



Using physicochemical properties to predict the impact of natural dissolved organic carbon on transepithelial potential in the freshwater rainbow trout (*Oncorhynchus mykiss*) at neutral and acidic pH

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Abstract Dissolved organic carbon (DOC) is a complex mixture of molecules that varies in composition based on origin as well as spatial and temporal factors. DOC is an important water quality parameter as it regulates many biological processes in freshwater systems, including the physiological function of the gills in fish. These effects are often beneficial, especially at low pH where DOCs mitigate ion loss and protect active ion uptake. DOCs of different compositions and quality have varied ionoregulatory effects. The molecular variability of DOCs can be characterized using optical and chemical indices, but how these indices relate to the physiological effects exerted by DOCs is not well understood. We tested the effects of five naturally sourced DOCs, at both pH 7 and pH 4, on transepithelial potential (TEP)

(a diffusion potential between the blood plasma and the external water) in rainbow trout. The five chosen DOCs have been well characterized and span large differences in physicochemical characteristics. Each of the DOCs significantly influenced TEP, although in a unique manner or magnitude which was likely due to their physicochemical characteristics. These TEP responses were also a function of pH. With the goal of determining which physicochemical indices are predictive of changes in TEP, we evaluated correlations between various indices and TEP at pH 7 and pH 4. The indices included: specific absorbance coefficient at 340 nm, molecular weight index, fluorescence index, octanol–water partition coefficient, molecular charge, proton binding index, % humic acid-like, % fulvic acid-like, and % protein-like components by parallel factor analysis on fluorescence data (PARAFAC). Our results demonstrate the novel finding that there are three particularly important indices that are predictors of changes in TEP across pHs in rainbow trout: specific absorbance coefficient at 340 nm, octanol–water partition coefficient; and proton binding index.

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Introduction

In freshwater ecosystems, dissolved organic carbons (DOCs) regulate both biotic and abiotic processes (Petersen 1991; Kullberg et al. 1993; Steinberg et al. 2006; Al-Reasi et al. 2011). The biological processes with which DOC molecules interact include ultraviolet screening, the carbon cycle, CO₂ generation and O₂ consumption via photo-oxidation, production of reactive oxygen species (ROS), acidification, nutrient transport, and metabolic processes (Kullberg et al. 1993; Sucker and Krause 2010; Wood et al. 2011; Johannsson et al. 2017, 2020; Holland et al. 2018). DOCs are also subject to seasonal and temporal changes in concentration and composition, further influencing aquatic ecosystems (Kullberg et al. 1993; Sucker and Krause 2010; Wood et al. 2011; Holland et al. 2018). The full extent of the ecological and biological significance of DOCs in freshwater systems is not yet understood. Nevertheless, there is now an abundance of evidence that DOCs affect gill function in freshwater organisms, particularly fish. It is well-recognized that DOCs are a mitigating factor against metal toxicity as they have the ability to complex and thereby reduce the bioavailability of metal cations (Playle et al. 1993; Hollis et al. 2001; Matsuo et al. 2004; Wood et al. 2011; Crémazy et al. 2016). Al-Reasi et al. (2013) proposed that the physicochemical properties of DOCs that are important in the protection against metal toxicity may be similar to those that exert physiological effects on the gills, though this has not been proven. These physiological effects are usually beneficial to the ionoregulation of the organism and are particularly important at low pH. These include protection of active ion uptake from inhibition and mitigation of the often-fatal increase in passive loss of major salt ions (Na⁺, Cl⁻) across the gills during low pH exposure (Gonzalez et al. 2002, 2006; Wood et al. 2003, 2011; Matsuo et al. 2004; Glover and Wood 2005; Glover et al. 2005; Duarte et al. 2016, 2018; Al-Reasi et al. 2016). At environmentally realistic low pH (i.e., pH 3.5–5), the general physiological response of non-acidophilic freshwater fish such as the rainbow trout is an elevation of passive ion loss and the inhibition of active Na⁺ and Cl⁻ uptake. This results in reduced plasma Na⁺ and Cl⁻ concentrations, low blood volume, and elevated plasma protein levels (McDonald et al. 1980; Wood and McDonald 1982; McDonald 1983; Wood 1989).

Circulatory failure is the eventual cause of mortality (Milligan and Wood 1982). Low pH also affects the transepithelial potential (TEP) across the gills. In freshwater fish, the TEP largely represents a diffusion potential between the blood plasma and the ambient water, predominantly due to the differential permeabilities of Na⁺ versus Cl⁻ across the gills (Potts 1984). An acidic environment alters this differential permeability of Na⁺ versus Cl⁻ and increases the diffusion gradient for proton entry. The resulting less negative TEP (blood plasma relative to the external water) favors Na⁺ efflux and opposes active Na⁺ influx. Fresh, natural DOCs have been found to cause hyperpolarization of TEP in freshwater fish, thereby making the blood side more negative relative to the water side (Galvez et al. 2008; Sadauskas-Henrique et al. 2019). This improves the electrochemical gradient for net Na⁺ uptake at the gills and is likely an important contributor to the positive effects of DOCs on ionoregulation (Wood et al. 2011; Morris et al. 2021).

Some DOCs are more effective at protecting against metal toxicity and exerting physiological effects than others (Al-Reasi et al. 2011, Al-Reasi et al. 2013; Sadauskas-Henrique et al. 2019) and the extent of the effects appear to vary with DOC type and pH (Wood et al. 2011; Morris et al. 2021). Specific physicochemical indices have been developed to describe the molecular variability of DOCs based on their optical and chemical properties. The optical indices include aromaticity (specific absorbance coefficient at 340 nm, Curtis and Schindler 1997), molecular weight (molecular weight index, Dahlén et al. 1996; Peuravuori and Pihlaja 1997), allochthonous versus autochthonous origin (fluorescence index, McKnight et al. 2001; DePalma et al. 2011), lipophilicity (octanol–water partition coefficient, Gjessing et al. 1999; Egeberg and Alberts 2002; Namjesnik-Dejanovic and Cabaniss 2004), and relative percent composition by parallel factor analysis (PARAFAC) (Kroonenberg and Heiser 1998; Stedmon and Bro 2008). Non-optical indices of chemical reactivity include the proton binding index and molecular charge (an indication of the ionizable fraction of the molecule) (Al-Reasi et al. 2013).

Although these indices are well described, how they relate to one another, and behave at different pHs was not understood, until a recent companion study (Morris et al. 2024), where we applied all

of the techniques outlined above to characterize five sources of DOC that were freshly isolated from two very different sites in Ontario, Canada (Luther Marsh and Lake Ontario) and three intermediate sites within the *rare* Charitable Research Reserve in Ontario, Canada (Preston Flats, Ancient Woods Swamp, and Ancient Woods Pond). Important relationships were detected among the various optical and chemical indices which were pH-dependent (Morris et al. 2024). In the present study, we have used these same, now well-characterized DOCs from the companion study, to systematically assess their effects on a sensitive indicator of biological effect; the TEP of the freshwater fish gill. Specifically, we have described the relationships between TEP effects and the physicochemical characteristics of DOCs at pH 7 and pH 4.

Our specific hypotheses were (i) DOCs that were more aromatic, more allochthonous, larger in molecular size, with greater % humic acid content, and with greater a proton binding index (PBI) would hyperpolarize TEP to a greater extent; (ii) the DOC effects on TEP would be greater at lower pH because ionoregulatory protection is more apparent at low pH; and (iii) one or more of the indices would strongly predict the effect of DOC on TEP at each pH. Our overall goals were to develop tools to predict the effects of DOCs with different physicochemical characteristics on TEP in fish, and in so doing better understand the nature of the interactions of DOC with fish gill function at circumneutral and acidic pH.

Methods

Experimental animals

Juvenile rainbow trout (*Oncorhynchus mykiss*) ($N=60$, 124 ± 5 g) were obtained from the Freshwater Fisheries Society of BC (Abbotsford, BC, Canada) and transferred to the University of British Columbia (UBC). Fish were held for several months prior to commencing experiments at 12 °C in flowing dechlorinated Vancouver tap water ($\text{Na}^+ = 0.09$, $\text{Cl}^- = 0.10$, $\text{Ca}^{2+} = 0.10$, $\text{Mg}^{2+} = 0.011$, $\text{K}^+ = 0.004$ mmol L^{-1} , hardness as $\text{CaCO}_3 = 3.3$ mg L^{-1} , $\text{pH} = 7.0$, $[\text{DOC}] = \sim 1.07$ mg C L^{-1}). Fish were fed to satiation every second day with commercial pellet food (BioTrout 2.0 mm, Bio-Oregon™, Long-view, WA, USA) and were fasted for at least 72 h prior to

experiments. All experiments were approved by the UBC Animal Care Committee (A18-0271 and A22-0195) and conformed to the national regulations of the Canada Council for Animal Care.

Experimental solutions

The five natural sources of dissolved organic carbon (DOC) were collected in Ontario, Canada. These were Luther Marsh (LM) ($43^\circ 57' 42.8\text{N}$, $80^\circ 24' 04.0\text{W}$), Lake Ontario (LO) ($44^\circ 14' 25.3\text{N}$, $76^\circ 27' 57.5\text{W}$) and three sites within the *rare* Charitable Research Reserve: Preston Flats (PF) ($43^\circ 23' 17.5\text{N}$, $80^\circ 22' 38.0\text{W}$), Ancient Woods Pond (AP) ($43^\circ 22' 30.4\text{N}$, $80^\circ 21' 55.5\text{W}$) and Ancient Woods Swamp (AS) ($43^\circ 22' 30.8\text{N}$, $80^\circ 22' 09.2\text{W}$). Throughout the paper, these are referred to by their site designations: LO, LM, PF, AP, and AS. The collection and treatment of these DOCs were exactly as described by Morris et al. (2024). Briefly, water was pumped from the source and pre-filtered through a 1- μm wound string filter. Then DOC was concentrated gradually (Sun et al. 1995) by a portable reverse osmosis unit fitted with a polyamide thin-film composite membrane (FilmTec™ BW30-4040, DuPont Water Solutions, Edina, MN, USA). The samples were then treated to remove cations that may have accumulated during reverse-osmosis with a cation exchange resin (Amberlite IR-118 (H), Sigma Aldrich, St. Louis, MO, USA). The concentrate was filtered using 0.45- μm pore size (47 mm), hydrophilic, polyethersulfone polymer membrane filters (MS® PES Membrane Solutions, Auburn, WA, USA), and stored in sealed bottles at 4 °C until used. DOC concentrations and absorbance measurements were taken to ensure that significant degradation of DOC was not occurring during the storage time, which was consistent with previous studies (Peacock et al. 2015). Exposure solutions for physicochemical characterization (Morris et al. 2024) and physiological testing were made by adding each of the natural DOC concentrates to dechlorinated Vancouver tap water to nominally equal 10 mg C L^{-1} and the pH was adjusted to nominally pH 7 or pH 4 using HNO_3 or NaOH and a sympHony™ pH electrode (C03243) and meter (SP70P) (VWR International, Radnor, PA, USA) (Supplementary Table S1). DOC concentrations of exposure waters were measured using a Shimadzu total carbon and nitrogen analyzer (TOC-L

Shimadzu, Mandel Scientific, Guelph, ON, CA). This physicochemical characterization of exposure solutions was done in a companion study (Morris et al. 2024) and is presented for reference in Supplementary Table S1 of the present study. Morris et al. (2024) also analyzed the following indices for each of the DOC sources and quantified how they are altered by a change in pH from 7 to 4, as well as their inter-relationships: specific absorbance coefficient at 340 nm (SAC_{340}), molecular weight index (MWI), fluorescence index (FI), the octanol–water partition coefficient (LogKow), molecular charge, PBI, and % humic acid-like, % fulvic acid-like, and % protein-like components by parallel factor analysis (PARAFAC) on fluorescence data. Values for these indices are tabulated in Supplementary Table S2 of the present study. The methods and calculation equations for the physicochemical indices of Morris et al. (2024) are briefly described here, in the Supplementary Information.

Transepithelial potential measurements

Transepithelial potential measurements (TEP) were made following the procedure outlined by Wood et al. (2020). TEP was measured using intraperitoneal catheters which yield identical data to those generated using more invasive vascular catheters employed in earlier studies (Wood and Grosell 2008). Trout were anesthetized using 0.2 mg L⁻¹ of tricaine methane sulfate (MS222) and buffered with NaOH to neutral pH (Sigma Aldrich). Gills were irrigated with a continuous flow of anaesthetic water to avoid hypoxic stress (Po and Wood 2022). A 5-cm section of polyethylene PE50 catheter (Clay Adams™, Becton Dickinson, Franklin Lakes, NJ, USA) filled with Cortland saline (Wolf 1963), was inserted into the peritoneal cavity as described by Wood and Grosell (2008). The catheter was anchored using a 2-cm PE160 sleeve secured with cyanoacrylate glue and silk sutures and sealed with a stainless-steel pin. Fish were allowed to recover overnight in separate aerated enclosures supplied with dechlorinated Vancouver tap water (no added DOC) before control TEP measurements were made.

TEP determinations employed 3 M KCl agar bridges connected via Ag/AgCl electrodes (World Precision Instruments, Sarasota, FL, USA) to a high impedance voltmeter (Radiometer pHm 82,

Radiometer-Copenhagen, Copenhagen, DK). The reference bridge was placed into the experimental solution of the measurement chamber and the measurement bridge was connected to the indwelling catheter. The individual measurement chambers were plastic containers filled with 2 L of solution held at 10 °C. Each individual fish was exposed to the appropriate solution for 2 min before the measurements commenced and then three measurements (with correction for junction potential) were completed within a further 2 min and averaged. Fish were exposed to the control solution without added DOC either at pH 7 or pH 4 in one measurement chamber and then carefully transferred into another measurement chamber containing the experimental solution. The background TEP (baseline TEP measured in the control solution) served as a baseline TEP for comparison with the TEP measured in the experimental solution. The Δ TEP was calculated as the change from baseline TEP caused by the presence of DOC. A paired statistical design was used, where each fish was its own control. TEP was expressed in the traditional manner as the inside voltage relative to the outside water as 0 mV. Directly following the experiments fish were euthanized by an overdose of neutralized MS222 (0.5 mg/L).

Statistical analysis

The effect of each DOC source on absolute TEP, compared to the control value within the same pH, was assessed using Student's paired *t*-test. The differences between the change in TEP (Δ TEP) amongst DOC sources within the same pH were assessed using one-way ANOVA with Tukey's post hoc test. Simple linear regressions ($p < 0.05$) were used to assess relationships between selected indices and TEP, and between these same indices and Δ TEP, at pH 7 and at pH 4. All statistical analyses and data plots were done using GraphPad™ Prism 7 (GraphPad Software, San Diego, CA, USA). A significance level of $p < 0.05$ (two-tailed) was used throughout.

Results

Characterization of the five natural docs at two pHs using physicochemical indices

As summarized in Supplementary Table S2 (data from Morris et al. 2024), at pH 7, LO DOC exhibited

the lowest aromaticity, as indicated by SAC_{340} , and this coincided with the highest fluorescence index (FI), indicating the most autochthonous origin, which would be expected for a DOC collected from a large natural lake. LO also exhibited the lowest apparent molecular weight as indicated by the MWI value. Note that MWI is an inverse scale, with smaller DOC molecules displaying higher MWI values. In contrast, AP and AS, collected from small ponds, exhibited the highest aromaticities but intermediate positions with respect to FI, whereas their low MWI values indicated large molecular weights. LM DOC, collected from a large swamp, exhibited intermediate aromaticity based on SAC_{340} , but was the most allochthonous in origin as indicated by the lowest FI value, and had a high apparent molecular weight as indicated by the second lowest MWI value. LO DOC was again noteworthy for its greatest lipophilicity indicated by the highest LogKow value, and lowest chemical reactivity as indicated by the lowest PBI, and second lowest molecular charge at pH 7. In contrast, LM demonstrated the lowest lipophilicity (lowest LogKow value), greatest reactivity (highest PBI value), and a high molecular charge. AP DOC was similar to LM DOC in these characteristics, but AS DOC actually carried the greatest molecular charge at pH 7. PAR-AFAC analysis at pH 7 revealed that fulvic acid-like fluorophores were the dominant component (> 65%) in all 5 DOC sources, whereas the humic acid-like fluorophores accounted for < 30% and the protein-like fluorophores were the smallest component (0–20%). Notably, LM exhibited the highest humic acid-like component, and lowest protein-like component, whereas the opposite was true for AP DOC. PF DOC, collected from a small wetland pond, was generally more similar to LO than to the other three in most characteristics.

When the pH was lowered from 7 to 4, some of the site-specific indices changed in order, but the general trends were for reductions in apparent aromaticity (except LM where SAC_{340} increased), greater apparent allochthonous origin (again except for LM where FI increased), lower apparent molecular weight (except for PF where MWI decreased), and universal increases in lipophilicity (higher LogKow values), molecular charge, % humic-acid like fluorophores, and reciprocal decreases in % fulvic acid-like fluorophores. The % contribution of protein-like fluorophores also tended to decrease.

It is well known that absorbance and fluorescence spectra are pH dependent (Yan et al. 2013); proton binding sites with pKa values near 4 will become more protonated compared to pH 7. Thus, changes in these optical indices are expected and do not imply the sample actually changed in terms of aromaticity, molecular weight, or its actual origin; this is why the term “apparent” is used in the discussion of the trends (see above). Lipophilicity is a strongly pH-dependent variable where the more neutral (less ionized) form of the molecule will be more lipophilic. As negative sites become protonated, going from pH 7 to pH 4 the lipophilicity will increase. The PBI utilizes data from all titratable pH values and is thus a pH-independent parameter.

The effects of DOC on transepithelial potential in trout

There was an overall significant difference between absolute transepithelial potential (TEP) at pH 7 and pH 4 in control, dechlorinated Vancouver tap water ($p < 0.0001$), with TEP becoming depolarized (i.e., less negative) at pH 4 (overall mean \pm SEM = -18.1 ± 0.7 mV, $N = 30$ at pH 7 versus -4.8 ± 1.0 mV, $N = 30$ at pH 4). This trend was seen consistently in every control group (Fig. 1).

In response to LO DOC at pH 7, TEP became less negative and at pH 4 it became more negative (Fig. 1A). Similarly, for PF DOC, TEP became less negative at pH 7 and more negative at pH 4 (Fig. 1B). However, TEP became more negative at both pH 7 and pH 4 in response to LM DOC (Fig. 1C), AP DOC (Fig. 1D), and AS DOC (Fig. 1E).

The Δ TEP (the change from baseline TEP caused by the presence of DOC) was not significantly different between LO and PF at pH 7 (Fig. 2A). The Δ TEP values caused by LM, AP, and AS DOCs were not significantly different from one another but were significantly different compared to the Δ TEP values caused by LO and PF DOCs at pH 7 (Fig. 2A). At pH 4, Δ TEP in AP was significantly larger compared to all other DOC sources except LM (Fig. 2B). The Δ TEP at pH 4 in LM was not significantly different from any of the other sources (Fig. 2B). The Δ TEP at pH 7 versus pH 4 was only significantly different for LO ($p < 0.0001$) and PF ($p < 0.0001$), as indicated by the asterisk symbol (Fig. 2).

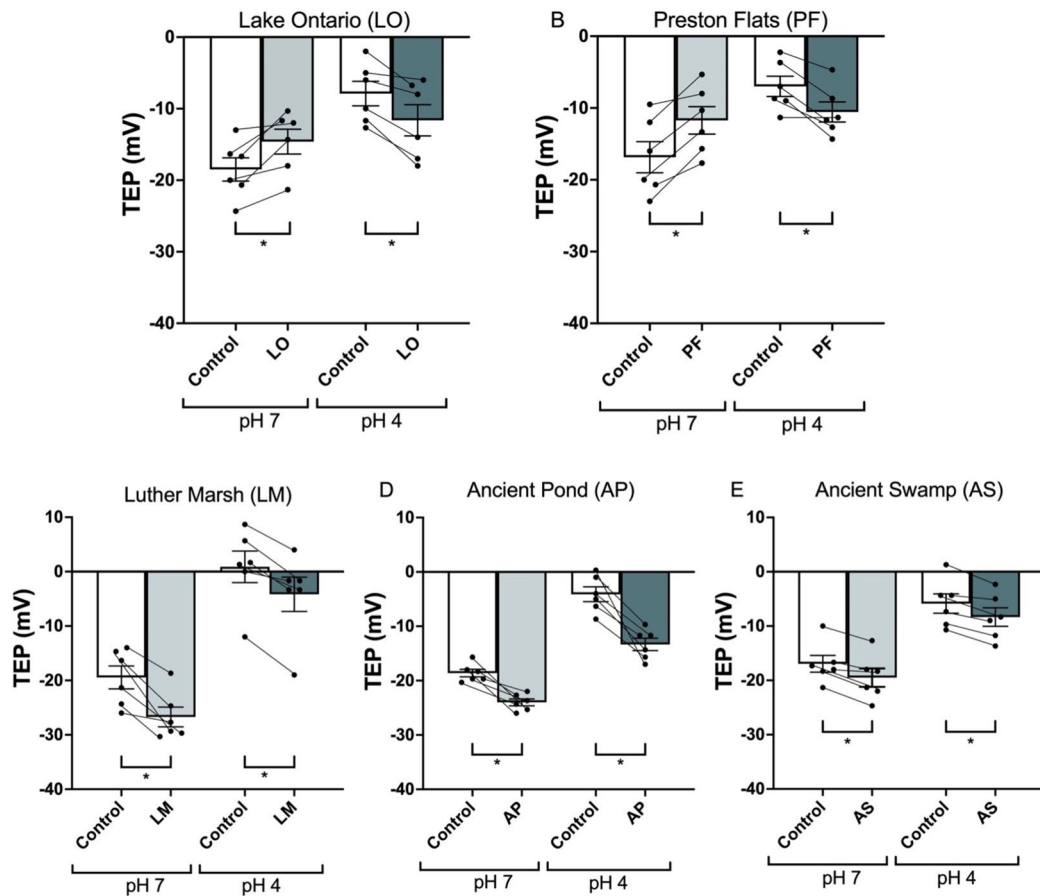


Fig. 1 The effect of natural DOC sources on absolute transepithelial potential (TEP). **A** Lake Ontario (LO), **B** Preston Flats (PF), **C** Luther Marsh (LM), **D** Ancient Woods Pond (AP), and

E Ancient Woods Swamp (AS) at pH 7 and pH 4. Values are mean \pm SEM, $N=6$. Asterisks indicate statistically significant differences ($p < 0.05$ by Student's paired t -test)

The relationships between TEP and the physicochemical characteristics of DOC

Absolute TEP values were regressed separately against specific absorbance coefficient at 340 nm (SAC_{340}), molecular weight index (MWI), fluorescence index (FI), the octanol–water partition coefficient (LogKow), molecular charge, proton binding index (PBI), relative % humic acid-like, relative % fulvic acid-like and relative %, protein-like components at pH 7 and pH 4 using simple linear regression; lines were fitted only for significant relationships (Figs. 3 and 4). At pH 7, when absolute TEP was regressed against SAC_{340} (Fig. 3A), absolute charge (Fig. 3I), and PBI (Fig. 4A), there were significant negative relationships. There were also significant positive relationships between absolute TEP and

FI (Fig. 3C) and absolute TEP and LogKow (Fig. 3G) at pH 7. At pH 4, there were significant negative relationships when absolute TEP was regressed against FI (Fig. 3D), LogKow (Fig. 3H), and relative % protein-like concentration (Fig. 4H) and with relative % fulvic acid-like concentration (Fig. 4F).

We reasoned that if the relationships were robust, they would persist when Δ TEP (Figs. 5 and 6) rather than absolute TEP (Figs. 3 and 4) was used in the same regressions because Δ TEP would compensate for inter-individual variations in baseline TEP that might unduly influence relationships. Again, lines were fitted only for significant relationships (Figs. 5 and 6). Indeed, at pH 7, the significant negative relationships with SAC_{340} (Fig. 5A), absolute charge (Fig. 5I), and FI (Fig. 5C) persisted, as did the significant positive relationships with PBI (Fig. 6A) and

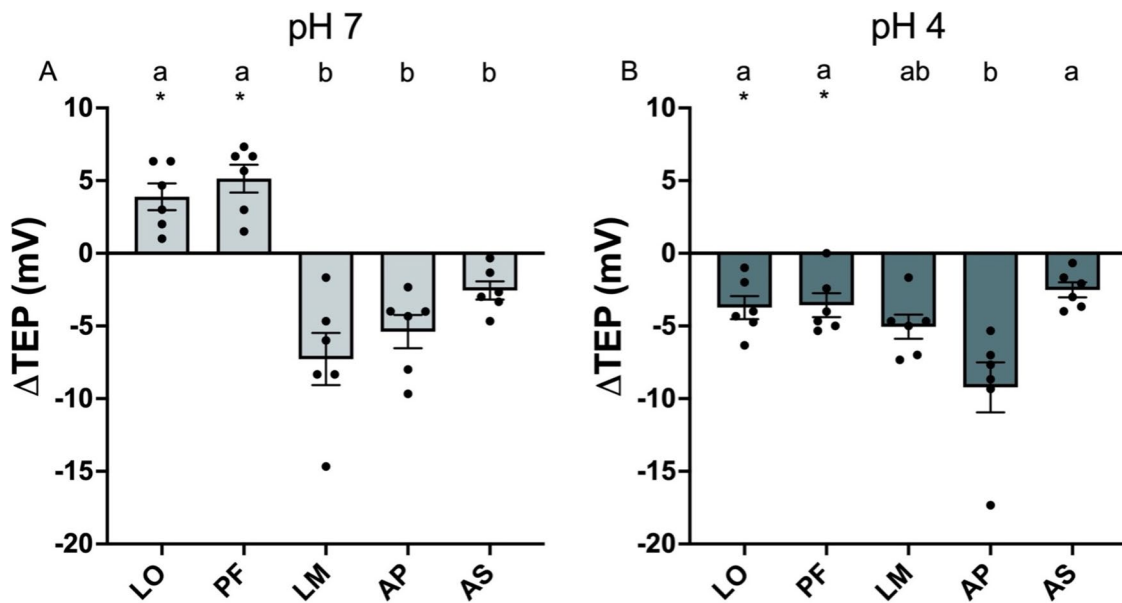


Fig. 2 The effect of natural DOC sources on delta transepithelial potential (ΔTEP). **A** Lake Ontario (LO), Preston Flats (PF), Luther Marsh (LM) Ancient Woods Pond (AP) and Ancient Woods Swamp (AS) at pH 7 and **B** Lake Ontario (LO), Preston Flats (PF), Luther Marsh (LM) Ancient Woods

Pond (AP) and Ancient Woods Swamp (AS) at pH 4. Values are mean \pm SEM, $N=6$. Letters that differ indicate statistically significant differences ($p < 0.05$ by one-way ANOVA plus Tukey's post hoc test). The asterisk symbol indicates the significant difference between the ΔTEP at pH 7 versus pH 4

LogKow (Fig. 5G). There was also now a significant positive correlation between relative % fulvic acid-like concentration and ΔTEP (Fig. 6E).

However, at pH 4, there was a loss of the formerly significant relationships with FI (Fig. 5D), MWI (Fig. 5F), and LogKow (Fig. 5H). The significant negative relationship between ΔTEP and relative % protein-like concentration persisted (Fig. 6H) and significant negative relationships between ΔTEP and SAC_{340} (Fig. 5B), ΔTEP and PBI (Fig. 6B) were now detected.

Discussion

Overview

Each of the DOCs significantly influenced TEP, although in a unique manner or magnitude which may be due to physicochemical differences between the DOCs. In support of our original hypothesis (i) AP and LM had the highest SAC_{340} values (more aromatic), the lowest FI values (more allochthonous), and the lowest MWI values (larger in molecular

size), and with greater PBI and did indeed hyperpolarize TEP to a greater extent, particularly at pH 7 (Supplementary Table S2, Figs. 1 and 2). However, the predicted relationships with % humic acid content by PARAFAC were not apparent, as discussed subsequently. While LM did have a higher % humic acid-like concentration, AP had amongst the lowest at pH 7 (Supplementary Table S2). At pH 4 all DOCs caused TEP to become more negative (i.e., protective) (Fig. 2). Therefore, our second hypothesis (ii) was only partially supported. The effects on TEP with two sources, LO and PF, went from positive (i.e., less protective) to negative (i.e., more protective); however, the magnitude of the hyperpolarization of the other three sources did not significantly change from pH 7 to pH 4 (LM: $p=0.2878$, AP: $p=0.0932$, AS: $p=0.960$). At pH 7, characteristics that appear to be predictive of a more negative TEP are SAC_{340} (more aromatic), LogKow (less lipophilic), FI (more allochthonous), molecular charge (higher charge), and PBI (higher value indicating more reactivity). However, not all these indices hold predictive power at pH 4 (Figs. 3, 4, 5, and 6). Therefore, only SAC_{340} , logKow, and PBI appear to be predictive characteristics

Fig. 3 Absolute TEP of natural DOC sources at pH 7 and pH 4 regressed against **A, B** specific absorbance coefficient; **C, D** fluorescence index; **E, F** molecular weight index; **G, H** LogKow; and **I, J** absolute charge. Values are mean \pm SEM. A fitted line indicates statistical significance ($p < 0.05$ by simple linear regression)

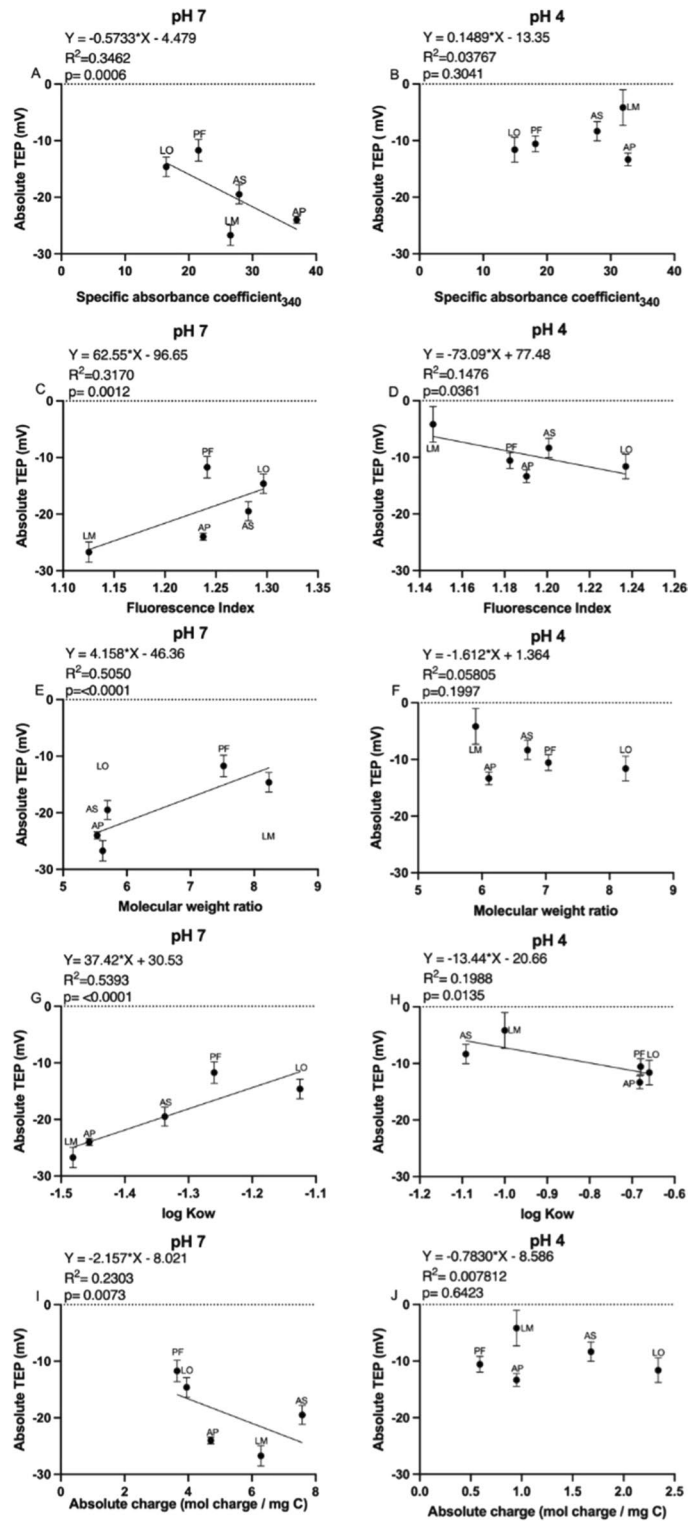


Fig. 4 Absolute TEP of natural DOC sources at pH 7 and pH 4 regressed against **A, B** proton binding index; **C, D** relative % humic acid-like concentration; **E, F** relative % fulvic acid-like concentration; and **G, H** relative % protein-like concentration. Values are mean \pm SEM. A fitted line indicates statistical significance ($p < 0.05$ by simple linear regression)

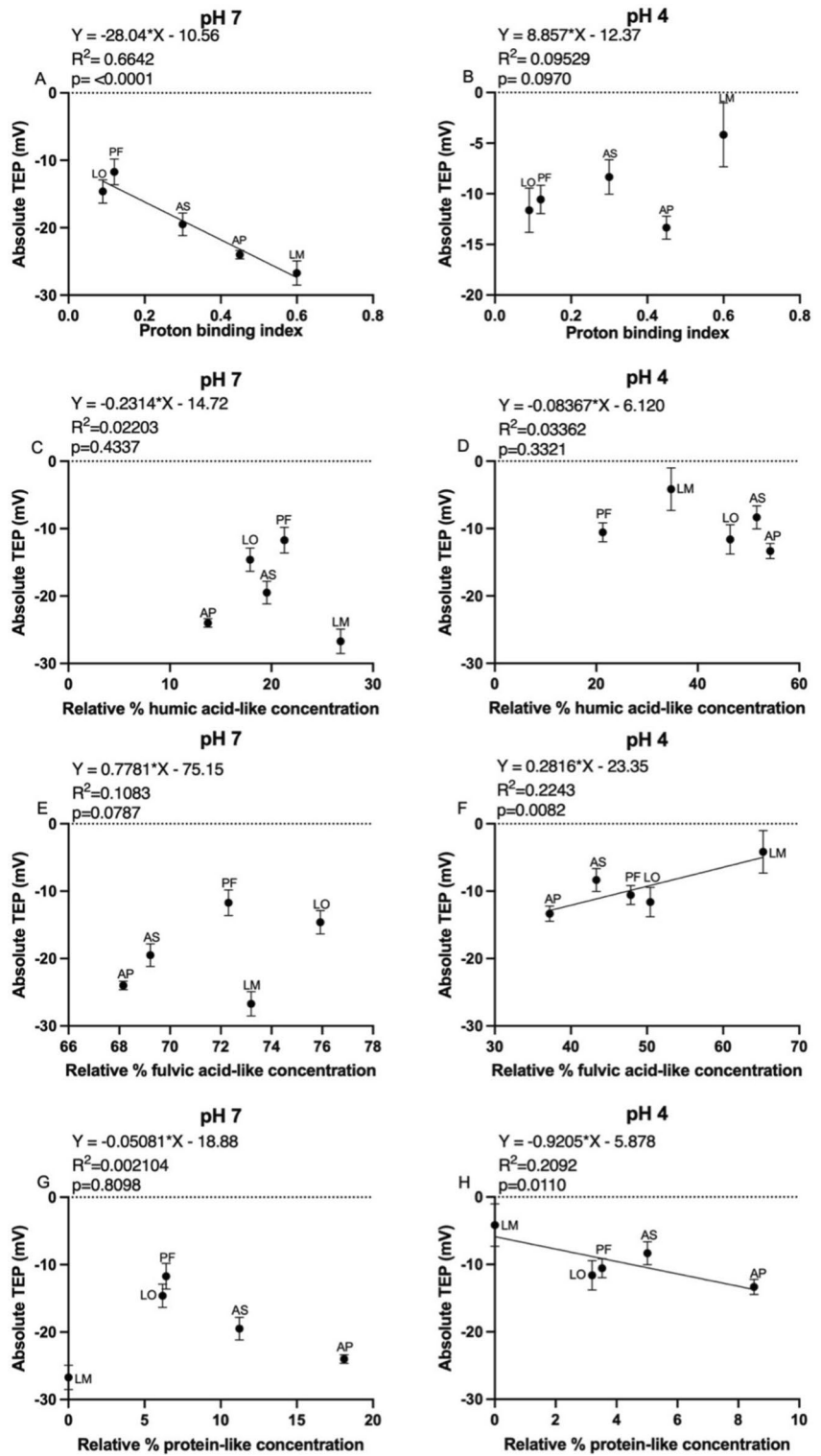


Fig. 5 Δ TEP of natural DOC sources at pH 7 and pH 4 regressed against **A, B** specific absorbance coefficient; **C, D** fluorescence index; **E, F** molecular weight index; **G, H** LogKow; and **I, J** absolute charge. Values are mean \pm SEM. A fitted line indicates statistical significance ($p < 0.05$ by simple linear regression)

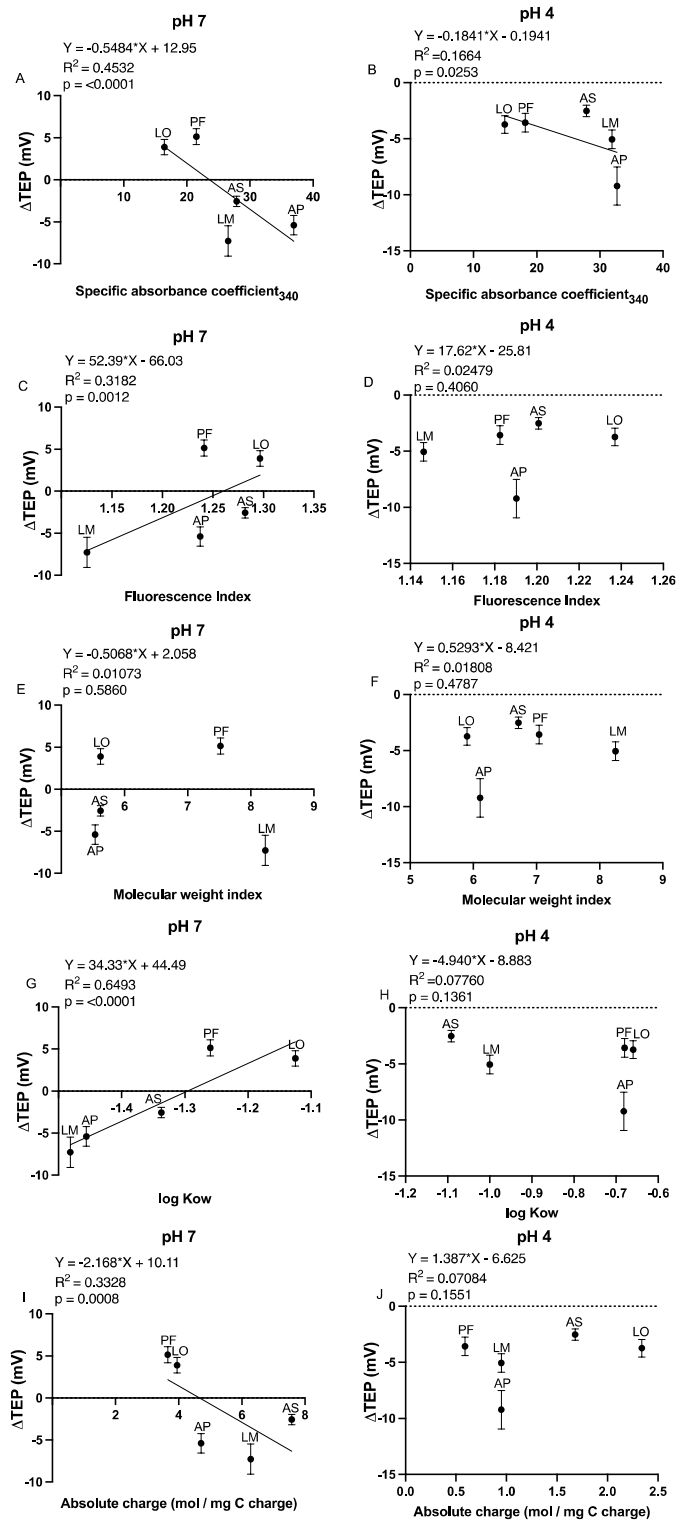
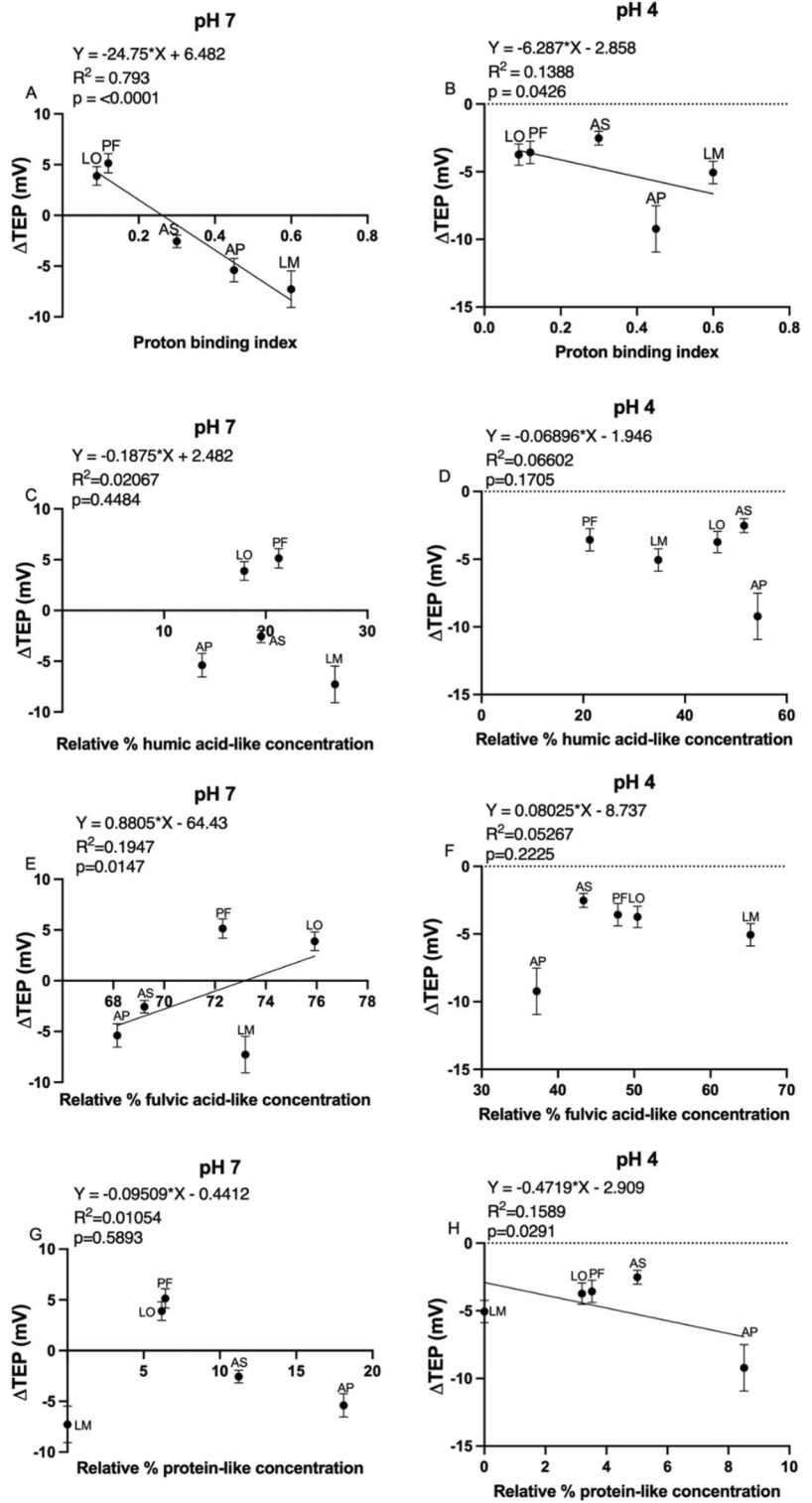


Fig. 6 Δ TEP of natural DOC sources at pH 7 and pH 4 regressed against. **A, B** proton binding index; **C, D** relative % humic acid-like concentration; **E, F** relative % fulvic acid-like concentration; and **G, H** relative % protein-like concentration. Values are mean \pm SEM. A fitted line indicates statistical significance ($p < 0.05$ by simple linear regression)



across pHs (Figs. 3, 4, 5, and 6). Overall, this analysis supports our hypothesis (iii) that one or more of the indices would strongly predict the effect of DOC on TEP at each pH.

DOCs sharing physicochemical characteristics show similar trends in TEP response

Our physicochemical data clearly shows two groupings of DOCs based on the similarity of characteristics; LO and PF as one group, and LM, AP, and AS as the other group. LO and PF were less aromatic, more autochthonous, and more lipophilic (Morris et al. 2024) (Supplementary Table S2). They also had smaller molecular weights, lower PBIs, and lower absolute charge. LM, AP, and AS had the opposite characteristics: they were more allochthonous, more aromatic, less lipophilic with higher molecular weights, and they had higher PBIs and absolute charges. The trends in TEP responses were likewise similar between LO and PF compared to LM, AP, and AS (Figs. 2 and 3). At pH 7 LO and PF caused depolarization (i.e., less negative/more positive TEP) and LM, AP, and AS caused hyperpolarization (i.e., more negative TEP). At pH 4 all sources caused hyperpolarization (Figs. 2 and 3), thereby likely providing more protection to ionoregulation (Wood et al. 2011; Morris et al. 2021).

Previously, it has been suggested that SAC_{340} may be an indicator of the ability of a DOC to influence ionoregulation in aquatic organisms (Al-Reasi et al. 2013; Duarte et al. 2016; Sadauskas-Henrique et al. 2019) and that the aromaticity of the DOC molecules directly influences the electrophysiology of the fish gill (Galvez et al. 2008). Our results are in support of this: AP, AS, and LM had the highest SAC_{340} values and induced negative ΔTEP values at pH 7 (Supplementary Table S2, Fig. 2). SAC_{340} is strongly correlated with molecular size, such that larger DOC molecules are more aromatic (Morris et al. 2024) likely causing a more negative ΔTEP . We did observe that DOCs with larger molecular weights were likewise the DOCs that had the greater influence on TEP; however, the only significant relationship for MWI was with absolute TEP at pH 4 (Fig. 3F). At pH 7, Galvez et al. (2008) reported a significant negative correlation between ΔTEP and SAC_{340} . Our simple linear regressions showed the same relationship at pH 7 between SAC_{340} and absolute TEP, and between

SAC_{340} and ΔTEP , and at pH 4 between SAC_{340} and ΔTEP (Figs. 3A, B and 5A, B). Galvez et al. (2008) suggested that the potency for hyperpolarization is a function of aromaticity. Our data agree with this at pH 7 but not entirely at pH 4. At low pH, SAC_{340} decreased for LO and PF but there was a coincident hyperpolarization of TEP (Supplementary Table S2, Fig. 3). It is possible that SAC_{340} is a slightly better predictor of TEP responses at pH 7 than at pH 4.

The more negative ΔTEP s at pH 7 for allochthonous DOCs were parallel to previous studies indicating that allochthonous DOCs are highly protective against metal toxicity (Al-Reasi et al. 2011, Al-Reasi et al. 2012, Wood et al. 2011), though this difference was no longer apparent at pH 4 (Supplementary Table S2, Fig. 3). Overall, this supports the idea (Al-Reasi et al. 2013) that DOCs that are important in the protection against metal toxicity may be similar to those that exert physiological effects on the gills. As allochthonous DOCs are generally larger in size, it is possible that molecular size may contribute to the physiological effect of DOC as mentioned above. There were significant positive correlations between FI and absolute TEP and ΔTEP at pH 7 but a significant negative relationship between absolute TEP only at pH 4. The fluorescence profiles changed with pH such that at pH 4 the FI indicated the DOCs to be more autochthonous-like in origin (Morris et al. 2024). This is, perhaps, not surprising as autochthonous DOCs are often less protective and the significant negative correlation by simple linear regression between FI and TEP did not hold true at low pH. The change in pH does not reflect a change in the DOC origin, of course, but rather in the fluorescence profile that we generally associate with a specific origin. The change in pH from 7 to 4 probably reduces the ionization of DOCs, altering the molecular orbital of excitable electrons (Laane 1980; Patel-Sorrentino et al. 2002), and causing the observed increases in LogKow values and reductions in molecular charge, or otherwise affecting the macromolecular conformation (Morris et al. 2024).

Although the overall conclusion appears to be that there is no relationship between lipophilicity and the ability to bind metals (Al-Reasi et al. 2012), it is certainly possible that the lipophilicity of a DOC may influence its ability to interact with the gill membrane and affect ionoregulatory processes. It is possible that the changes from TEP

depolarization at pH 7 to TEP hyperpolarization at pH 4 with LO and PF were due to the changes in charge and lipophilicity (Figs. 2 and 3). This greater lipophilicity might favor interaction with the cell membranes of the gills, and this may explain the differences in the physiological effects of these two DOCs at pH 7 versus pH 4. At low pH much of the negative charge on both the gills and the DOC molecules will be removed by H⁺ titration, resulting in ligands that on an overall basis would be more hydrophobic and lipophilic, thereby promoting interaction. LogKow was significantly positively correlated to absolute TEP and Δ TEP at pH 7 but negatively correlated to absolute TEP only at pH 4 (Figs. 3G, H and 5G, H) whereas the relationships of absolute TEP and Δ TEP to absolute charge were only significant at pH 7. These results indicate that at pH 7 logKow is correlated to TEP responses that are not protective of Na⁺ transport but at pH 4, logKow is correlated to TEP responses that are protective of Na⁺ transport. In the companion study by Morris et al. (2024), logKow was not correlated to charge at pH 7 (-0.57) but was very strongly correlated to charge at pH 4 (-0.85 , threshold for significance, $+/-0.87$). It appears that, at pH 4, DOCs with lower charge and higher lipophilicity are beneficial to TEP and in turn Na⁺ transport. In contrast at pH 7, the reduced lipophilicity may decrease the ability of DOCs to interact with the gill compared to pH 4, as described above, and the higher charge could increase TEP in a manner similar to cations such as Na⁺ and K⁺ (Wood et al. 2020; Po and Wood 2022).

PBI is an indicator of the chemical reactivity of DOC and was developed by Al-Reasi et al. (2013) for predicting its metal binding capacity; higher PBI was protective towards metal toxicity and is thought to represent the potential for “tridentate” metal binding sites in DOC molecules. These authors proposed that PBI may also be useful in predicting the effects of DOC on physiological processes, but did not test the idea experimentally. PBI, because it uses data from the entire titratable range, is a pH-independent parameter. Our study shows, for the first time, that PBI is a useful tool in predicting the TEP response to natural DOCs in freshwater fish. DOCs with higher PBIs generally had a larger effect on Δ TEP (Supplementary Table S2, Fig. 2). PBI was significantly negatively correlated to absolute

TEP at pH 7 (Fig. 4A) and to Δ TEP at pH 7 and pH 4 (Fig. 6A, B).

With regards to the PARAFAC components, relative % humic acid-like concentration was not a useful predictor of TEP response. There were no significant relationships between relative % humic acid-like concentration and absolute TEP or Δ TEP at either of the tested pHs (Figs. 4C, D and 6C, D). These results were surprising because, in general, DOCs that have a high humic acid concentration are also more aromatic and have higher molecular weights. As such, SAC₃₄₀ has previously been positively correlated with relative % humic acid-like concentration (PARAFAC component) and negatively correlated with the relative % fulvic acid-like concentration (Al-Reasi et al. 2012). As described by Morris et al. (2024) at pH 4, the sources we used in the present study were consistent with this observation: AP had the highest SAC₃₄₀ value, the highest humic-like concentration, and the lowest fulvic-like concentration (Table S2). However, at pH 7 this was not the case. Morris et al. (2024) reported a significant correlation between SAC₃₄₀ and MWI and we did observe that DOCs with larger molecular weights were likewise the DOCs that had the greater influence on TEP. However, the only significant relationship between MWI was with absolute TEP at pH 7 (Fig. 4E). For relative % fulvic acid-like concentration, absolute TEP was positively correlated at pH 4 (Fig. 4F) and Δ TEP was positively correlated at pH 7 (Fig. 6E), making it a better predictor of TEP effects. Again, it was surprising that the best PARAFAC component for predicting TEP response appeared to be relative % protein-like concentration but only at pH 4, where there was a significantly negative relationship with both absolute TEP and Δ TEP (Figs. 4H and 6H). However, it must be remembered that PARAFAC components are user-defined and the results depend on the quality and number of samples input which is likely why our PARAFAC components show different relationships with SAC₃₄₀ at pH 7 than previously reported, even though the quality and number of samples were acceptable in both Al-Reasi et al. (2012) and the present study. Overall, we conclude that the PARAFAC results must be interpreted with caution, as they are sample-dependent. It is possible that with a different set of natural DOC sources, PARAFAC components may provide more useful predictors of TEP responses than seen in the current study.

Overall interpretation

The TEP response to natural DOCs appears to be directly related to their physicochemical characteristics. The most effective predictors of DOC effects on TEP were SAC₃₄₀, logKow, and PBI across pHs. Each of these indices had at least three significant relationships within the simple linear regression plots (Figs. 3, 4, 5, and 6). The changes in these three indices with the change in pH from pH 7 to pH 4 likely reflect real molecular or structural changes in the DOC as described above and by Morris et al. (2024). This contrasts with other indices where there are changes in the optical signature that likely do not reflect changes in the actual DOC molecule. For example, the difference in the SAC₃₄₀ value from pH 7 to pH 4 may reflect actual changes in the conformation and aromaticity of the DOC molecule, through condensing and protonation/deprotonation reactions (Pace et al. 2012) and this could be the cause of the altered TEP response for a given DOC source. Whereas, FI also had three significant relationships within the simple linear regressions, however, as described by Morris et al., (2024), and above, changes in FI with pH do not reflect changes in what the index is thought to represent. Morris et al (2024) showed that the origin of the DOC does not change with a change in pH, rather, the fluorescence profile and in turn how we interpret that value changes. Therefore, we reject FI as being truly a good predictor of TEP effects across pHs. The natural water pHs of our sites were near circumneutral with a range of 6.83–7.35 (Morris et al. 2024); however, these sites could be subjected to acidification through climate change events especially those situated in swamp or bog areas. The dissociation of protons from allochthonous DOC is a major cause of the acidity in natural blackwaters so as DOC concentrations increase, pH often decreases.

The hyperpolarization of TEP caused by DOC improves Na⁺ uptake and opposes Na⁺ efflux at the gills and is therefore supportive of ionoregulatory function. DOCs that are more aromatic, more chemically reactive, and less lipophilic may influence the paracellular permeability of the gills, mitigating diffusive ion loss. This, in turn, could alter the differential permeability of Na⁺ versus Cl⁻ at the gill, directly influencing TEP where a more negative

TEP creates a favorable electrochemical gradient for net Na⁺ retention. This is even more important at low environmental pH where the diffusion gradient for proton entry is increased (see the “Introduction” section). Our short exposure times were designed to understand how changes in water chemistry impact TEP at a mechanistic level. Our experimental exposures were very acute; however, changes in TEP from acute exposures have been shown to persist and remain stable for 24 h (Wood et al. 2020) and even 96 h (Po and Wood 2022). Further investigation will be necessary to determine whether TEP responses are sustained beyond 96 h. Sustained changes in TEP from the baseline would likely have profound effects on overall ionoregulatory processes and homeostasis. While our data clearly show that some physicochemical properties of DOC are better predictors of TEP outcome than others, further work is required to explore which functional moieties of DOC are influencing TEP at the gills at circumneutral and acidic pH. For example, here, we show that aromaticity (SAC₃₄₀), lipophilicity (logKow), and chemical reactivity (PBI) are important characteristics of DOC in predicting the TEP responses, but future studies are needed to determine which particular chemical components associated with these properties are causing the TEP responses. DOC concentrations are rising and pH levels are falling in freshwaters with climate change (Sucker and Krause 2010; Pagano et al. 2014; Morris et al. 2021) which will likely have profound effects on ecosystems. The roles of DOCs within an ecosystem are widespread, spanning from UV light screening and changing microbial activity to influencing the bioavailability of contaminants, all of which may influence the health of freshwater fish. Therefore, a mechanistic understanding of not only the interaction between the physicochemical properties of DOC and ionoregulation in fish but also how these physicochemical characteristics influence other environmental parameters is crucial for the future monitoring of aquatic environmental health. Future studies should aim to integrate the effects of DOC on other physiological endpoints in freshwater organisms with the physicochemical properties of DOC while simultaneously considering other water quality parameters such as pH, hardness, alkalinity, major ion concentrations, and potential pollutants.

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Data availability Data is provided within the manuscript or supplementary information files.

Code availability Not applicable.

Declarations

Conflict of interest The authors declare no competing interests.

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