



Optical and electrochemical measurements characterize different functional fractions of aquatic humic substances

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Abstract. The extent to which the metal-binding functionality of dissolved organic matter (DOM) can be inferred from its conventional optical properties remains largely unknown. Optical measurements of dissolved organic matter (DOM), including chromophoric (CDOM) and fluorescent (FDOM) properties, are commonly used as proxies for humic substances, yet it remains unclear whether these approaches also reflect the same metal-binding fraction quantified by recently developed
15 electrochemical techniques. In this study, electroactive humic substances (eHS), CDOM, FDOM, and dissolved trace metals were measured across a humic-rich tropical terrestrial-marine continuum, and the results were compared with a previous dataset from a less humified estuarine system. Principal component analysis revealed that eHS was decoupled from conventional optical indices, including the absorption coefficient at 254 nm (a_{254}) and the humification index (HIX), but was closely associated with dissolved manganese and cobalt. Despite the contrasting environmental settings, a similar separation
20 between eHS and optical DOM properties was observed, indicating this decoupling is a general feature rather than a site-specific phenomenon. These findings show that optical proxies alone do not adequately characterize the fraction of humic substances responsible for trace metal complexation and transport, whereas electrochemical measurements selectively quantify a chemically distinct, metal-reactive fraction of the DOM pool. Integrating optical and electrochemical approaches therefore provides complementary perspectives on DOM composition and functionality, offering a more complete framework for
25 improving our understanding of trace metal-DOM interactions in aquatic environments.

1 Introduction

Dissolved organic matter (DOM) is a major component of aquatic carbon pools and plays a vital role in regulating biogeochemical processes in highly productive terrestrial-marine interface region (Ittekkot, 1988; Hedges et al., 1997;
30 Middelburg and Herman, 2007). Among its many ecological and geochemical functions, DOM strongly influences the transport, speciation, and bioavailability of trace metals through organic complexation (Davis, 1984; Lu and Allen, 2002;



Baken et al., 2011). Humic substances, derived primarily from the decomposition and transformation of terrestrial plant material, constitute one of the most important metal-binding fractions of DOM, and can facilitate the transport of terrestrially derived trace metals from rivers and estuaries into coastal environments (Mantoura et al., 1978; Tipping et al., 2002; Yang and
35 van den Berg, 2009).

Despite their importance in aquatic biogeochemistry, direct measurements of humic substances remain difficult because their isolation and molecular-level characterization are time consuming and often require highly specialized analytical techniques such as Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS) (Lv et al., 2016; Xu et al.,
40 2020). Therefore, optical characterization methods have been widely used for assessing DOM composition and transformation in natural waters. In particular, chromophoric dissolved organic matter (CDOM), characterized by ultraviolet-visible absorbance measurements (Boyd and Osburn, 2004; Lu et al., 2016), and fluorescent dissolved organic matter (FDOM), characterized by fluorescence spectroscopy (Yamashita and Tanoue, 2008, Huguet et al., 2009), are frequently employed to investigate the sources, distributions, and biogeochemical cycling of DOM. The various optical parameters derived from
45 CDOM and FDOM measurements, including absorbance coefficients, specific ultraviolet absorbance (SUVA₂₅₄), humification indices (HIX), fluorescence components identified by parallel factor (PARAFAC) analysis, have been widely used to infer the chemical properties of humic substances (Stedmon and Bro, 2008; Yamashita et al., 2008; Murphy et al., 2013).

50 More recently, electrochemical approaches have emerged as powerful tools for the quantification of humic substances because they specifically target the fraction of humic substances involved in trace metal complexation, thereby enabling investigations into the role of humic substances in controlling the speciation, solubility, and transport of trace metals, particularly iron, in aquatic environments (Laglera et al., 2007; Hassler et al., 2025). Electrochemical approaches quantify humic substance concentrations based on their ability to form complexes with metal ions such as iron or copper, thereby
55 targeting the metal-binding fraction of the humic pool, operationally defined as electroactive humic substances (eHS). Because optical and electrochemical methods probe different physicochemical characteristics of humic substances, they may not necessarily quantify the same fraction of the DOM pool. The extent to which commonly used optical proxies, including CDOM and FDOM parameters, reflect the abundance and distribution of eHS remains poorly understood (Lee et al., 2023).

60 Tropical peatlands are among the largest sources of terrestrially derived organic matter to aquatic systems and export substantial quantities of humic-rich DOM to adjacent rivers and coastal waters (Gandois et al., 2014; Sanwlani et al., 2022). During the terrestrial-marine continuum, DOM undergoes extensive transformation, including microbial degradation, photochemical alteration, flocculation, and mixing with marine-derived organic matter, which can modify its optical characteristics and metal-binding properties (Gandois et al., 2020; Nichols and Martin, 2021). Therefore, peatlands, its
65 surrounding blackwaters, estuaries, and coastal regions, provide an ideal natural gradient for evaluating the electrochemical



and optical properties of humic substances and assessing whether CDOM and FDOM parameters can reliably represent the abundance and distribution of eHS across diverse aquatic environments.

In this study, we evaluate the relationship between eHS and optical proxies of DOM along a humic-rich tropical terrestrial-marine continuum. Specifically, we characterize the spatial distributions of eHS and optical DOM properties across a diverse range of aquatic environments, including peatland, blackwater river, estuary, and coastal waters, and assess the extent to which CDOM and FDOM parameters reflect the abundance and distribution of eHS. By directly comparing optical and electrochemical measurements of humic substances, we examine whether these approaches characterize the same functional components of the aquatic DOM pool and evaluate the implications of their relationship for the use of optical proxies in studies of metal-binding humic substances dynamics and trace metal biogeochemistry.

2 Materials and Methods

2.1 Sample Collection

Surface water samples were collected from 18 locations across the Bengkulu River catchment and adjacent coastal waters in August 2025 (Fig. 1). Water was collected using an acid-washed polypropylene bucket and immediately transferred into acid-clean 5 L cubic containers. Salinity was determined on-site using a conductivity sensor immediately after collection. The samples were protected from light during transport to University of Bengkulu, where they were filtered on the day of collection through an acid-cleaned 0.2 μm pore size capsule filter (Acropak-200, Pall Industries).

Filtered samples were subsequently divided according to analytical requirements. Aliquots for eHS measurements were stored in 250 mL fluorinated polyethylene (FLPE) bottles, while those designated for CDOM, and FDOM measurements were transferred to 40 mL in acid-washed, pre-combusted amber glass vials and preserved at -20°C until analysis. For dissolved trace metal determination, filtered samples were collected in low density polyethylene (LDPE) bottles, acidified to $\text{pH} < 2$ using ultrapure HCl (20%, Tamapure AA-100, Tama Industries), sealed in double plastic bags, and stored in room temperature for more than 6 months before analysis.

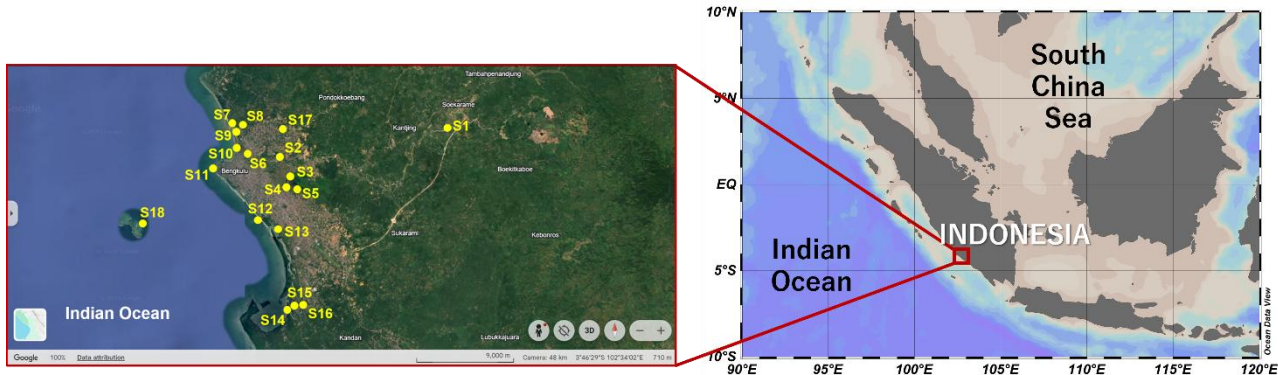


Figure 1: Map showing the sampling locations of the 18 stations in the Bengkulu River catchment and adjacent coastal waters, western Sumatra, Indonesia. Details of the sampling stations and their geographic coordinates are provided in Table S1. Maps data: © Google Earth 2026.

2.2 Measurements of Dissolved Trace Metals

Dissolved trace metal concentrations of cadmium (dCd), cobalt (dCo), copper (dCu), manganese (dMn), nickel (dNi), lead (dPb), and zinc (dZn) concentrations were determined by inductively coupled plasma mass spectrometry after offline pre-concentration using a chelating resin, following the method of Wong et al., (2021). All pre-concentration procedures were performed in a trace metal-clean laminar flow bench using a four-channel manifold equipped with 10 mL syringe cartridges packed with 250 mg NOBIAS PA-1 chelating resin (Hitachi Hi-Tech).

Before use, each resin column was cleaned by sequentially passing 10 mL of acetone (Wako Special Grade, Wako Chemicals) and 10 mL of 2 M ultrapure HNO₃ (Tamapure AA-100, Tama Chemicals) through the resin under gravity. The columns were subsequently conditioned with 15 mL of deionized water (Milli-Q, Merck Millipore, 18.2 MΩ·cm), followed by 10 mL of 0.05 M ammonium acetate buffer (pH 6.0-6.2) prepared from ultrapure acetic acid (Tamapure AA-100, Tama Chemicals) and ultrapure ammonia solution (Tamapure AA-100, Tama Chemicals).

To minimize interference from naturally occurring organic ligands on metal extraction, acidified samples were exposed to ultraviolet irradiation prior to preconcentration according to Wiwit et al. (2025). Briefly, 40 mL aliquots were transferred into 50 mL perfluoroalkoxy (PFA) beakers fitted with quartz covers and irradiated directly beneath a 400 W ultraviolet lamp (365 nm).

Following ultraviolet treatment, the sample pH was adjusted to 6.0 - 6.2 using 3.6 M ammonium acetate buffer and ultrapure ammonia solution before loading onto the conditioned chelating resin columns under gravity. After sample loading, the columns were rinsed with 30 mL of 0.05 M ammonium acetate buffer to remove residual seawater. The retained metals were



then eluted with 5 mL of ultrapure HNO_3 into 15 mL acid cleaned LDPE bottles, resulting in an 8-fold enrichment of the original sample. Procedural blanks were evaluated by processing 0.05 M ammonium acetate buffer in place of the samples.

120 Detailed procedural blank values and recoveries for each trace metal have been reported previously by Wong et al. (2024).

2.3 Measurements of Electroactive Humic Substances

eHS were determined by CSV using a voltammetric analyzer (797 VA Computrace, Metrohm) following the method described by Lee et al. (2023). Measurements were carried out in a Teflon voltametric cell equipped with a hanging mercury drop working electrode, an Ag/AgCl reference electrode, and a glassy carbon auxiliary electrode.

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Prior to analysis, dissolved oxygen was removed by purging each sample with high-purity nitrogen gas (99.99995%) for 5 min. eHS were then complexed with Cu by adding 50 nM Cu (CuSO_4 prepared in 0.1 M ultrapure HCl), producing Cu-humic substance complexes (Cu-HS). The Cu-HS complexes were accumulated onto the hanging mercury drop electrode at a deposition potential of 0.05 V while stirring the solution at 1400 rpm. Depending on the eHS concentration, deposition times between 60 s to 180 s were employed. A mercury drop size of 7 was used throughout the measurements.

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After the deposition step, differential pulse voltammograms were recorded over a potential range of 0 to -0.75 V. Instrumental parameters were as follows: equilibration time, 9 s; pulse amplitude, 0.05 V; pulse time, 0.04 s; voltage step, 0.005 V; voltage step time, 0.1 s. Three replicate scans were performed per sample. A 1 s potential jump to -0.2 V was applied immediately before each scan to suppress interfering signals. Under these conditions, the Cu-HS complex generated a characteristic peak at approximately -0.17 V.

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Quantified was based on external calibration using Suwannee River Fulvic Acid (SRFA) reference material (batch 2S101F) obtained from the International Humic Substances Society (IHSS). A 1.0 g/L stock solution was prepared in 0.1 M sodium hydroxide solution (trace metal grade, Sigma) and working standards were freshly prepared by serial dilution with deionized water immediately before analysis.

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2.4 Optical Properties of Dissolved Organic Matter

Ultraviolet-visible absorbance spectra were measured using a UV-Vis spectrophotometer (V-630, JASCO, Japan) with a 1 cm quartz cuvette. Spectra were acquired from 200 to 1000 nm at 0.5 nm intervals, and Milli-Q water blanks were measured and subtracted from sample spectra before calculation. To reduce the influence of residual baseline drift at low absorbance, spectra were baseline-corrected by subtracting the mean absorbance over 700–800 nm. Blank- and baseline-corrected absorbance spectra were converted to Napierian absorption coefficients according to:

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$$a(\lambda) = 2.303A(\lambda)/L$$

where $a(\lambda)$ is the measured absorbance at wavelength λ and L is the optical pathlength (m). The absorption coefficients at 254 and 320 nm were used as proxies for UV-absorbing CDOM and aromatic DOM abundance, while the absorption coefficients at 412, 443, and 450 nm represented visible-light CDOM absorption associated with colored humic-rich DOM. The spectral slopes between 275 and 295 nm ($S_{275-295}$), and between 350 and 400 nm ($S_{350-400}$) were calculated from log-transformed
155 absorption spectra following Helms et al. (2018).

Fluorescence excitation-emission matrices (EEM) were measured using a spectrofluorometer (RF-5300PC, Shimadzu, Japan) with a 1 cm fluorescence-free quartz cuvette. Excitation and emission wavelengths were both scanned from 220 to 600 nm, with excitation and emission intervals of 5 and 2 nm, respectively. The humification index (HIX) was calculated from the
160 corrected fluorescence EEM following Ohno (2002). HIX was determined as the ratio of the integrated fluorescence intensity between emission wavelengths of 435-480 nm to the sum of the fluorescence intensities between 300-345 nm and 435-480 nm at an excitation wavelength of 254 nm.

2.5 Statistical Analysis

165 Multivariate analyses were performed to investigate patterns in DOM composition and environmental variability among sampling sites. Principal component analysis (PCA) was conducted using standardized variables (z-score normalization) to identify the major factors controlling the distribution of eHS, CDOM, and FDOM properties across the terrestrial-marine continuum.

170 3 Results

3.1 Classification of Study Sites

PCA was conducted using salinity, eHS, a_{254} , HIX, $S_{275-295}$ and trace metal (dMn and dCo) concentration to examine the similarities in environmental conditions among sampling stations. The first two principal components explained 73.5% of the total variance, with PC1 and PC2 accounting for 45.2% and 28.3%, respectively (Fig. 2). PC1 was characterized by positive
175 loadings of trace metal concentration, $S_{275-295}$, and eHS, while exhibiting a negative relationship with a_{254} and HIX. Meanwhile, PC2 was dominated by $S_{275-295}$ and salinity, with negative loadings of HIX and trace metal concentrations (Table S2).

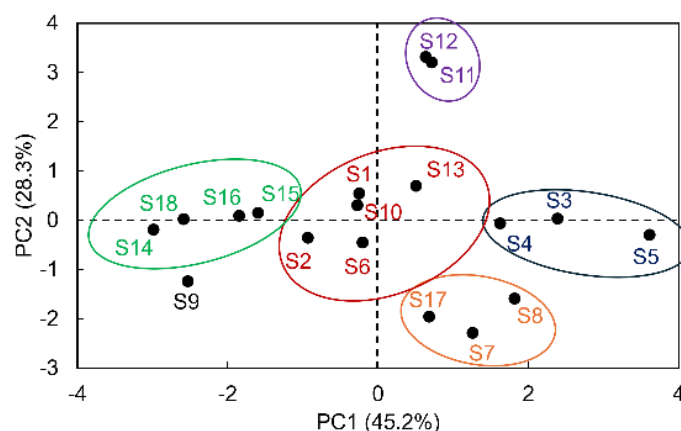


Figure 2: Principal Component Analysis score plot showing the classification of 18 sampling stations based on salinity, electroactive humic substances (eHS), dissolved manganese (dMn), dissolved cobalt (dCo), HIX, a_{254} , and $S_{275-295}$. Based on the distribution in the PCA space, the stations were classified into five environmental groups: peat blackwater sites (orange), humic-rich freshwater sites (blue), transitional waters (red), marine waters (green), and coastal beach waters (purple).

The PCA revealed a clear separation of sampling sites along the terrestrial-marine continuum according to their hydrographic and biogeochemical characteristics (Fig. 2). Based on their positions in the PCA plot, the samples were grouped into five distinct categories. The first group comprised of the peat blackwater sites (S7, S8, S17), which were characterized by elevated dissolved trace metal and eHS concentrations and low salinity, indicating strong terrestrial and peat-derived influences. The second group consisted of S3, S4, and S5, which formed a distinct cluster that was clearly separated from all other stations. Despite their low salinities, these stations exhibited different DOM characteristics and relatively low trace metal concentrations compared to the peat blackwater sites, representing a humic-rich freshwater system.

The third group included S1, S2, S6, S10, and S13, which occupied an intermediate position between the freshwater and marine endmembers. These stations likely represent transitional waters influenced by varying degrees of terrestrial runoff and estuarine mixing. S14, S15, S16, and S18 formed a fourth group (marine waters) characterized by low eHS concentrations and relatively high salinity, indicating a strong marine influence. Lastly, the fifth group consisted of S11 and S12, which are clearly separated from the other marine stations by their high PC2 scores. As these sites were located along sandy beaches, they were classified as coastal beach waters. S9 did not cluster with any of the five groups, suggesting that it is an intermediate site with mixed environmental characteristics.

3.2 eHS and Optical DOM Properties

eHS concentrations and optical DOM characteristics showed distinct patterns among the five environmental groups. Notably, eHS concentrations were highest in the humic freshwater group, which also exhibited relatively high $S_{275-295}$ values



compared to the other stations (Fig. 3; Table S3). Meanwhile, the marine waters displayed the highest a_{254} values and moderately elevated HIX values despite relatively low $S_{275-295}$ values and comparable eHS concentrations. These contrasting patterns suggest that the spatial distribution of eHS is not directly coupled to conventional optical indices of DOM abundance (a_{254}) and humification.

The apparent decoupling between eHS and a_{254} suggests that eHS do not track the abundance of CDOM, and the lack of correspondence between eHS and HIX further indicates that highly humified DOM is not necessarily enriched in eHS. Instead, the similarity between the distributions of eHS and $S_{275-295}$ suggests that eHS may be associated with specific molecular characteristics rather than with the overall quantity of DOM.

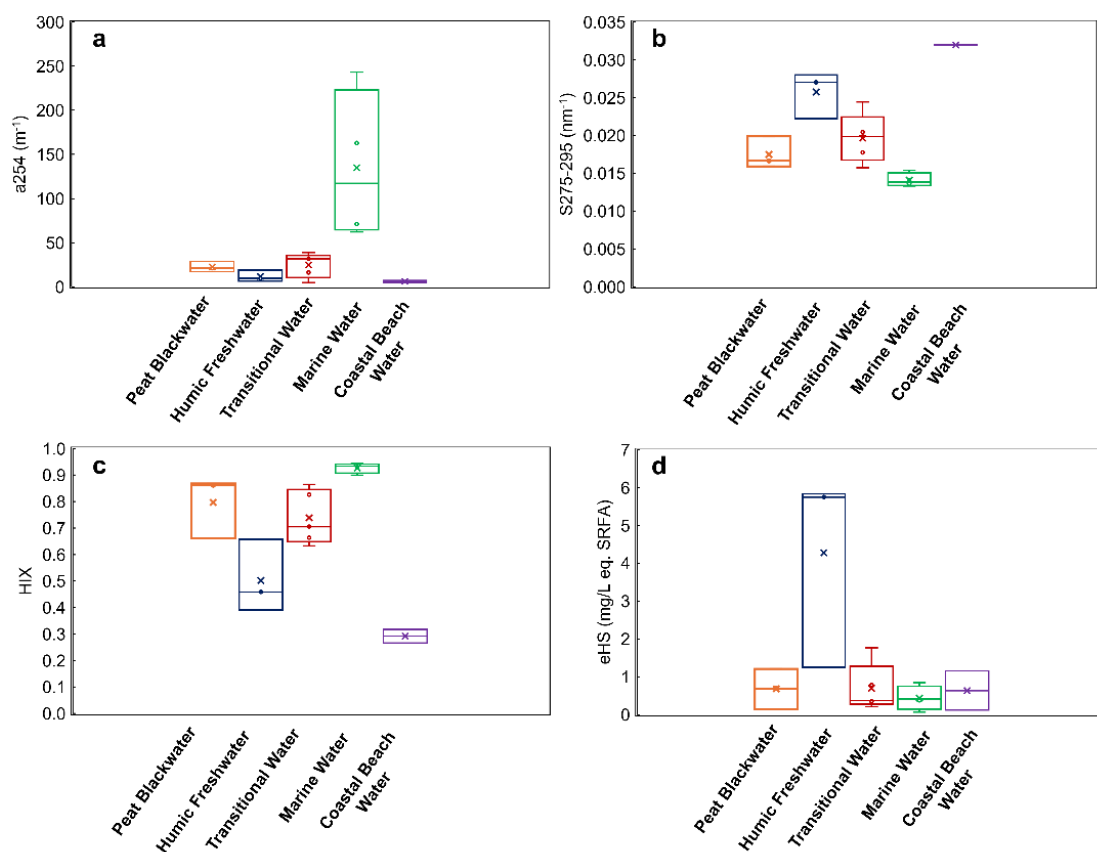


Figure 3: Distribution of (a) a_{254} , (b) $S_{275-295}$, (c) HIX, and (d) electroactive humic substances (eHS) among the five environmental groups identified by PCA.



3.3 Distributions of Dissolved Trace Metals

dCo and dMn concentrations exhibited significant differences among the five PCA-derived groups. The peat blackwater and humic freshwater groups showed the highest concentrations of both metals, with concentrations substantially exceeding those observed in all other sites (Fig. 4; Table S3). Meanwhile, Transitional waters had slightly higher metal concentrations compared to the marine sites. This pattern indicates a significant terrestrial source of dCo and dMn and a sharp decrease in metal concentrations along the freshwater-marine continuum due to estuarine mixing.

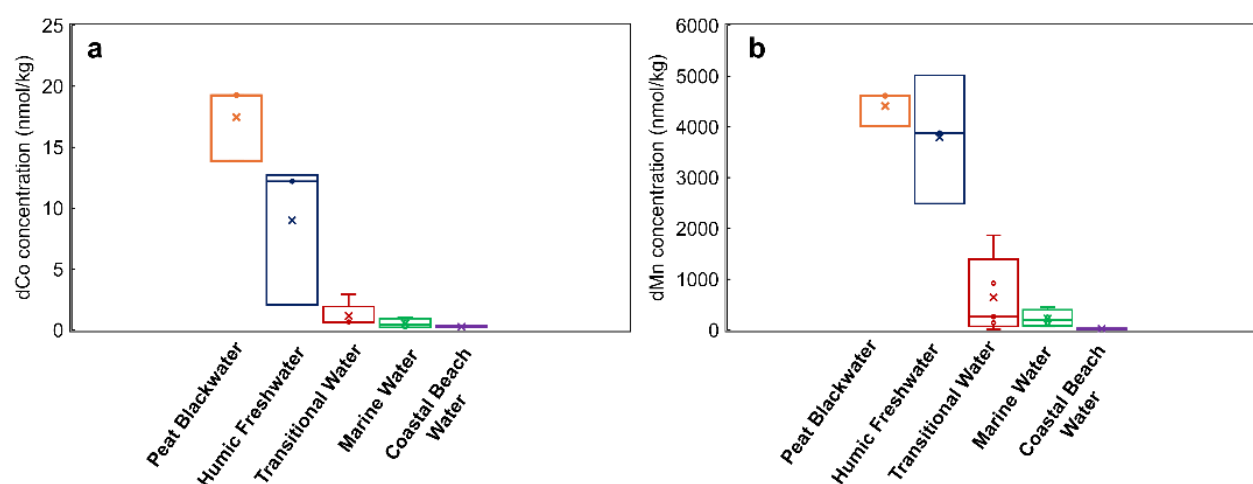


Figure 4: Distribution of (a) dissolved cobalt (dCo) and (b) dissolved manganese (dMn) among the five environmental groups identified by PCA.

4 Discussion

4.1 Decoupling of eHS with optical DOM properties

The PCA loading revealed a clear separation between eHS and conventional optical DOM indices such as a_{254} and HIX (Fig. 5). Instead, eHS was positively associated with dCo and dMn along PC1, indicating that eHS may play an important role in the mobilization and transport of trace metals in terrestrial and estuarine environments. These findings suggest that eHS was not directly coupled to DOM abundances or humification but rather represents a chemically distinct metal-reactive fraction of DOM that is not fully captured by conventional optical proxies.

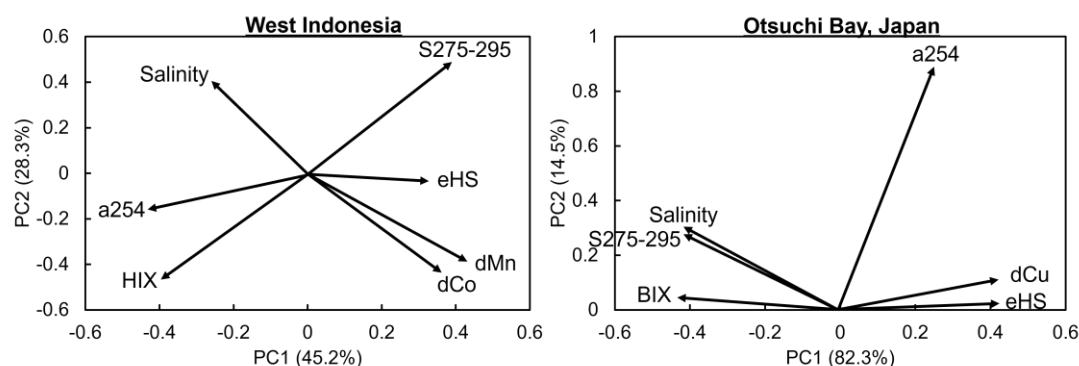


Figure 5: PCA loading plots showing the relationships among electroactive humic substances (eHS), optical DOM properties, salinity, and dissolved trace metals in Western Indonesia (left) and Otsuchi Bay, Japan (right). In both regions, eHS was closely associated with dissolved trace metals.

To further evaluate this observation, we also compared our results with those reported in Otsuchi Bay, Japan, one of the very few studies that simultaneously quantified eHS and optical DOM proxies (Fig. 5, Lee et al., 2023). Similar to this study, the PCA of the Otsuchi dataset showed a close association between eHS and dCu, whereas *a254* exhibited little relationship with eHS. In addition, eHS was negatively associated with *S275-295* and the biological index (BIX), which is an indication of biological or microbial production of DOM, indicating that eHS was not linked to microbial production. Despite the substantial difference between the two systems - Western Indonesia representing a highly humified peat-influenced environment and Otsuchi Bay representing a biologically influenced estuarine system – both datasets consistently demonstrate that eHS is more closely associated with trace metal distributions than with bulk optical DOM proxies.

4.2 Implication for Trace Metal Stabilization and Transport in Aquatic Systems

Recently, eHS has gained attention as a proxy for investigating trace metal stabilization and transport, particularly for dissolved iron (dFe) (Whitby et al., 2020; Mahieu et al., 2024; Hassler et al., 2025) and dCu (Mellet and Buck, 2020; Hollister et al., 2021) stabilization and transport in oceanic and estuarine environments. In the open ocean, where external Fe inputs are limited and dFe concentrations are low, eHS has been shown to constitute a major component of the Fe-binding ligand pool and contribute significantly to the stabilization of dFe by preventing oxidation, aggregation, and precipitation under oxic conditions (Whitby et al., 2020; Mahieu et al., 2024; Hassler et al., 2025).

In estuarine regions, however, the relationship between eHS and dFe appears to be weaker (Mellet and Buck, 2020), suggesting that multiple processes may influence metal speciation during river-ocean transport. Although dFe was not measured in this study, the strong association observed between eHS and dMn and dCo in the PCA suggests that eHS may play a broader role in facilitating the mobilization and stabilization of multiple trace metals in estuarine systems. This



interpretation is supported by previous studies reporting strong relationships between humic substances and multiple trace metals such as dMn, dFe, dNi, dCu, and dZn in estuarine mixing zones, although the specific metals associated with humic substances vary among aquatic environments (Wang et al., 2017; Chen et al., 2021; Muller et al., 2024).

Interestingly, HIX did not track eHS in this study despite both parameters being associated with humic substances. Previous studies have shown that the humic-like components of FDOM are often positively correlated with trace metals like dFe (Yamashita et al., 2020; Lagozzo et al., 2023; Seo et al., 2026) and dCu (Wong et al., 2019; Luz-Santos et al., 2025). Since HIX is widely interpreted as an indicator of DOM humification, the lack of correspondence between eHS and HIX suggests that eHS represents a chemically distinct, metal-reactive subset of humic DOM, which may overlap with the humic-like fluorescent components identified by FDOM analyses, rather than the bulk humified DOM pool. Therefore, while the optical properties such as HIX provide valuable information on DOM source and humification, eHS offers complementary information on the metal-binding functionality of humic substances.

5 Conclusion

Overall, this study demonstrates that eHS exhibit spatial distributions that are fundamentally different from those described by conventional DOM optical proxies across a humic-rich tropical terrestrial-marine continuum. Although optical parameters such as a₂₅₄ and HIX have been widely used as indicators of humic substances and DOM humification, neither parameter consistently tracked eHS concentrations. Instead, eHS was closely associated with dissolved trace metals, particularly dCo and dMn, indicating that electrochemical measurements selectively quantify a chemically distinct, metal-reactive fraction of humic substances rather than the bulk humified DOM pool.

The consistency of this relationship with observations from less humified environment of Otsuchi Bay suggests that the decoupling between eHS and optical DOM properties is not unique to peat-influenced tropical waters but may represent a more general characteristic of aquatic DOM. Consequently, while optical measurements remain valuable for characterizing DOM sources and transformation processes, they should not be assumed to represent the metal-binding functionality of humic substances. Electrochemical measurements of humic substances therefore provide important complementary information that is directly relevant to trace metal stabilization, complexation, and transport. Integrating electrochemical and optical approaches will provide a more comprehensive understanding of DOM functionality and improve assessments of trace metal cycling and aquatic biogeochemical process.



Data availability

The data supporting the findings of this study are available in the supplement.

295 Author contributions

Wiwit and Wong conceived and designed the study. Wiwit, Wong, Negishi, Yanuar, Minamiyama, and Ni conducted the laboratory analyses, while Wiwit, Yanuar, Nurhamidah, and Oktiarni carried out the field investigation and performed in-situ measurements. Wiwit, Wong, Ni, Iwata, Mashio, and Hasegawa validated the data. Wiwit and Wong prepared the original manuscript draft. Iwata, Mashio, and Hasegawa reviewed and edited the manuscript. All authors reviewed the manuscript and
300 approved the final version.

Competing interests

The authors declare that they have no conflict of interest.

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