). Recent studies have shown that DON is considered as a bioavailable nitrogen source for phytoplankton and bacteria in coastal waters (Bronk et al., 2007; Jani & Toor, 2018; Yan et al., 2021). Contrary to early models of conservative dilution mixing, ON behavior is often non-conservative, exhibiting net "addition" or "removal" from the dissolved phase. This results from the interplay of biological processes (e.g., microbial degradation, uptake) and physicochemical removal mechanisms (e.g., adsorption, flocculation) (Jani & Toor, 2018; Yan et al., 2022; Zhou et al., 2016). While biological activity alters ON's chemical characteristics, physicochemical processes can transfer it to the particulate phase (Zhou et al., 2021; Yan et al., 2023; Li et al., 2024). However, how and what these processes interact and collectively determine the net transformation and bioavailability of the ON pool remains a critical knowledge gap.

Given the complex structure of DON and its compositional overlap with a significant proportion of dissolved organic matter (DOM), Ultraviolet-visible spectroscopy (UV-Vis) and Excitation-emission matrix - Parallel factor analysis (EEM-PARAFAC) were used to characterize DON (Hounshell et al., 2017; Li et al., 2024). Humic-like (terrestrial and microbial sources) and protein-like components are considered as the dominant terrestrial DON components entering the sea. The terrestrial humic-like components, characterised with complex and high molecular weight and lower bioavailability, is preferentially removed through polymerization (Asmala et al., 2014; Pan et al., 2020; Shimabuku et al., 2017), while microbial humic-like component could be partially utilized by organisms (Cole et al., 2007; Yao et al., 2024). Moreover, protein-like components, featured low-molecular-weight, is more easily utilized by microorganisms (Li et al., 2017; Zhang et al., 2015; Zhang et al., 2024), thereby promoting microbial activity. Research shows the significant correlation between DON bioavailability and protein-like fluorescence, the latter could be used as an alternative means to estimate the bioavailability of DON pool (Hu & Ren, 2016; Li et al., 2019). Despite these advances, limitations remain in understanding

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how different DON components respond to the interplay of physicochemical and biological processes across the estuarine salinity gradient, and how these transformations influence their bioavailability.

The stable isotopic composition of PON (δ^{15}N-PON) is widely used to trace its sources and behavior (Kodama et al., 2021; Zhu et al., 2021). Similar to DON, PON exhibits non-conservative behavior in estuaries due to continuous physicochemical and biological alterations (Wu et al., 2016; Dähnke et al., 2022; Ghosh et al., 2024). Consequently, the δ^{15}N-PON signature reflects not only its source but also subsequent transformations, such as isotopic fractionation during nitrate assimilation or DON utilization, which can alter the δ^{15}N of the residual nitrogen pool (Yan et al., 2021). Although combining fluorescence and isotopic techniques has advanced our understanding of ON dynamics (Yan et al., 2022; Yao et al., 2024; Zhong et al., 2022), a framework linking these transformations to ecological outcomes (e.g., algal blooms) is lacking. Specifically, the relative importance of biological versus physicochemical processes in regulating the overall fate of ON across estuarine gradients remains a key uncertainty. Here, we address this gap by isolating and quantifying coupled physicochemical–biological pathways that directly alter ON partitioning and bioavailability during mixing, providing mechanistic constraints that can be evaluated against field gradients and incorporated into estuarine nitrogen budget models.

In this study, estuaries are considered not merely as a passive "nitrogen filter", but rather an active "nitrogen transformer", where complex processes jointly shape the cycling pathways of ON. Accordingly, this study aims to investigate the roles of biological and physicochemical processes in driving the transformation and bioavailability of ON during estuarine mixing. To this end, estuarine mixing experiments were conducted across salinity gradients under two treatments: biologically active mixing (BAM; combined processes) and biologically inhibited mixing (BIM; physicochemical processes only). Tangential flow filtration, ON content, optical characteristics, and δ^{13}C and δ^{15}N isotopes were analyzed to elucidate the mechanisms controlling ON fate and its impact on coastal nitrogen cycling.

2 Materials and methods

2.1 Sampling

River water (salinity = 0.49 PSU) and seawater (salinity = 31.52 PSU) (Table S1) were collected in July 2024 using polyethylene containers from the Ziya Xinhe River (117.25°E, 38.60°N), and from Bohai Bay, China (118.18°E, 38.66°N), approximately 40 km downstream of the river mouth, respectively. Phytoplankton biomass typically peaks during summer in the study area, increasing both the concentration and molecular complexity of DON in its riverine and marine environments. Water temperature, salinity (S), pH, and dissolved oxygen (DO) were measured in Table S1 using a portable multiparameter analyzer (YSI ProPlus, USA). Samples were promptly transported to the laboratory, stored at low temperature in the dark, and homogenized prior to analysis. Additional physicochemical background information, including nutrient concentrations, suspended particulate matter (SPM), and its mineral composition, is also presented in Table S1.

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2.2 Experiments

River water and seawater were mixed at the following seven volume ratios (v/v) to prepare 5 L of solution: 10:0, 8:2, 6:4, 5:5, 4:6, 2:8, and 0:10. Two experimental treatments were performed. Biologically active mixing (i.e., biological and physicochemical processes occurring concurrently, BAM): untreated mixtures to assess biological and physicochemical processes. Biologically inhibited mixing (i.e., physicochemical processes mixing only, BIM): mixtures amended with 0.1% (v/v) chloroform to inhibited biological activity and isolate physicochemical processes. All treatments were performed in triplicate. Both treatments were incubated for 3 days under a 12-hour light/12-hour dark cycle at 20°C and agitating at 180 rpm. After incubation, samples were vacuum-filtered through pre-combusted (450 °C, 4 h) 0.45 μm GF filters to separate PON from DON. The filtrate (< 0.45 μm) was subsequently fractionated by tangential flow filtration (TFF) into colloidal (CON; 1 kDa–0.45 μm; also referred to as the high molecular weight (HMW) fraction) and truly dissolved (tDON; < 1 kDa; also known as the low-molecular-weight (LMW) fraction) fractions. Further TFF details are provided in Text S1 and Fig. S1.

2.3 Sample analysis

Concentrations of dissolved organic carbon (DOC), total dissolved nitrogen (TDN), and dissolved inorganic nitrogen (DIN, including nitrite (NO_2^-), nitrate (NO_3^-), and ammonium (NH_4^+)) were measured using a TOC analyzer (multi N/C 3100, Analytik Jena) and a UV-Vis spectrophotometer (TU-1901, Persee), respectively (Ye et al., 2018; Wu et al., 2023). DON was calculated as the difference between TDN and DIN. The same procedures were used to determine nitrogen concentrations in the truly dissolved fraction (<1 kDa), and CON was calculated by difference (DON - tDON), as detailed in the Text S1 (Yang et al., 2021). Particulate organic carbon (POC), particulate nitrogen (PN), and stable isotopes ($\delta^{13}C$, $\delta^{15}N$) were analyzed on an elemental analyzer-isotope ratio mass spectrometer (DELTA V Advantage, Thermo Fisher Scientific) after acid treatment to remove carbonates. Particulate inorganic nitrogen was determined via HCl extraction (Zuo et al., 2016) and found to be negligible (<5% of total PN); therefore, PN was considered equivalent to PON. All analyses were performed in triplicate. Measurement uncertainties from subtractions were estimated using error propagation Cornell et al. (2003). Further details on analytical precision, detection limits, and quality control are provided in the Text S2.

Purified colloidal fraction (1 kDa–0.45 μm) and truly dissolved fraction (< 1 kDa) water samples (3 mL aliquots) were transferred to 1-cm quartz cuvettes for optical characterization using a UV-Vis spectrophotometer (TU-1901, Persee, China) and a fluorescence spectrometer (FS5, Edinburgh Instruments, UK). EEM fluorescence spectra were recorded across excitation wavelengths of 200–500 nm (5 nm intervals) and emission wavelengths of 250–700 nm (2 nm intervals), with a fixed spectral bandwidth of 5 nm. Milli-Q water blanks were analyzed regularly to ensure instrument stability.

Additional water quality parameters including S, pH, SPM, and chlorophyll-a (Chl-a) were measured. The chemical composition of DON was analyzed using Fourier-transform infrared spectroscopy (FTIR, Nicolet iS10, Thermo Fisher Scientific, USA) after freeze-drying. Particle size distribution, polydispersity index (PdI), and zeta potential were measured using a Zetasizer Nano ZS90 (Malvern Instruments, UK). Activities of enzymes related to denitrification and organic matter

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degradation (nitrite reductase (NIR), succinate dehydrogenase (SDH), and chitinase (CHI); Solarbio, China) were quantified using commercial assay kits. ATPase activity was also measured with a commercial kit (Solarbio, China) to verify the effectiveness of chloroform-induced biological inhibition (Fig. S2a). Potential physicochemical interference from chloroform was assessed by monitoring particle properties in a separate control experiment. Zeta potential analysis showed no significant changes in average particle size, zeta potential, or Phl following chloroform addition (Fig. S2, $p > 0.05$), indicating minimal interference in the observed ON behavior during the experiments.

2.4 Statistical analyses

Statistical analyses were performed using IBM SPSS 20.0, with results presented as mean ± standard deviation ($n = 3$). Group differences were assessed by one-way ANOVA with Tukey's post hoc tests. Deviations of nitrogen concentration, component fluorescence intensity, and stable-isotope ratios from the theoretical conservative mixing line between the river water and seawater endmembers are defined as nonconservative behavior (addition or removal). PARAFAC was conducted in MATLAB (R2018b) using the DOMFluor toolbox (Stedmon and Bro, 2008). Spectra were corrected for inner-filter effects and Raman normalized following Murphy et al. (2010). A three-component model was selected based on residual analysis and split-half validation. Component fluorescence intensity was normalized to the total intensity per sample. Relationships between parameters were evaluated using regression analysis. A significance level of $p < 0.05$ was used for all tests.

3 Results

3.1 DON in estuarine mixing

DON, comprising tDON and CON, was the dominant form of ON under both mixing conditions, accounting for over 71% of total ON, with CON contributing 19–41% (Fig. S3). Along the increasing salinity gradient, the concentrations of both CON and tDON declined (Fig. 1; Table S2) causing also the proportion of DON relative to ON to decrease (Fig. S3). These concentration trends for CON and tDON deviated from the conservative mixing curve (Fig. 1), indicating non-conservative behavior. Specifically, tDON showed significant addition in the BAM treatments and removal in the BIM treatments, while CON exhibited removal under both BAM and BIM treatments. A significant difference was thus observed by including and excluding biological processes between the two mixing treatments ($n = 21$, $p < 0.05$).

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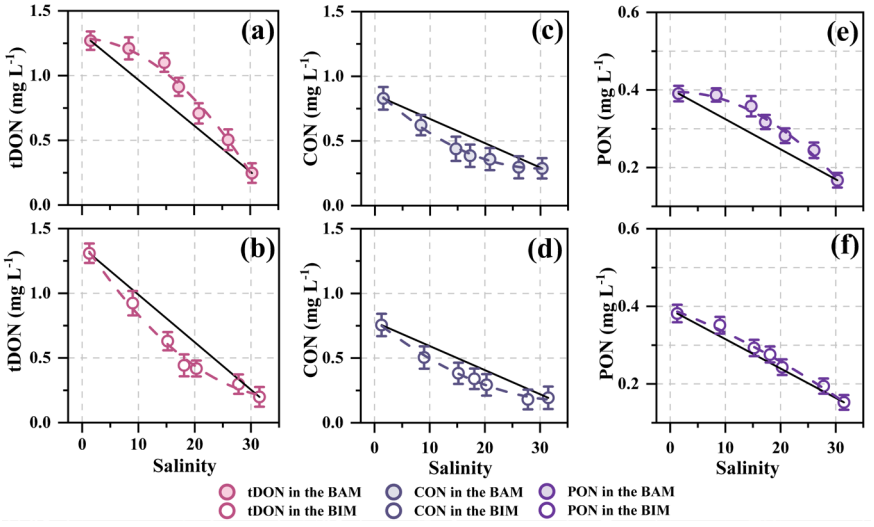

Figure 1. Variations in the concentrations of truly dissolved organic nitrogen (tDON) (a, b), colloidal organic nitrogen (CON) (c, d) and particulate organic nitrogen (PON) (e, f) across different river-seawater mixing ratios in the biologically active mixing (BAM) and biologically inhibited mixing (i.e., physicochemical mixing only, BIM) treatments. Conservative mixing curves (solid lines) were calculated using a two-end-member model (Langmuir et al., 1978), while dashed lines represent smoothed fits to the observed data.

PARAFAC analysis identified the three fluorescent components (Fig. 2; Table S3) commonly observed in natural DOM: two humic-like components (C1: terrestrial; C3: microbial) and one protein-like component (C2). All components decreased with increasing salinity (Fig. S4; Table S2), yet showed distinct non-conservative behaviors. C2 and C3 were particularly influenced by biological processes. Compared to theoretical fluorescence values derived from the conservative two-end-member mixing model, all three components were reduced in the BIM treatments (Fig. 2). In contrast, in the BAM treatments, C1 and C3 were reduced, while C2 exhibited a net addition (Fig. 2).

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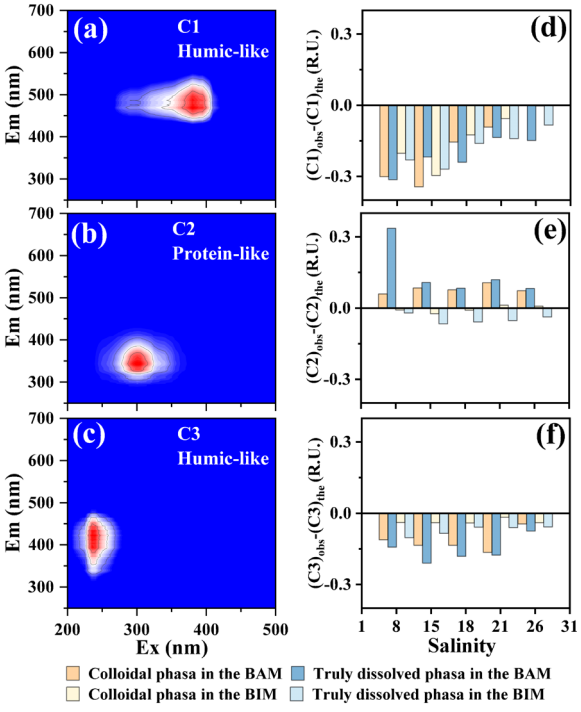

Figure 2. Variations in dissolved organic nitrogen (DON) components in the biologically active mixing (BAM) and biologically inhibited mixing (i.e., physicochemical mixing only, BIM) treatments. Panels a–c show the three fluorescence components identified by parallel factor analysis (PARAFAC): C1 - terrestrial humic-like ON, C2 - microbial protein-like ON, and C3 - microbial humic-like ON. Panels d–f display the difference in relative fluorescence intensity of C1, C2, and C3, respectively, plotted against salinity. These differences are calculated as the observed values ("obs") under mixed salinity conditions minus the theoretical values ("the") predicted by the two-end-member model. All Y-axis values in d–f is expressed in arbitrary units (R.U.), corrected using water Raman normalization.

3.2 PON in estuarine mixing

PON generally followed a conservative mixing trend, but there was a significant difference between the two treatments (n = 21, $p < 0.05$). In the BIM treatments, there was only a slight addition in PON, while the BAM treatments gave a substantial addition in PON (Fig. 1e, f). In the BAM treatments, $\delta^{13}C$ and $\delta^{15}N$ of PON ranged from −28.3‰ to −26.5‰, and 1.1‰ to 8.8‰, respectively. While in the BAM treatments, $\delta^{13}C$ and $\delta^{15}N$ ranged from −26.1‰ to −25.9‰, and 6.2‰ to 7.2‰, respectively (Fig. 3a, b; Table S2). Both $\delta^{13}C$ and $\delta^{15}N$ values generally declined along the salinity gradient, with significant positive correlations observed between them (r = 0.93, $p < 0.001$ and r = 0.63, $p < 0.001$, respectively; Fig. S6). The $\delta^{15}N$ values in this study were within the range reported in previous studies (Dong et al., 2022; Zhu et al., 2021).

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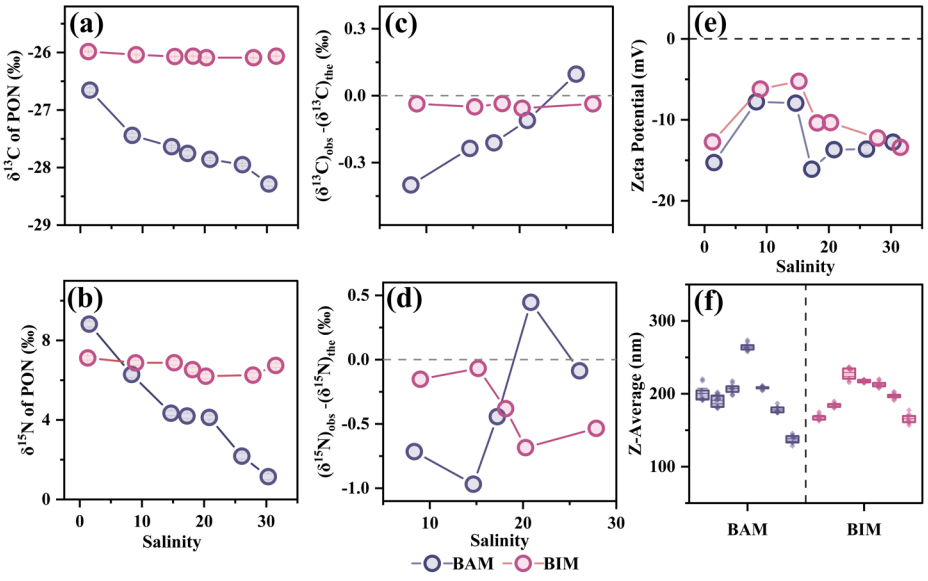

Figure 3. Variations of $\delta^{13}C$ values of particulate organic nitrogen (PON) (a), $\delta^{15}N$ values of PON (b), zeta potential (e), and mean particle size (f) along the salinity gradient in the biologically active mixing (BAM) and biologically inhibited mixing (i.e., physicochemical mixing only, BIM) treatments. Panels c and d show the deviations of $\delta^{13}C$ and $\delta^{15}N$ values of PON, respectively, calculated as the difference between the observed values ("obs") under mixed salinity conditions and the theoretical values ("the") predicted by the two-end-member model.

4 Discussion

4.1 Non-conservative behavior of organic nitrogen

The non-conservative behavior of DON during its transport from estuaries to sea is primarily driven by a combination of biological activities and physicochemical interactions (Yan et al., 2022; Yan et al., 2024; Zhong et al., 2022). Adsorption, flocculation, and biological transformations of DON have been documented as important governing processes in major estuarine systems such as the Yangtze and Mississippi Rivers (Zhang et al., 2021; Zhou et al., 2021; Zhou et al., 2016). The non-conservative changes in tDON, CON, and PON are govern by physicochemical processes under abiotic conditions (i.e., biologically inhibited mixing treatments). The turbidity zone, characterized by high SPM concentrations (Fig. S7b), showed negative zeta potentials ($\zeta < 0$; Fig. 3e), which promote the adsorption of DON onto SPM surfaces, leading to PON enrichment (Sun et al., 2020; Zhang et al., 2020). As zeta potential values approached neutrality, colloidal destabilization facilitated particle aggregation and sedimentation, promoting CON conversion to PON by increased particle size (Fig. 3f). 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

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and Hur, 2016). In the absence of external ON inputs, the non-conservative ON behavior observed in the BIM treatments was primarily induced by salinity-driven flocculation and adsorption, resulting in dynamic exchanges between DON and PON pools. Importantly, the loss of CON during estuarine mixing implies that HMW fractions are preferentially removed from the dissolved phase by physicochemical processes (Fig. 1).

The contrasting DON dynamics between BAM and BIM treatments suggests the critical role of biological processes in estuarine DON transformation. In the BAM treatments, the removal of CON with a concurrent increase in tDON and PON (Fig. 1) aligns with observations in major global estuaries (Li et al., 2023; Thibodeau et al., 2017; Yan et al., 2022; Yan et al., 2024). This suggests that the active biological processing of DON is a widespread estuarine phenomenon, where DON serves as a key nitrogen source for microbial growth (Chen et al., 2022, 2024; Yan et al., 2024; Zhang et al., 2024). In our study, phytoplankton were a primary driver of this transformation. This is evidenced by elevated Chl-a, which strongly correlated with both PON formation and the assimilation of NO_3^- (Fig. S7). The preferential utilization of LMW-DON, consistent with previous findings in the region (Li et al., 2020; Zhang et al., 2024), further supports this conclusion. Since phytoplankton taxa exhibit varied preferences for inorganic nitrogen and ON substrates (Moschonas et al., 2017; Yan et al., 2022), DON availability becomes a critical factor in shaping community structure. This is particularly relevant for the Bohai Sea, where phytoplankton communities are reportedly shifting from NO_3^--preferring diatoms to DON-preferring dinoflagellates (Reinl et al., 2022; Chen et al., 2024). Our findings thus underscore that DON availability not only fuels productivity but also regulates phytoplankton succession and the potential for harmful algal blooms.

Microbial degradation is considered the dominant removal pathway for allochthonous DON input through the estuaries to the Bohai Sea (Li et al., 2017). Although larger organic molecules require enzymatic hydrolysis for assimilation, HMW DON has been shown to degrade more rapidly than LMW fractions (Perliński and Mudryk, 2018), and serve as a superior bacterial substrate (Li et al., 2020). In this study, NH_4^+ enrichment and elevated activities of SDH and CHI enzymes the 3-day experimental period of the BAM treatments (Figs. S7e and S8b, c) further supported the biological reactivity of DON, particularly the CON fraction, indicating potentially bacterial degradation pathways. These processes were likely promoted by DIN-depleted high-salinity conditions (Fig. S7). This is similar to patterns observed in the Elbe Estuary, where DON consumption and NH_4^+ regeneration occur concurrently (Schlarbaum et al., 2010). Overall, these results confirm that estuarine DON exhibits non-conservative behavior driven by active biological processes, consistent with previous observations from the Yangtze (Yan et al., 2024) and Pearl River estuaries (Li et al., 2023).

4.2 Enhancement of DON bioavailability

The transformation of ON during estuarine mixing is governed by interacting factors such as proportion of saltwater and freshwater, ON bioavailability, and particle characteristics , typically involving coupled biological and physicochemical mechanisms. The fluorescence EEM quantified compositional changes in DON across the estuarine mixing gradient (Fig. 2). The fluorescence intensity of the terrestrial humic-like component (C1) decreased similarly in both BAM and BIM treatments ($p > 0.05$; Fig. S5a). This indicates that the phase transfer of this refractory DON fraction (RDON) is primarily

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regulated by physicochemical processes, with minimal influence from biological activity (Shutova et al., 2014). In contrast, the significant differences ($p < 0.001$) in the behavior of the protein-like (C2) and microbial humic-like (C3) components between treatments reveals their susceptibility to both biological degradation and physicochemical interactions (Fig. S5b, c). Moreover, a strong positive correlation between DOC and DON ($n = 21$, $r > 0.97$, $p < 0.001$; Fig. S9a, b) indicates their coupling in the estuarine mixing processes. In the BAM treatments, accumulation of tDON was observed (Fig. 1a), alongside increases in the fluorescence index (FI; Fig. 4a) and biological index (BIX; Fig. 4c), with a concurrent decrease in aromaticity ($SUVA_{254}$; Fig. 4b). These optical changes collectively suggest enhanced DON bioavailability during estuarine mixing.

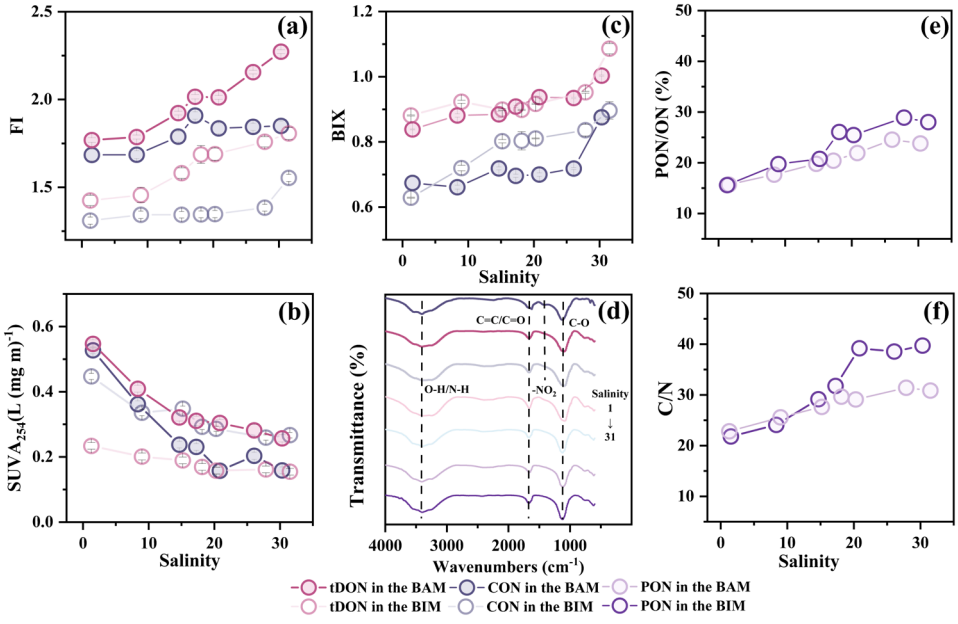

Figure 4. Changes in the compositions of dissolved organic nitrogen (DON) and particulate organic nitrogen (PON) along the salinity gradient in the biologically active mixing (BAM) and biologically inhibited mixing (i.e., physicochemical mixing only, BIM) treatments. Panels a–d show variations in DON properties with increasing salinity: (a) Fluorescence Index (FI); (b) Specific UV absorbance at 254 nm ($SUVA_{254}$); (c) Biological Index (BIX); and (d) FTIR spectra (shown for the BAM treatments only). Panels e–f display changes in particulate organic carbon (POC) characteristics with increasing salinity: (e) POC content and (f) C/N ratios.

Physicochemical processes drive the significant non-conservative removal of humic-like components (C1 + C3), causing deviations of 10–21% from conservative mixing (Fig. 2). These abiotic processes alone accounted for the majority (63 ± 11%) of the total humic-like removal observed in the BAM treatments, with the refractory terrestrial component (C1) being a primary contributor. This finding aligns with observations in the Yangtze Estuary (Yan et al., 2024). Salt-induced flocculation during estuarine mixing is shown to remove both protein-like and humic-like components. HMW humic-like

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components (Asmala et al., 2014) are preferentially removed, consistent with the observed loss of CON (Fig. 1). Moreover, particulate matter including colloids adsorb humic-like or aromatic DOM (Pan et al., 2020; Shimabuku et al., 2017; Yan et al., 2022), likely enhanced by the local mineralogy. The suspended matter in this study was rich (greater than 73%) in illite and quartz (Table S1), which have a known affinity for aromatic DOM (Krettek et al., 2023). Therefore, particle adsorption and salt-induced flocculation were the dominant physicochemical removal pathways for humic-like DON.

In the BAM treatments, humic-like components (C1 + C3) showed similar removal trends as in the BIM treatments, but the fluorescence attenuation is greater, deviating more strongly from the theoretical dilution curve (Fig. 2, Table S2). Conversely, the protein-like component (C2) increased significantly along the salinity gradient, opposite to its removal in the BIM treatments.

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 BIM treatments (Fig. 4d). These spectral trends thus reflect partial removal of humic-like components and concurrent enrichment of protein-like component. 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. S9c). 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). 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). 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). Moreover, NH_4^+ accumulation and elevated expression of DON degrading enzymes, such as SDH and CHI, in the BAM treatments (Figs. S7e and S8b, c), further confirm the increased biological reactivity and transformation of DON. The strong linkage between microbial activity and terrestrial DON suggests that humic-like components were utilized as substrates to generate bioavailable LDON, resulting in a relative increase in C2 fluorescence intensity by 6% to 12% from the theoretical dilution curve (Fig. 2, Table S2). This process led to net tDON enrichment and enhanced overall DON bioavailability in the BAM treatments.

DON remained the dominant component of the ON pool (greater than 71%; Fig. S3), although its proportion decreased along the estuarine salinity gradient. As discussed above, the combined effects of physicochemical removal and biological degradation of humic-like components (C1 + C3) enhanced the relative abundance of the protein-like component (C2, LDON) within the residual pool. This processing also resulted in a net addition of tDON (LMW-DON), causing a positive deviation from the conservative two-end-member mixing model (Fig. 1). These observations demonstrate that the interplay between physicochemical and biological processes during estuarine mixing enhances the bioavailability of DON, especially

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its LMW fraction. These findings highlight the importance of biological processes in regulating DON recycling, further demonstrating their dominant role in shaping the bioavailability of DON. The increased bioavailability of DON has implications for the size, stability, and reactivity of the dissolved nitrogen pool in estuarine and coastal systems, potentially affecting primary productivity and nitrogen retention capacity.

4.3 Accumulation of refractory PON

The proportion of PON increased with rising salinity in both BAM and BIM treatments (Fig. 4e), exceeding theoretical dilution predictions by 14.5% to 24.1% and 2.34% to 8.76%, respectively (Fig. 1e–f). Concurrently, the C/N ratio also increased across all incubation experiments (Fig. 4f). As discussed, this increase in PON can be attributed to two main processes: (1) adsorption and flocculation of DON onto particulate matter, and (2) biological production of PON by planktonic organisms. Flocculation and particle adsorption predominantly target dissolved humic-like/aromatic components (C1 + C3), with the extent of removal influenced by ON composition, concentration, and interactions with mineral surfaces (Krettek et al., 2023). Field studies have shown that settling particles in dynamic sedimentation–resuspension systems can become coated with refractory organic matter (Liu et al., 2024; Roland et al., 2008), facilitating the accumulation of refractory PON in aquatic environments

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). Typically, phytoplankton-derived PON shows lower $\delta^{15}N$ values, and mixing or dilution tends to further decrease these values. However, in the BAM treatments, $\delta^{15}N$ values deviated from the expected dilution trend, instead showing enrichment (Fig. 3d). At intermediate salinities, $\delta^{15}N$ remained relatively stable, and both $\delta^{13}C$ and $\delta^{15}N$ exceeded theoretical predictions (Fig. 3d), with $\delta^{13}C$ enrichment of + 0.10‰ and $\delta^{15}N$ enrichment of + 0.45‰ (Fig. 3b, c, d). These isotopic enrichments suggest that selective adsorption of ON with elevated $\delta^{15}N$ values offset the expected dilution-induced decrease. 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, changes in PON composition likely resulted from the in-situ formation of PON and/or selective re-adsorption of refractory microbial DON (RDON'). This RDON′ is characterized by a negative $\delta^{13}C$ signature, elevated $\delta^{15}N$ and a high C/N ratio, collectively contributing to the accumulation of refractory ON in the PON pool.

This study highlight that estuarine mixing processes significantly reshape the composition and quantity of PON mainly through biological modifications and adsorption and flocculation of refractory DON, these processes account for at least 44-71% of the removal of humic-like components. These transformation pathways result in particle-associated accumulation of refractory ON, a process often overlooked in studies of estuarine particle dynamics. Given rising anthropogenic ON inputs to river–estuary systems (Yan et al., 2025), the relative proportion of PON within suspended particles is likely to increase further. Moreover, the biological and physicochemical characteristics of particle-associated ON, having undergone extensive

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degradation and interaction with mineral surfaces, may differ substantially from newly produced ON (Roland et al., 2008). This transformation process likely enhances the potential for ON burial during transport from river to sea, contributing to long-term nitrogen sequestration.

4.4 Mixing-driven organic nitrogen transformation and fate

Estuarine ON exhibits both conservative and non-conservative behavior during its transport to the sea (Fig. 5a, b), with variability driven to differing extents by biological and physicochemical processes. This study shows that salt-induced flocculation and adsorption during estuarine mixing preferentially removed ON, especially HMW humic-like components (C1 + C3), from the dissolved pool, transferring them to particulate phases and promoting the accumulation of refractory components (Fig. 6a, Pathway 1). In parallel, microbial utilization of terrestrial DOC and DON as a nutrient source enhanced biological activity, leading to degradation of humic-like components (C1 + C3) and subsequent enrichment of NH_4^+ and LMW-DON (tDON, Fig. 6b, Pathway 2), increasing the bioavailability of dissolved nitrogen. In turn, the selective re-adsorption process of refractory DON (RDON′) derived from microbial transformation onto particles further promotes the formation of particle-associated refractory nitrogen (Fig. 6c, Pathway 3).

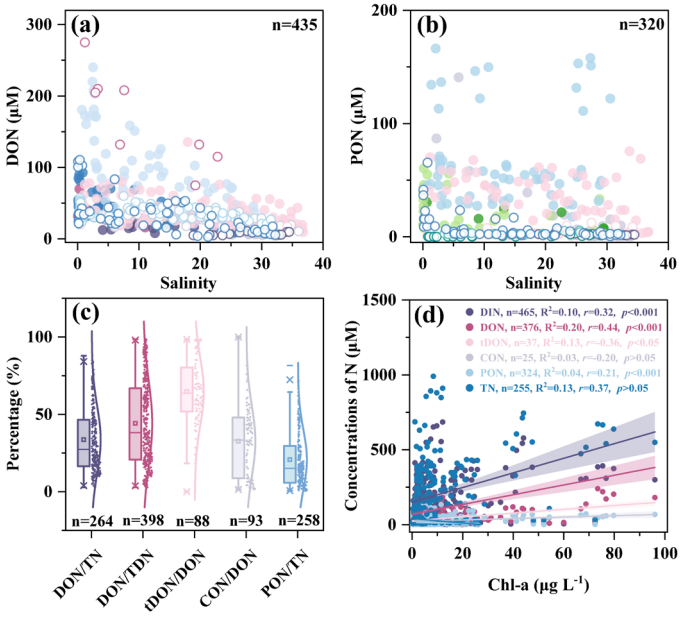

Figure 5. Non-conservative behavior of dissolved organic nitrogen (DON) (a) and particulate organic nitrogen (PON) (b) along the salinity gradient in estuarine mixing zones of rivers discharging into global ocean. Different colors and symbols (hollow and solid) represent different estuaries; detailed site information is provided in Table S4. (c) Boxplots showing the proportions of DON and PON relative to total nitrogen (TN), DON relative to total dissolved nitrogen (TDN), and the distribution of truly dissolved organic nitrogen (tDON) and colloidal organic nitrogen (CON) within DON. (d) Correlation analysis between organic nitrogen species and chlorophyll-a (Chl-a) concentrations in the water column.

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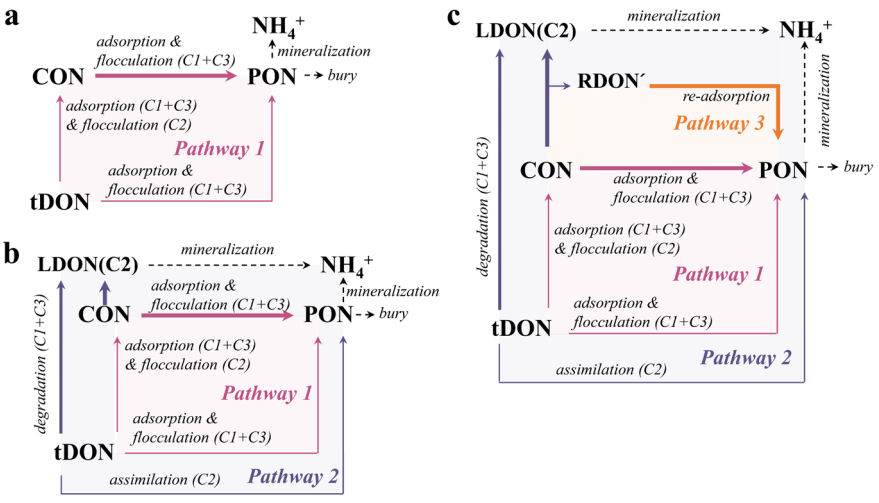

Figure 6. Conceptual pathways (1-3) illustrating the transformation of organic nitrogen (ON) during estuarine mixing. Pathway 1 (a): Adsorption and flocculation of humic-like (C1 + C3) and protein-like (C2) components from truly dissolved organic nitrogen (tDON) and colloidal organic nitrogen (CON) onto particulate phases. Pathway 2 (b): Microbial assimilation and degradation of DON, where DON serves as a carbon and nitrogen source, leading to the formation of labile DON (LDON), which may be mineralized or buried. Pathway 3 (c): Re-adsorption of refractory DON (RDON') generated or modified through microbial processes. The thick arrow represents the main pathways.

Estuarine mixing reshapes nitrogen cycling and rebalances the estuarine nitrogen budget through multiple transformation pathways. Similar mechanisms likely occur across estuaries worldwide, with their relative intensities modulated by regional hydrodynamics, sediment characteristics, and the strength of coupled biological (microbial degradation, phytoplankton uptake) and physicochemical (adsorption/flocculation) processes. In physiochemically dominated estuaries, like the northern Gulf of Mexico (Zhou et al., 2016), ON transformation is mainly governed by dilution, flocculation, and sedimentary re-mineralization, leading to the transfer or burial of terrestrial nitrogen (Fig. 7a). However, biologically active estuaries operate differently. For example, humic-like components could be transformed into protein-like or inorganic nitrogen through microbial activity and enzymatic processes in the Yangtze Estuary (Yan et al., 2021; Yao et al., 2024), highlighting the significance of biologically mediated pathways (Fig. 7b, Pathway 2). The interplay of biological and physicochemical processes during mixing can produce component effects. In the Pearl River Estuary, coupled mechanisms shape DON distribution (Li et al., 2023; Ye et al., 2018), while studies in the Yangtze Estuary have shown selective adsorption of microbially modified and 15N-enriched DON onto particles, supporting RDON′ burial (Chen et al., 2023; Yan et al., 2022; Zhou et al., 2021). Collectively, these findings, along with the results from the current study, suggest that coupled biological and physicochemical interactions during mixing not only enhance nitrogen sequestration through a top-down PON pump (Fig. 7b, Pathway 1 & 3), but also alter DON composition and bioavailability through biological activity (Fig. 7b, Pathway

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2). Specifically, ON undergoing estuarine mixing is likely removed primarily through particle formation, the resulting PON may subsequently be buried or mineralized during transport. Nevertheless, DON remains the dominant fraction of the ON pool (greater than 71%) during DIN-depleted periods or long residence time, and its modified forms can serve as a nitrogen reservoir that fuels coastal algal blooms.

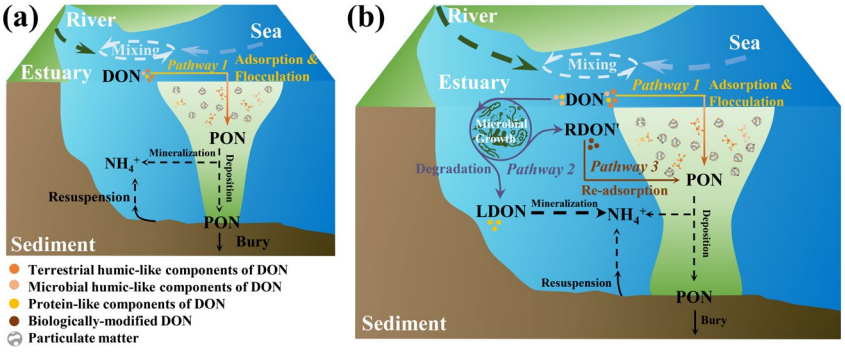

Figure 7. Conceptual model of nitrogen cycling under estuarine mixing, highlighting the role of organic nitrogen (ON) transformation. (a) Classical nitrogen pump model dominated by physicochemical processes. (b) Enhanced nitrogen pump model integrating microbial transformations with physicochemical processes to shape ON dynamics.

Different ON forms contribute variably to TN, with marked variation in their composition and bioavailability (Fig. 5c). Changes in ON composition influence phytoplankton community structure, primary productivity, and the frequency of algal blooms (Hammer, 1993; Sethna & Royer, 2024; Zhang et al., 2024). For example, the increased availability of ON may lead to its transport into coastal waters, where it serves as a nitrogen source, thereby increasing the risk of algal blooms. These effects can cascade through food webs, altering the ecological stability and function of estuarine and coastal ecosystems (Atkinson et al., 2024). While TN is commonly used as a metric in eutrophication assessments (Browning and Moore, 2023), this study show that Chl-a was more strongly correlated with DIN, DON, and tDON than with TN (Fig. 5d), indicating that nitrogen bioavailability, and thus eutrophication risk, is better predicted by nitrogen fractionation than by TN alone. Furthermore, the increased bioavailability of protein-like components during mixing underscores the importance of ON composition in regulating biological utilization. This suggests that both ON speciation and bulk composition jointly control its role in supporting productivity. The potential role of ON should be considered in the increased frequency and distribution of algal blooms in coastal regions.

4.5 Limitations and future perspectives

This study uses a controlled laboratory mixing framework to resolve coupled physicochemical and biological pathways of ON transformation; here we outline the main limitations that may influence representativeness and scaling to natural estuaries. First, the use of freshwater and seawater end-members collected from a single sampling event during the summer

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high-biomass period (July 2024) for shaken-flask incubations under constant temperature and light provide a controlled framework to isolate key mechanisms, but they inevitably simplify natural estuarine complexity and may not fully represent in situ conditions. This approach overlooks the influence of multiple water sources (e.g., groundwater, tidal creek return flow, wastewater inputs, and various tributaries), tidal dynamics, stratification-resuspension cycles, and seasonal variability. Second, the short 3-day incubation period, while enough to capture rapid processes like flocculation, adsorption, and initial microbial transformations, is inadequate to encompass slower, long-term mineralization sedimentation, and burial processes. Consequently, this may lead to an underestimation of PON burial and the long-term formation and stability of RDON′. Moreover, current analytical limitations prevent full characterization of the complex molecular diversity of DON. Most studies, including this study, only partially resolve DON bioavailability using discrete fluorescence or chemical components. Despite these constraints, the controlled design allows us to disentangle rapid salinity-driven partitioning from concurrent biological processing, which are difficult to separate in the field, and thus provides a mechanistic baseline for interpreting non-conservative ON behavior along estuarine salinity gradients.

Although derived from a controlled two-end-member experiment, the three pathways identified here (salt-induced adsorption/flocculation, biological conversion to LMW-DON and NH_4^+, and re-adsorption of microbially modified RDON′) provide testable and model-ready constraints on ON fate during mixing. In particle-rich low-salinity mixing zones, our results indicate that adsorption/flocculation can account for ~44–71% of humic-like component removal, enhancing particle-associated ON accumulation and the potential for downstream settling and burial. In parallel, biological processing increased protein-like fluorescence by ~6–12% above conservative mixing and regenerated NH_4^+, implying a shift toward more bioavailable dissolved N forms that can be rapidly utilized when DIN becomes limiting. These shifts are consistent with our observation that Chl-a relates more strongly to bioavailable N fractions (DIN, DON, tDON) than to TN, underscoring that ON speciation, not bulk TN, better captures eutrophication risk. A practical next step is to incorporate (i) a salinity-dependent partitioning term for humic-like DON between dissolved and particulate pools and (ii) a parameterized biological conversion term for residual humic-like DON to LMW-DON + NH_4^+ into estuarine box models; coupling these terms with residence time and SPM/mineral surface properties would allow quantitative predictions of ON export versus retention across estuaries.

Moreover, as coastal phytoplankton blooms increase in intensity and frequency (Dai et al., 2023), it becomes increasingly important to understand how biological processing modulates ON fate in estuarine systems. Future work should therefore incorporate controlled experiments to disentangle the influence of microbial activity and environmental variables on ON transformations. This is particularly crucial since our focus on summer conditions in the Northern Hemisphere likely captures a scenario of peak biological processing, whereas physicochemical transformations may dominate ON fate during periods of suppressed microbial activity, such as winter. In addition, advancements in high-resolution analytical techniques, such as Fourier-transform ion cyclotron resonance mass spectrometry (FT-ICR MS) (Yan et al., 2024) and single-cell stable isotope analysis, are opening new opportunities for detailed DON characterization (Arandia-Gorostidi et al., 2024). These tools can be used to quantify DON transformation dynamics across estuarine gradients. Nevertheless, multiple external

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factors, such as hydrodynamics, residence time, nutrient loading, and climate variability differently affect ON behavior across estuaries, making it difficult to develop a universal framework. Long-term, in situ observations and comparative studies are therefore essential for quantifying and generalizing ON biogeochemical processes under natural conditions.

5 Conclusions

This study reveals a critical partitioning mechanism for ON during estuarine mixing, which simultaneously alters its 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, effectively creating a long-term sink. Concurrently, biological activity degrades the remaining labile humic-like fraction, releasing highly bioavailable nitrogen forms such as low-molecular-weight DON and NH_4^+. Crucially, our isotopic evidence that biologically-modified DON re-adsorbs onto particles, further enhancing their refractoriness. This transformation effectively increases the bioavailability of the residual dissolved nitrogen pool, thereby elevating the potential risk of coastal eutrophication. These results underscore the necessity for future environmental monitoring to extend beyond inorganic and total nitrogen to the characterization of ON composition. We recommend future research focus on the molecular and microbial mechanisms that regulate the bioavailability of specific ON components to better predict their impact on coastal ecosystems.

Data availability

Data of nutrients and isotopes generated by this study can be found in the Supplement.

Supplement link

The supplement related to this article is available online at XXX.

Author contributions

CH led the study conceptualization and methodology, curated the data, produced the visualizations, and wrote the original draft; SQ contributed to methodology development; YK and XL curated the data; RDV contributed to manuscript review and editing; XL provided supervision and funding acquisition and contributed to manuscript review and editing.

Competing interests

The authors declare there are no conflicts of interest for this manuscript.

https://doi.org/10.5194/egusphere-2026-216
Preprint. Discussion started: 9 February 2026

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Financial support

This work was supported by the National Key R&D Program of China (Grant numbers: 2024YFF1306800).

Review statement

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