Journal of Comparative Physiology B
https://doi.org/10.1007/s00360-024-01580-2

ORIGINAL PAPER

The effects of dissolved organic carbon and model compounds (DOC analogues) on diffusive water flux, oxygen consumption, nitrogenous waste excretion rates and gill transepithelial potential in Pacific sanddab (*Citharichthys sordidus*) at two salinities

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Received: 30 May 2024 / Accepted: 16 August 2024
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Abstract

Many flatfish species are partially euryhaline, such as the Pacific sanddab which spawn and feed in highly dynamic estuaries ranging from seawater to near freshwater. With the rapid increase in saltwater invasion of freshwater habitats, it is very likely that in these estuaries, flatfish will be exposed to increasing levels of dissolved organic carbon (DOC) of freshwater origin at a range of salinities. As salinity fluctuations often coincide with changes in DOC concentration, two natural freshwater DOCs [Luther Marsh (LM, allochthonous) and Lake Ontario (LO, autochthonous) were investigated at salinities of 30 and 7.5 ppt. Optical characterization of the two natural DOC sources indicate salinity-dependent differences in their physicochemistry. LO and LM DOCs, as well as three model compounds [tannic acid (TA), sodium dodecyl sulfate (SDS) and bovine serum albumin (BSA)] representing key chemical moieties of DOC, were used to evaluate physiological effects on sanddabs. In the absence of added DOC, an acute decrease in salinity resulted in an increase in diffusive water flux (a proxy for transcellular water permeability), ammonia excretion and a change in TEP from positive (inside) to negative (inside). The effects of DOC (10 mg C L^{-1}) were salinity and source-dependent, with generally more pronounced effects at 30 than 7.5 ppt, and greater potency of LM relative to LO. Both LM DOC and SDS increased diffusive water flux at 30 ppt but only SDS had an effect at 7.5 ppt. TA decreased ammonia excretion at 7.5 ppt. LO DOC decreased urea-N excretion at both salinities whereas the stimulatory effect of BSA occurred only at 30 ppt. Likewise, the effects of LM DOC and BSA to reduce TEP were present at 30 ppt but not 7.5 ppt. None of the treatments affected oxygen consumption rates. Our results demonstrate that DOCs and salinity interact to alter key physiological processes in marine flatfish, reflecting changes in both gill function and the physicochemistry of DOCs between 30 and 7.5 ppt.

Keywords Marine flatfish · Dissolved organic carbon · Osmoregulation · Diffusive water flux · Ammonia excretion · Transepithelial potential

Communicated by Bernd Pelster.

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Published online: 09 September 2024

Introduction

There is now abundant evidence that dissolved organic carbon (DOC) affects gill function in freshwater organisms, particularly fish. The effects are usually beneficial to the osmoregulation of the organism (Gonzalez et al. 2002; Wood et al. 2003, 2011; Matsuo et al. 2004; Al-Reasi et al. 2016), although exceptions exist (Steinberg et al. 2003, 2006). The mechanisms remain largely unknown, but it is clear that DOC affects unidirectional and net ion flux rates, ammonia and urea-N excretion rates, and transepithelial potential (TEP) (Gonzalez et al. 2002; Wood et al. 2003; Matsuo et al. 2004; Galvez et al. 2008; Al-Reasi et al. 2013b, 2016; Duarte et al. 2016). DOCs may also influence the electrical properties of the gills, hyperpolarizing the inside- negative TEP (Galvez et al. 2008; Sadauskas-Henrique et al. 2019). The TEP is a diffusion potential in freshwater fish (Potts 1984). However, as yet, there appears to be no information on whether DOC can affect diffusive water flux across the gills, the "water aspect" of osmoregulation.

Some DOCs are more effective at exerting physiological effects than others (Al-Reasi et al. 2011, 2013a; Saudaskas-Henrique et al., 2019). It has been proposed, but not yet proven, that the chemical properties or functional moieties of DOC that are important in the protection against metal toxicity are similar to those that exert physiological effects on the gills (Wood et al. 2011; Al-Reasi et al. 2013a). Optical indices based on absorbance, fluorescence, and parallel factor analysis (PARAFAC) have been used to describe molecular variability of DOC (Al-Reasi et al., 2011; Wood et al. 2011). Indices that have shown promise in predicting protection by DOCs against metals are: (i) the specific absorbance coefficient 340 (SAC_{340}) (Curtis and Schindler, 1997) with higher values representing greater aromaticity; (ii) the absorbance ratios at 254 nm to 365 nm (molecular weight index, MWI), with lower values indicating a greater molecular weight (Dahlén et al., 1999), (iii) the fluorescence index (FI) which is the ratio of fluorescence $intensity_{450nm}$/fluorescence $intensity_{500\ nm}$ at an excitation wavelength of 370 nm, with higher values indicating an autochthonous origin, and lower values, an allochthonous origin (McKnight et al. 2001); and (iv) PARAFAC, a computational model based on the fluorescence excitation-emission fingerprint of a DOC sample that uses a multivariate statistical approach to yield a relative fractionation into user-defined components such as % humic acid-like, % fulvic-acid-like, and % protein-like composition (Kroonenberg and Heiser 1998; Stedmon and Bro 2008). Generally, aromatic rich, high molecular weight allochthonous DOCs with high % humic acid- like fluorescence are highly protective against metal toxicity (Al-Reasi et al., 2011).

Based on studies to date, limited entirely to marine invertebrates, it is unclear whether the same optical indices predict the protective abilities of DOCs in seawater (Nadella et al. 2009, 2013; DePalma et al. 2011; Cooper et al. 2014; Tait et al. 2016; Blewett et al. 2016, 2018, 2021; Nogueira et al. 2017, 2018). Nevertheless, these studies confirm that DOCs can protect against metal toxicity in seawater although the degree of protection varies among DOC sources, salinity, and aquatic species. It remains to be seen whether DOCs that protect best against metal toxicity also have the greatest physiological effects on marine organisms, and specifically whether such effects occur in marine fish.

With this background in mind, we evaluated the effects of two natural freshwater DOCs and three model compounds (reflective of specific properties of DOC) on gill function in the Pacific sanddab (*Citharichthys sordidus*).

The sanddab is euryhaline, like many other flatfish (Evans 1984; Rogers et al. 1984; Minami and Tanaka 1992; Gibson 1994; Burke et al. 1995). They spawn, metamorphose, and feed in highly dynamic estuaries with salinity ranging from full seawater (30 ppt) to freshwater (0 ppt), depending on the seasonal river discharge (Thornburgh 1980; Durkin et al. 1981; Bottom et al. 1984; Armor and Herrgesell 1985; Rackowski and Pikitch 1989; Rooper et al. 2006; Sobocinski et al. 2018). Previous studies have established that gill function in *Citharichthys sordidus* is indeed very sensitive to changes in salinity, with marked effects on both ammonia excretion and diffusive water flux, a proxy for gill transcellular water permeability (Onukwufor and Wood 2022; Morris and Wood 2023). In these estuaries on the Pacific coast of North America, sanddab will also be exposed to freshwater DOCs of allochthonous and/or autochthonous origin (McNicol et al. 2023), such that interactive effects with salinity may occur.

The two natural DOCs chosen for investigation were freshly isolated from two sources. These sources have been well studied in freshwater fish, and their physico-chemical properties have been well characterized (Al-Reasi et al. 2012, 2013a, b, 2016; Duarte et al. 2018; Morris et al. 2024), but they differ greatly. Lake Ontario (LO) DOC is an autochthonous DOC that is less aromatic, and smaller in molecular size, with lower % humic acid-like content when compared to Luther Marsh (LM) DOC which is allochthonous, highly aromatic, with a larger molecular weight and higher % humic content. As such, LM DOC appears to have greater supportive effects on ionoregulation in freshwater fish than does LO DOC (Galvez et al. 2008; Wood et al. 2011; Al-Reasi et al. 2013a).

The choice of model compounds used in the present study was based on the criteria that they structurally resemble and functionally behave like certain components of natural DOCs. Tannic acid (TA) has been a widely used as a model

allochthonous carbon compound because of its high aromatic content and similarity to humic substances (Moreno-Castilla et al. 2004; Lin and Baoshan 2008; Campinas et al. 2013). Sodium dodecyl sulfate (SDS), often called sodium lauryl sulfate, is a commonly used anionic surfactant (Holmberg et al. 2007), and was chosen to mimic the amphiphilic properties of DOCs with hydrophilic and hydrophobic moieties (von Wandruszka 2000; Young and von Wandruszka 2001). Autochthonous DOCs may be richer in amino acids such as tyrosine (Thurman 1985). Therefore, bovine serum albumin (BSA) was chosen to represent tyrosine-rich proteinaceous material (Heidari et al. 2018).

We chose several endpoints known to be affected by DOCs in freshwater organisms, including ammonia and urea-N excretion, and TEP. Notably, TEP has a rather different origin in marine fish, with both diffusive and electrogenic components of ion fluxes, whereas in freshwater fish, there is no electrogenic component (Potts 1984). We did not study ion flux rates themselves, because these are extremely challenging to measure due to the high background ion levels in seawater which would necessitate use of very large amounts of expensive radioisotopes, and the available techniques lack precision to detect small differences (Giacomin et al. 2020). Instead, we evaluated diffusive water flux rate, where the technology is well established. Previous studies have shown it to be sensitive to salinity, hypoxia, and temperature in marine flatfish (Onukwufor and Wood 2022; Morris and Wood 2023). We chose to study two different salinities because rapidly reduced salinity is an environmentally realistic osmotic challenge when flatfish first enter dilute estuaries, where effects of DOC may become more apparent. Additionally, the effects of acute low salinity exposure in the absence of added DOC have previously been documented in this species (Onukwufor and Wood 2020; Morris and Wood 2023) for comparison. As in these previous studies, routine oxygen consumption rate ($\dot{M}O_2$) was also monitored to check whether observed responses were secondary effects of changes in aerobic metabolism.

Our specific hypotheses were (i) As ionic strength and pH changes the molecular conformation of the DOC, the effects of DOC would be greater at lower salinity; (ii) as LM DOC is more aromatic and larger in size than LO DOC, the effects of LM DOC would be greater than those of LO DOC; (iii) as the origin of TEP is different at the two tested salinities, the TEP responses to DOC would differ between salinities; and (iv) as the model compounds shared specific key chemical structures with the natural DOCs, the effects of the model compounds would help illuminate which specific properties of the natural DOCs were contributing to the observed responses.

Methods

Animals

Pacific sanddab (*Citharichthys sordidus*) ($N = 90$, 0.389 ± 0.0129 kg) were caught in July, 2022, by angling off Brady's beach, near the Bamfield Marine Sciences Center (BMSC), Bamfield, BC, Canada, under Fisheries and Oceans Canada collection permit XR 119–2022. At BMSC, flatfish were held in aerated fiber glass tanks supplied with flowing seawater (30 ppt, 12 ºC) and a layer of sand at the bottom into which the fish buried during the day. Fish were allowed to rest for at least 72 h after capture. The fish were fed small chunks of salmon every second day and were fasted for at least 72 h before experiments commenced. Seawater was pumped from the nearby ocean to both BMSC holding tanks and the experimental set-up. Experimental fish were transferred to individual aerated chambers slightly longer and wider (35 cm X 25 cm) than each individual fish. The chambers were supplied with running seawater (30 ppt), and rested in a water bath to maintain a control temperature of 12 °C when seawater flow was stopped during experiments. Sand was absent in the experimental vessels, as a previous study showed no significant differences in diffusive water flux or oxygen consumption rates in the presence and absence of sand (Onukwufor and Wood 2022). All procedures used in this investigation were in accordance with the Canadian Council on Animal Care guidelines and were approved by the University of British Columbia Animal Care Committee (AUP A18-0271) and the BMSC Animal Care Committee (RS-22-08).

Experimental series

Six experimental series were carried out.

- *Series 1* measured diffusive water flux rates, oxygen consumption rates, ammonia-N and urea-N excretion rates and the nitrogen quotient in response to two sources of natural DOC (LM and LO) at 30 ppt, together with their own control group at 30 ppt.
- *Series 2* evaluated the effect of these same natural DOCs on the same endpoints at 7.5 ppt, together with their own control group at 7.5 ppt.
- *Series 3* tested three model compounds (TA, BSA and SDS) at 30 ppt on diffusive water flux rates, oxygen consumption rates, ammonia-N and urea-N excretion rates and the nitrogen quotient, together with their own control group at 30 ppt.
- *Series 4* tested these same three model compounds at 7.5 ppt on the same endpoints, together with their own control group at 7.5 ppt.

- *Series 5* measured transepithelial potential (TEP) in response to both natural DOCs and model compounds at 30 ppt, using each fish as its own control.
- *Series 6* measured TEP in response to both natural DOCs and model compounds at 7.5 ppt, again using each fish as its own control.

Experimental solutions

Two natural sources of DOCs were collected, from Luther Marsh (LM, 43°57′N, 80°26′W) and Lake Ontario (LO, 44°14’N, 76°28’W) in Ontario, Canada. For both locations, DOC concentrates were obtained by a portable reverse osmosis unit which concentrated organic matter gradually (Sun et al. 1995). Following reverse osmosis, samples were treated with a cation exchange resin (Amberlite IR-118 (H), Sigma Aldrich, St. Louis, MO, USA) to remove cations that may have accumulated during reverse-osmosis. The concentrate was then filtered using hydrophilic, polyethersulfone polymer membrane filters with a 0.45-µm pore size (MS® PES Membrane Solutions, Auburn, WA, USA), and were stored and transported in sealed 1 L bottles at 4ºC until use.

Salinity was measured using a WTW Portable Conductivity Meter (ProfiLine Cond3310; Xylem Analytics, Welheim, Germany). Solutions with a salinity of 7.5 ppt were made by diluting seawater with BMSC freshwater (Na^+ 0.3, Cl^- 0.2, K^+ 0.005, Ca^{2+} 0.1, Mg^{2+} 0.05 mM) until the desired salinity was reached. All experimental solutions were maintained at 12 °C.

Natural DOCs (LO and LM) and model compounds (TA, BSA, and SDS, Sigma-Aldrich, St. Louis, Missouri, USA) were added to and dissolved in the appropriate salinity to equal nominally 10 mg C L^{-1}. Approximately, 10 mg C L^{-1} has been recorded as a global average DOC concentration within freshwater streams, river tributaries and main stems (Liu and Wang, 2022) and is within range of coastal waters with DOC input via rivers (Maxey et al. 2020).

DOC concentrations in the exposure waters were measured using a Shimadzu total carbon and nitrogen analyzer (TOC-L, Shimadzu, Mandel Scientific, Guelph, Canada) (Table 1). All DOC levels in samples were well above the instrument detection limit of approximately 0.1 mg C L^{-1}. The measured concentrations of the model compounds differ somewhat from the nominal concentrations, likely because of the formation of aggregates caused by interaction with the natural seawater. We believe that the presence and concentration of the model compounds is sufficient for our mechanistic comparison with the natural DOCs. Importantly, the nominal and measured concentrations of the natural DOCs are quite close. The pH range among the control and experimental solutions was typically less than 1.0 pH unit (Table 1). The recorded pHs were those that resulted naturally from mixing DOCs from the different sources with the two salinities, making the pH values environmentally relevant. Adjusting the pHs could have introduced another confounding factor. Solutions were allowed to equilibrate overnight, and exposures occured the following morning. Water samples were collected directly after concluding the experiment and were stored for a short time in the dark at 4 °C prior to characterization.

Optical characterization of experimental solutions

An Aqualog (HORIBA Scientific, Kyoto, Japan) was used to measure the fluorescence and absorbance of each of the experimental solutions. This instrument measures excitation and emission simultaneously, and provides the required excitation emission matrices such that parallel factor analysis (PARAFAC) of fluorophores (Stedmon and Markager 2005; Stedmon and Bro 2008) can be conducted. All characterizations were done in the actual exposure solutions at the appropriate salinity (i.e. 30 ppt or 7.5 ppt).

Specific absorbance coefficient at 340 nm (SAC_{340}), an index of aromatic composition of DOCs, was estimated based on the method described by Curtis and Schindler

Table 1 Characterization of experimental water samples in terms of pH, DOC concentration, specific absorbance coefficient at 340 nm (SAC_{340}), fluorescence index (FI) and molecular weight index (MWI) in exposures at two different salinities, 30 ppt and 7.5 ppt

Source	Salinity	pH	[DOC]	SAC_{340}	FI	MWI
Control	30 ppt	7.96	1.71	NA	NA	NA
Control	7.5 ppt	7.06	2.2	NA	NA	NA
Lake Ontario	30 ppt	6.84	9.74	15.6	1.34	4.61
Lake Ontario	7.5 ppt	6.53	9.44	9.65	1.37	6.61
Luther Marsh	30 ppt	7.23	8.34	21.61	1.21	4.48
Luther Marsh	7.5 ppt	7.71	11.97	22.72	1.21	4.39
Tannic acid	30 ppt	7.47	4.44	NA	NA	2.02
Tannic acid	7.5 ppt	7.36	5.53	NA	NA	1.84
Bovine serum albumin	30 ppt	7.73	12.3	NA	NA	NA
Bovine serum albumin	7.5 ppt	7.32	9.36	NA	NA	NA
Sodium dodecyl sulphate	30 ppt	7.75	15.8	NA	NA	NA
Sodium dodecyl sulphate	7.5 ppt	7.73	14.3	NA	NA	NA

(1997) by examining the absorbance of samples at a wavelength of 340 nm using a 1-cm quartz cuvette (Helma Canada Ltd., Concord, ON, Canada) in the Aqualog instrument. The instrument was “blanked” using ultrapure water. Briefly, SAC_{340} ($cm^2\ mg^{-1}$) was calculated as:

$$SAC_{340} = \frac{(2.303 \times \ Abs_{340})/\text{pathlength}}{[DOC]\ /1000} \quad (1)$$

Fluorescence index (FI) is an indicator of the DOC source with high values indicating an autochthonous origin, and low values, an allochthonous origin (McKnight et al. 2001). FI was calculated by previously described methods (McKnight et al. 2001):

$$FI\,(\text{at Ex370}) = \frac{Em450}{Em500} \quad (2)$$

where Ex370 is the excitation intensity at 370 nm, and Em450 and 500 refer to emission intensities at 450 nm and 500 nm.

Absorbance measurements at 254 and 365 nm may be used as indirect indicators of relative molecular weight of DOC (Dahlén et al., 1999). Note that this molecular weight index (MWI) is an inverse ratio, with high values indicating lower molecular weight, and low values indicating a higher molecular weight:

$$MWI = \frac{Abs_{254}}{Abs_{365}} \quad (3)$$

PARAFAC modelling was conducted in MATLAB (MathWorks, Natick, MA, USA). PARAFAC is a computational model that uses a multivariate statistical approach to define the components and quality of a sample based on the fluorescence excitation-emission matrix and known predetermined fluorophores (Stedmon and Markager 2005; Stedmon and Bro 2008). In the present study, our categories were humic acid-like, fulvic acid-like, and protein-like fluorophores. As some of the model compounds do not have these key functional moieties (therefore lack fluorescence in the appropriate range), they were excluded from the PARAFAC analysis.

Diffusive water flux

In *Series 1*, *2*, *3*, and *4*, the procedure outlined by Onukwufor and Wood (2020) was used for measuring diffusive water flux rates. Briefly, the loading period began 12 h before the experiment started. Fish (6 per experiment) were set in individual aerated vessels maintained at 12 °C and each were loaded with ^3H_2O radioactivity by exposure to 30 μCi of ^3H_2O (PerkinElmer, Woodbridge, ON, Canada) in the control solution (2–4 L), which was either 30 ppt (*Series 1* and *3*) or 7.5 ppt (*Series 2* and *4*) with no added DOC. Therefore. fish exposed to 7.5 ppt were not acclimated to this salinity, but rather had been acclimated to 30 ppt. Their exposure to 7.5 ppt started immediately at the onset ofthe 12-h loading period with ^3H_2O and continued during the approximately 4-h experimental period. This approach was adopted as our goal was to evaluate how fish would respond acutely when entering lower salinity water, rather than when resident in low salinity water.

After the 12-h loading period, each individual fish and chamber were quickly rinsed with clean seawater with care to minimize disturbance to the fish. The fish was immediately placed back into the aerated, temperature-controlled chamber containing 2–4 L (exact volume recorded) of the appropriate control or experimental solution without ^3H_2O. Each fish was provided with enough solution so that they were covered by several cm. The experimental solutions were either 30 ppt or 7.5 ppt supplemented with natural DOCs (LO or LM) or model compounds (TA, BSA, or SDS) at nominally 10 mg C L^{-1}.

A 4-ml aliquot of the control or experimental solution was taken at time 0, marking the start of the washout period, and every 5 min thereafter for 60 min. A final sample was taken at 12 h when the external and internal ^3H_2O pools were once again in equilibrium. The 12-h sample was used to calculate the original amount of radioactivity in the fish and the 0–60 min samples were used to determine the diffusive water flux rates.

Oxygen consumption rate measurements

Directly following the end of the 60 min diffusive water flux experiment, oxygen consumption rates ($\dot{M}O_2$) were measured as per Onukwufor and Wood (2022), on the same fish. At the start of the measurements PO_2 was > 80%. Aeration was suspended, and the vessels were sealed with Styrofoam covers cut to the exact size of the chambers with a sealable hole specifically designed to fit the diameter of the oxygen probe (YSI 55 Handheld polarographic O_2 meter, Yellow Springs, OH, USA). Preliminary experiments demonstrated that the Styrofoam covers adequately blocked the entry of O_2. The decrease in PO_2 was measured every 5 min until it reached 50% saturation, a PO_2 that is well above the critical PO_2 where $\dot{M}O_2$ becomes dependent on PO_2 in this species (O.E. Johannsson and C.M. Wood, unpublished results). This period ranged from 10 to 60 min within the various treatments.

Ammonia and urea-N excretion rate measurements

Immediately following the end of the $\dot{M}O_2$ measurements, aeration was re-established and 4 ml water samples were taken from each vessel representing time 0, and additional samples were drawn every 30 min for 2 h thereafter. Preliminary experiments demonstrated that this time interval was adequate to detect reliable changes in ammonia and urea-N levels in the water. Concentrations of total ammonia (salicylate hypochlorite assay, Verdouw et al. 1978) and total urea-N (diacetyl monoxime assay, Rahmatullah and Boyde 1980) in water samples were measured colorimetrically using a SpectraMax 190 microplate reader (Molecular Devices, San Jose, CA, USA), with standards made up in the appropriate salinities. Based on a preliminary test, none of the experimental solutions with added DOC imparted significant colour to the water. Note that while we did not directly measure whether any ammonia was added to the experimental waters by the addition of DOC extracts, our assays of ammonia concentrations in the starting water samples of our ammonia excretion trials indicated that it was negligible. It would not have contributed to our ammonia excretion measurements.

Transepithelial potential measurements

In *Series 5* and *6*, transepithelial potential measurements (TEP) were conducted according to the procedure outlined by Wood et al. (2020). TEP was measured using intraperitoneal catheters which earlier studies have shown to yield identical data to those generated using more invasive vascular catheters (Wood and Grosell 2008). Fish were fasted for at least 72 h prior to surgery and anesthetized using 0.5 mg L^{-1} of tricaine methane sulfate (MS222). To avoid hypoxic stress, gills were irrigated with a continuous flow of anaesthetic water (Po and Wood 2021). A 10-cm section of polyethylene PE50 catheter (Clay Adams™, Becton Dickinson, Franklin Lakes, NJ, USA) filled with Cortland saline (Wolf 1963), set to pH ~ 7.7 and adjusted with an additional 20 mM NaCl to mimic the composition of the marine fish plasma, was inserted into the peritoneal cavity as described by Wood and Grosell (2008). The catheter was anchored using a 3-cm PE160 sleeve secured with cyanoacrylate glue and silk suture. The catheter was sealed with a stainless-steel pin until experiments were conducted. After overnight recovery in separate aerated enclosures supplied with DOC-free water (12ºC) at either 30 ppt (*Series 5*) or 7.5 ppt (*Series 6*) as appropriate, 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, Copenhagen, Denmark). The reference bridge was placed into the experimental solution of the measurement chamber and the measurement bridge was connected to the indwelling catheter. For every solution, three measurements were made over a 2-min period (with correction for the junction potential) and averaged. The background TEP was similarly measured in control solution (30 ppt or 7.5 ppt) in each animal and served as a baseline TEP for comparison with the TEP measured in the experimental solution. A paired statistical design was used, where each fish was its own control. Each individual fish was exposed to the experimental solution (DOC or model compounds) at the appropriate salinity for 2 min before the measurements commenced and the three measurements described above were completed in about 2 min. The total time during which each fish was exposed to the experimental solution (including the time to take the measurements) was less than 5 min. 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 MS222.

Analytical procedures and calculations

As described by Onukwufor and Wood (2018), the concentration of ^3H_2O was measured by adding 8 ml of Optiphase 3 fluor (Perkin-Elmer, Wellesley, MA, USA) to the 4-ml water sample, shaking vigorously and loading it into a scintillation counter (LS6500, Beckman Coulter, Fullerton, CA, USA). Preliminary tests showed that quenching and chemiluminescence were negligible. The rate constant of ^3H_2O efflux (k) was calculated employing the protocol outlined by Onukwufor and Wood (2020). Briefly, using the final 12-h wash-out sample, the initial amount of ^3H_2O in the fish was determined. It was then possible to calculate the amount of ^3H_2O left in the fish at each sampling time from 0-60-min based on the measured amounts that appeared in the external solution at each time point and the known volume of the system. Next, the natural logarithm of the total ^3H_2O in the fish at each sample time was regressed against time in minutes on a linear scale. The fractional rate constant k for water turnover was the slope of this line. Multiplying the rate constant k by 60 yielded the fraction of the body water pool turned over per hour. Generally, the total exchangeable water pool of a fish is 800 ml kg^{-1} (Holmes and Donaldson 1969; Isaia 1984; Olson 1992). To calculate the diffusive water flux rate in ml/fish/h, the weight of the fish in kg was multiplied by 800 ml kg^{-1} and then by the fraction of the body water pool turned over per hour.

$\dot{M}O_2$ calculations followed the procedure of Onukwufor and Wood (2020). Briefly, using salinity and temperature-dependent solubility coefficients (Boutilier et al. 1984), PO_2 values were converted to O_2 concentrations (µmol L^{-1}). The

Table 2 Allometric mass scaling for diffusive water flux, oxygen consumption, ammonia and urea-N excretion rates for Pacific sanddab (*Citharichthys sordidus*) at 30 ppt and 7.5 ppt

Measurement	Salinity	Regression equation	R^2	*p*-values
Diffusive water flux rate*	30 ppt	Y = 0.8388*X − 0.4993	0.6326	0.002
Diffusive water flux rate*	7.5 ppt	Y = 0.7704*X − 0.2453	0.7569	0.0002
Oxygen consumption rate	30 ppt	Y = 0.5554*X + 1.350	0.1262	0.2571
Oxygen consumption rate	7.5 ppt	Y = -0.08703*X + 2.777	0.02585	0.6176
Ammonia excretion rate*	30 ppt	Y = 0.7844*X − 0.05554	0.4643	0.0147
Ammonia excretion rate*	7.5 ppt	Y = 0.6871*X + 0.2709	0.2481	0.0993
Urea-N excretion rate	30 ppt	Y = 0.3910*X + 0.05888	0.03803	0.5436
Urea-N excretion rate*	7.5 ppt	Y = 0.8806*X − 0.9213	0.3357	0.0484

Y = log rate and X = log body mass

$p<0.10$ was used for statistical significance

*indicates parameters for which allometric scaling was applied

Table 3 The mean values ± SEM of the two control groups at each salinity combined (*Series 1* and *3* at 30 ppt versus series *2* and *4* at 7.5 ppt) and the Student's t test unpaired comparison (p value, $p<0.05$ is significant) for diffusive water flux rate, oxygen consumption rate, ammonia excretion rate, urea-N excretion rate, nitrogen quotient and transepithelial potential

Physiological endpoint	30 ppt	7.5 ppt	*p*-value
Diffusive water flux rate (ml/kg/h)	131.8 ± 6.3	162.5 ± 8.5	0.0084
Oxygen consumption rate (μmol/kg/h)	1375.4 ± 115.5	1654.4 ± 171.5	0.1917
Ammonia excretion rate (μmol/kg/h)	278.0 ± 22.1	369.2 ± 57.0	0.0219
Urea-N excretion rate (μmol/kg/h)	94.4 ± 10.9	68.9 ± 9.1	0.0862
Nitrogen quotient	0.241 ± 0.027	0.327 ± 0.028	0.0367
Transepithelial potential (mV)	+11.9 ± 0.7	-9.2 ± 1.3	< 0.0001

rate of decline of O_2 concentration was then multiplied by the known volume of the experimental vessel, and divided by time (h), to give $\dot{M}O_2$ in µmol fish^{-1} h^{-1}.

Ammonia-N and urea-N excretion rates (note: 1 N per ammonia molecule, 2 N per urea molecule) were calculated as per Wood (1992) whereby the change in concentration in the water (µmol-N L^{-1}) was divided by the time of the flux period (h), multiplied by the volume of the experimental container (L) to give the excretion rate as µmol-N fish^{-1} h^{-1}. The four 30-min rates were averaged to yield a single value for each fish.

Scaling coefficients for diffusive water flux, $\dot{M}O_2$, ammonia excretion and urea-N excretion rates, were calculated using the control data for fish within each salinity group (i.e. 30 ppt or 7.5 ppt) in parallel to the approach used by Onukwufor and Wood (2022) and Morris and Wood (2023) in Pacific sanddab. The size ranges for each experimental group were: 30 ppt natural DOCs 0.135–0.495 kg, 30 ppt model compounds 0.130–0.610 kg, 7.5 ppt natural DOCs 0.135–0.525 kg and 7.5 ppt model compounds 0.130–0.605 kg. The logarithms of the individual rates (expressed as units per fish per h) were plotted against the logarithms of the individual fish weights (Tables 2 and 3). For each statistically significant result (Tables 2 and 3), an allometric mass scaling coefficient was obtained by using the equation $Y = aM^b$ where Y is the rate of interest (units fish^{-1} h^{-1}), M is the body weight (kg) and b is the scaling coefficient. The allometric mass scaling coefficient was used to adjust the measured rates of each individual fish to that of a standard 0.25 kg of flatfish, and then divided by 0.25 kg to yield rates expressed per kg per h. In cases where no scaling coefficient was applied because the regressions were not significant (see Results), each individual rate expressed per fish per h was divided by weight in kg, to yield rates expressed per kg per h.

The nitrogen quotient (NQ) is the ratio of the total measured nitrogenous waste excretion ($\dot{M}_N$) (the sum of the ammonia-N excretion and the urea-N excretion) to the $\dot{M}O_2$. Therefore, the NQ was calculated as NQ= ($\dot{M}_N/\dot{M}O_2$) for each individual fish at the control salinity and in the experimental solutions. The unscaled rates were used for each measurement in the calculations. By metabolic theory (Van Den Thillart and Kesbeke 1978), an NQ of 0.27 means that 100% of aerobic metabolism is fueled by protein.

Statistical analyses

Data have been expressed as mean ± standard error (SEM; n) where n represents the number of animals sampled. All data passed normality tests (Shapiro-Wilk). In *Series 1*, *2*, *3*, and *4*, data that also passed the homogeneity of variance tests were analysed using one-way analysis of variance (ANOVA) with Tukey's post hoc test (multiple comparisons). If data did not pass the Brown-Forsythe test for homogeneity of variances, data were analysed using the Brown-Forsythe and Welch ANOVA test with Dunnett's T3 multiple comparison test. Differences in mean control rates at 30 ppt and 7.5 ppt for diffusive water flux, $\dot{M}O_2$, ammonia and urea-N excretion were assessed by unpaired Student's

t-tests. For TEP measurements, each fish was used as its own control, so experimental effects relative to the control measurements were evaluated by paired Student's t-tests. Differences in mean control TEPs at 30 ppt and 7.5 ppt were assessed by unpaired Student's t-tests.

All statistical analyses and data plots were done using GraphPad™ Prism 7 (GraphPad Software, San Diego, CA, USA). A significance level of $p < 0.05$ (i.e. two-tailed) was used throughout except for allometric scaling coefficients where $p < 0.10$ was employed to avoid a Type 2 error. We adopted this approach based on our prior prediction that positive allometric scaling effects would occur for the parameters of interest, based on earlier findings with this and other species, and therefore a one-tailed rather than a two tailed approach was used. In contrast, for physiological effects of the various experimental treatments, we made no prior prediction of the directionality of the effects, and therefore a two-tailed approach (i.e. lower alpha value) was employed.

Results

Measured DOC concentrations and the optical characterization of natural DOCs and model compounds at two salinities

Measured DOC concentrations approximated the nominal value of 10 mg C L^{-1}, ranging from about 8.3 to 12 mg C L^{-1}, relative to natural background levels of ~2 mg C L^{-1} in the 30 ppt and 7.5 ppt seawater solutions (Table 1). However, the measured concentrations of model compounds ranged from about 4.4 to 15.8 mg C L^{-1} (Table 1). The three optical characterization methods used here were designed to characterize natural dissolved organic matter consisting of humic acid-like, fulvic acid-like, and protein-like components. At both salinities the general trends were for higher FI (i.e. more autochthonous, less allochthonous origin), lower SAC_{340} (i.e. lower aromaticity), and higher MWI (i.e. lower mean molecular weight) in LO than in LM samples, though the effects of salinity were not consistent between the two sources.

Consistent with our earlier study (Morris et al. 2024) where PARAFAC was performed on these same DOC sources in freshwater, when they were tested in 30 ppt seawater (Fig. 1), fulvic acid-like components (69.8% for LM DOC and 75.3% for LO DOC dominated in both, humic-acid like components were intermediate (30.1% for LM DOC and 17.9% for LO DOC), and protein -like components were low (0% for LM DOC and 6.8% for LO DOC) (Fig. 2E). LM DOC exhibited a moderately higher humic acid-like, lower fulvic acid-like, and lower protein-like composition than LO DOC. For both sources, the effects of lower salinity (7.5 ppt) were minor decreases in the % humic-like and the % fulvic-like components, and corresponding increases in the % protein-like components. Most notably, for LM DOC, the protein-like component increased from 0% at 30 ppt to 5.6% at 7.5 ppt.

Although the model compounds were also examined using SAC_{340}, FI and MWI (Table 1), interpreting them using these methods alone may not be appropriate in some cases (e.g. negative absorbances). These tools appear to be better suited to natural DOCs.

Allometric scaling coefficients

The allometric regressions in the control fish were significant at $p < 0.10$ for diffusive water flux and ammonia excretion rates at both salinities in *Series 1*, *2*, *3*, and *4*, and for urea-N excretion at 7.5 ppt only in *Series 2* and *4*, as indicated in Table 2. Therefore, allometric scaling was applied in subsequent analyses for those groups. Allometric scaling was not applied for $\dot{M}O_2$ excretion in any series, or for urea-N excretion rate in *Series 1* and *3*.

Control values as a function of salinity

At 30 ppt, the control values were not significantly different (Supplementary Table S1) between *Series 1* (DOC studies) and *Series 3* (model compound studies). Similarly, at 7.5 ppt, there were no significant differences between the control values between *Series 2* (DOC studies) and *Series 4* (model compound studies).

When the flux data for the two control groups at each salinity were combined and compared, (*Series 1* and *3* at 30 ppt versus *Series 2* and *4* at 7.5 ppt), it was clear that exposure to the lower salinity had a marked effect on the control rates (Table 3). Control rates for diffusive water flux and ammonia excretion were 23% and 33% higher respectively at the lower salinity, and the nitrogen quotient was 36% higher at the lower salinity. However, there were no significant differences in control $\dot{M}O_2$ or control urea-N excretion rates between the two salinities. The negative TEP in 7.5 ppt was significantly different from the positive value in 30 ppt (Table 3).

The effects of DOCs and model compounds on diffusive water flux rates at two salinities

At 30 ppt, exposure to LM DOC resulted in a significant increase of 38% in diffusive water flux rate (Fig. 2A). The 29% increase associated with exposure to LO DOC was not significantly different from either the control or the response to LM DOC.

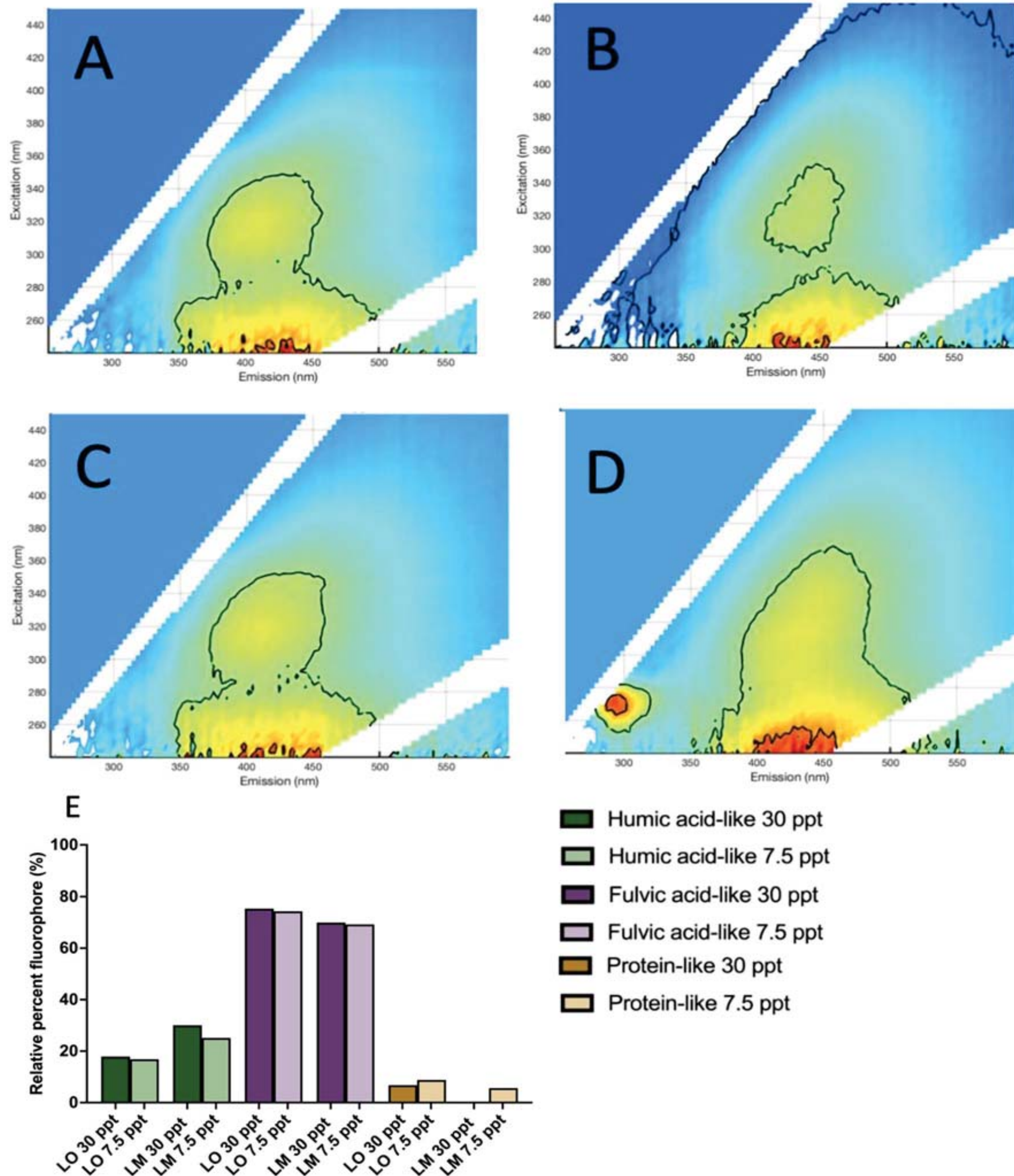

Fig. 1 Fluorescence excitation-emission matrices for natural DOCs at 30 ppt, (**A**) - Lake Ontario, (**B**) - Luther Marsh and at 7.5 ppt, (**C**) - Lake Ontario, (**D**) - Luther Marsh and (**E**) the relative % fluorophores of humic acid-like, fulvic acid-like and protein-like components for Lake Ontario (LO), and Luther Marsh (LM) at 30 ppt and 7.5 ppt

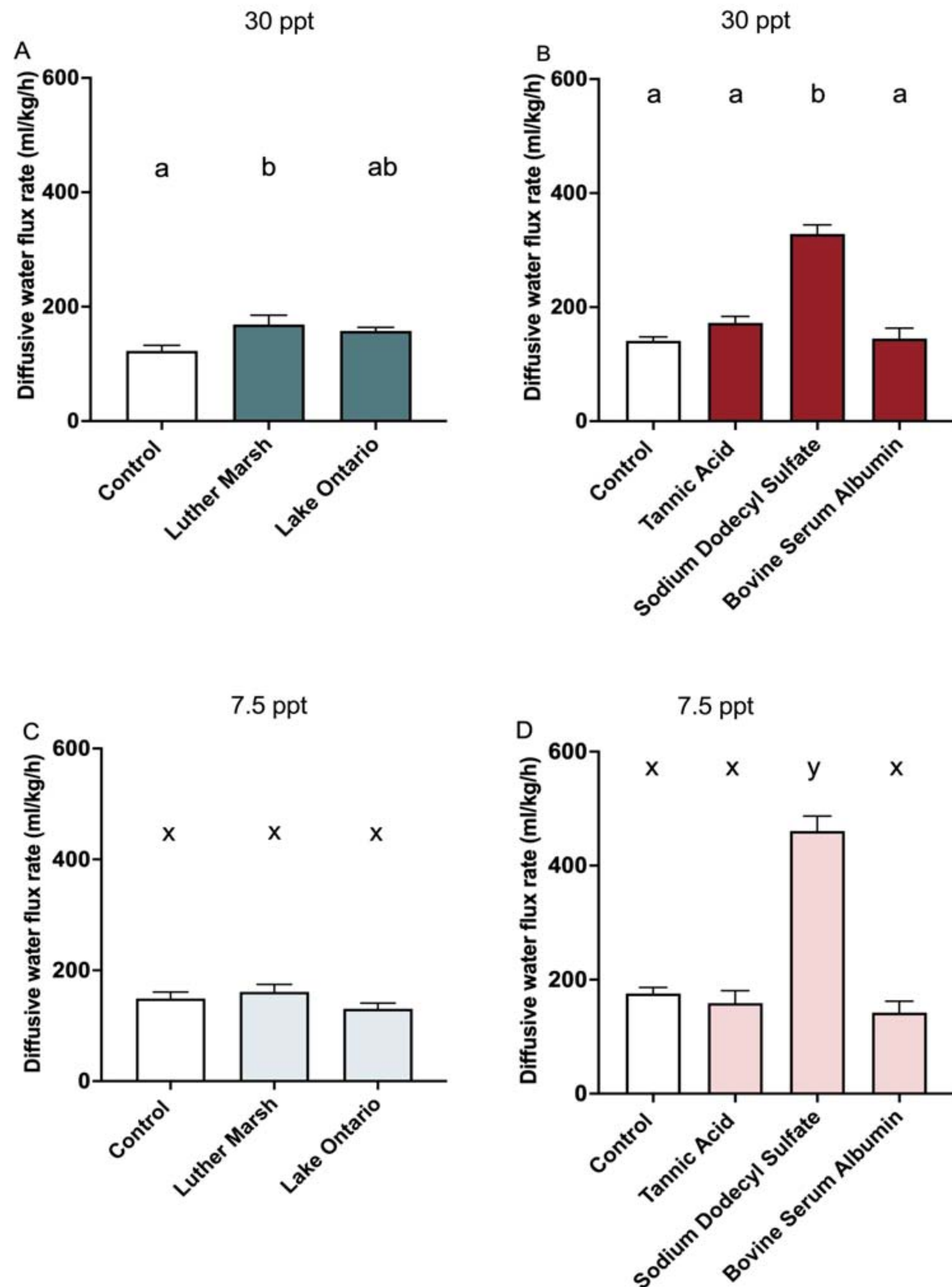

Fig. 2 The interactive effects on diffusive water flux rate (ml/kg/h) of (**A**) 30 ppt and natural DOCs (Luther Marsh and Lake Ontario), $n=6$ (*Series 1*), (**B**) 30 ppt and model compounds (tannic acid, sodium dodecyl sulfate and bovine serum albumin), $n=6$ (*Series 3*), (**C**) 7.5 ppt and natural DOCs (Luther Marsh and Lake Ontario), $n=6$ (*Series 2*), and (**D**) 7.5 ppt and model compounds (tannic acid, sodium dodecyl sulfate and bovine serum albumin), $n=5$–6, (*Series 4*) of Pacific sanddab (*Citharichthys sordidus*). Data are means ± SEM. Bars not sharing the same letters are significantly different ($p<0.05$) (analysis of variance with Tukey's test)

However, compared to the control condition, there was a large significant increase (133%) in diffusive water flux rate in response to SDS, whereas small elevations with TA and BSA were not significant (Fig. 2B).

At 7.5 ppt, there were no significant changes in diffusive water flux rates in response to the natural DOCs (Fig. 2C). However, as with 30 ppt, the acute transfer from the control solution to SDS at 7.5 ppt caused a large significant increase (162%) in diffusive water flux rate, whereas the other compounds were without effect (Fig. 2D).

The effects of DOCs and model compounds on $\dot{M}O_2$ at two salinities

There were no significant changes in $\dot{M}O_2$ in response to any of the natural DOCs or model compounds tested at 30 ppt or 7.5 ppt (Fig. 3A, B, C and D).

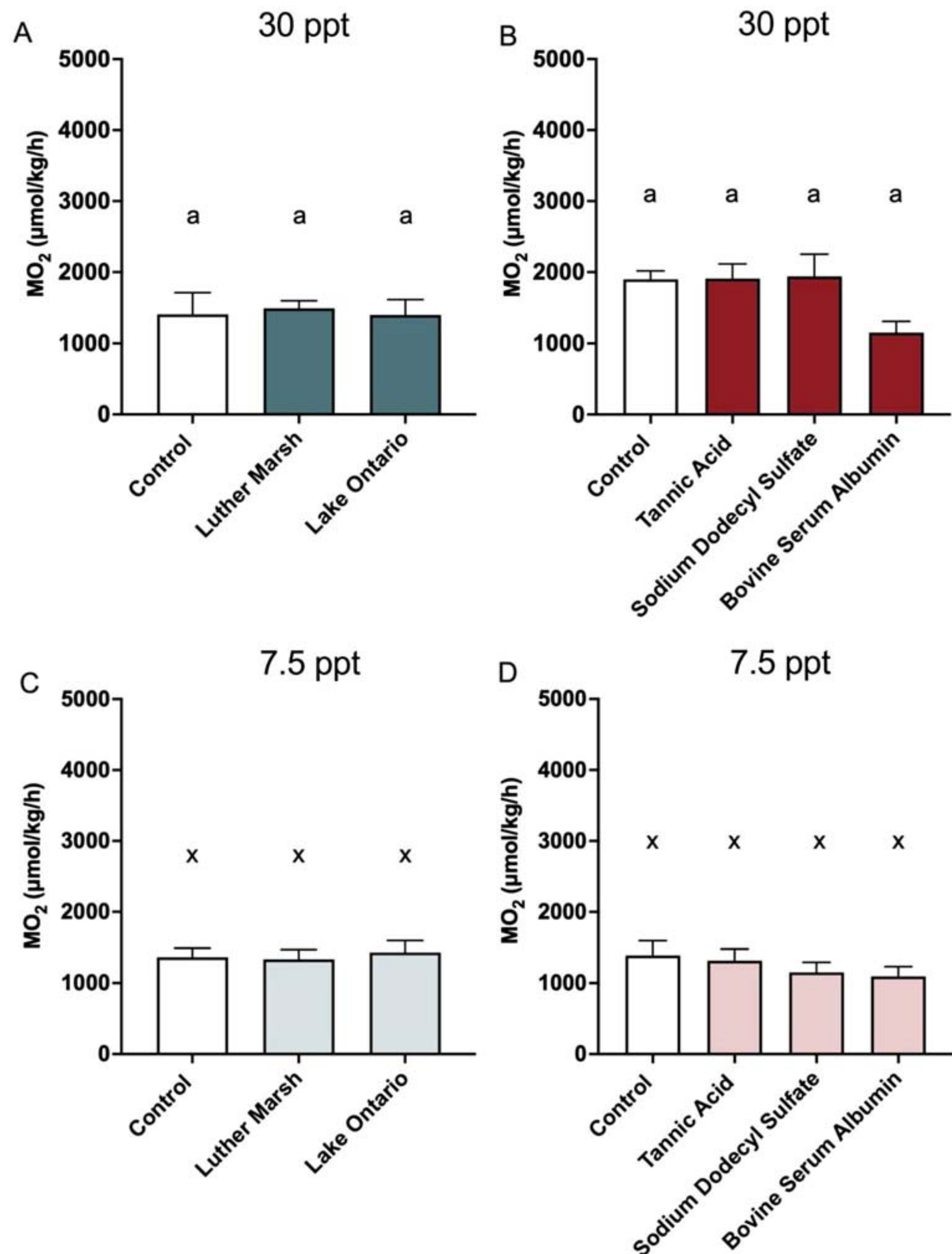

Fig. 3 The interactive effects on O_2 consumption rate ($\dot{M}O_2$, µmol/kg/h) of (**A**) 30 ppt and natural DOCs (Luther Marsh and Lake Ontario), $n=6$ (*Series 1*), (**B**) 30 ppt and model compounds (tannic acid, sodium dodecyl sulfate and bovine serum albumin), $n=6$ (*Series 3*), (**C**) 7.5 ppt natural DOCs (Luther Marsh and Lake Ontario), $n=6$ (*Series 2*), and (**D**) 7.5 ppt and model compounds (tannic acid, sodium dodecyl sulfate and bovine serum albumin), $n=5$–6, (*Series 4*), of Pacific sanddab (*Citharichthys sordidus*).). Data are means ± SEM. Bars not sharing the same letters are significantly different ($p<0.05$) (analysis of variance with Tukey's test)

The effects of DOCs and model compounds on ammonia and urea-N excretion rates at two different salinities

At 30 ppt, although not significantly different ($p=0.076$) from the control, there was a 40% increase in ammonia excretion rate in response to LO DOC (Fig. 4A), whereas exposure to LM DOC resulted in a slight but non-significant fall (23%, $p=0.4415$) in ammonia excretion rate. Therefore, there was a significant 85% difference between the rates in LM and LO DOC at 30 ppt (Fig. 4A). There were no significant differences in ammonia excretion rate in response to the model compounds compared to the control rate (Fig. 4B).

At 7.5 ppt, there were no significant differences in ammonia excretion rates in response to the natural DOCs (Fig. 4C). However, compared to the 7.5 ppt control, there was a significant 32% decrease in response to acute exposure to TA, similar to the non-significant fall seen at 30 ppt with TA, although the other model compounds were without effect at 7.5 ppt (Fig. 4D).

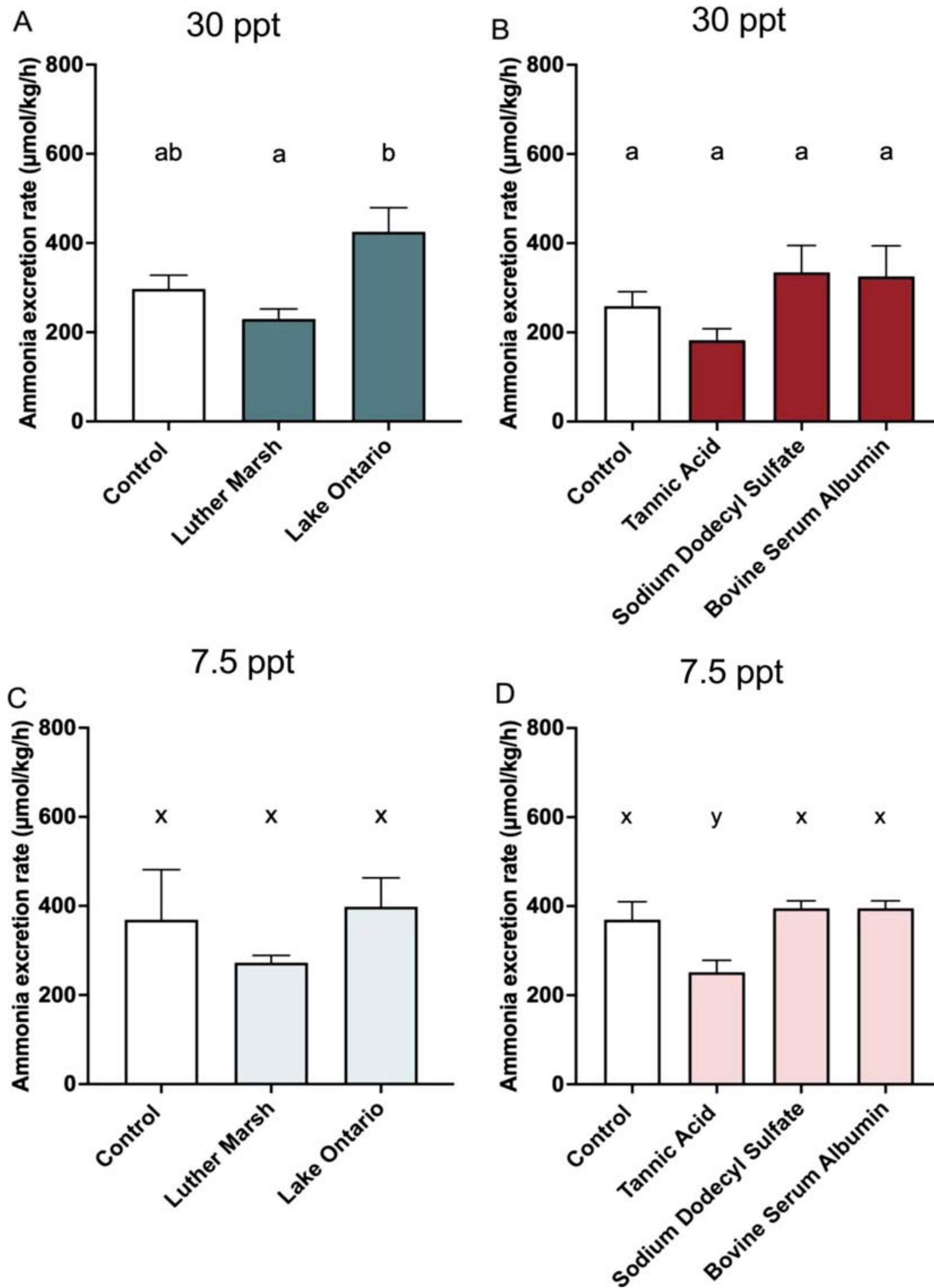

Fig. 4 The interactive effects on ammonia excretion rate (µmol/kg/h) of (**A**) 30 ppt and natural DOCs (Luther Marsh and Lake Ontario), $n = 6$ (*Series 1*) (**B**) 30 ppt and model compounds (tannic acid, sodium dodecyl sulfate and bovine serum albumin), $n = 6$ (*Series 3*) (**C**) 7.5 ppt natural DOCs (Luther Marsh and Lake Ontario), $n = 6$ (*Series 2*) (**D**) 7.5 ppt and model compounds (tannic acid, sodium dodecyl sulfate and bovine serum albumin), $n = 5$–6, (*Series 4*) of Pacific sanddab (*Citharichthys sordidus*). Data are means ± SEM. Bars not sharing the same letters are significantly different ($p < 0.05$) (analysis of variance with Tukey's test or Brown-Forsythe and Welch ANOVA test with Dunnett's T3 multiple comparison test)

At 30 ppt, there were significant decreases in urea-N excretion rates in response to both LM DOC (47%) and LO DOC (39%) (Fig. 5A). Exposure to BSA resulted in a significant increase in urea-N excretion rate by 119%, whereas the other model compounds were without effect (Fig. 5B).

At 7.5 ppt, there was a significant 61% decrease in urea-N excretion rate in response to LO DOC, whereas the smaller decrease (22%) associated with exposure to LM DOC was not significant relative to either the control or LO values (Fig. 5C). There were no significant changes in urea-N excretion rates in response to any of the model compounds tested (Fig. 5D).

The effects of DOCs and model compounds on nitrogen quotients at two different salinities

At 30 ppt, there were no significant differences in NQ relative to the control values in response to either of the natural DOCs. However, the NQ tended to fall slightly for LM and rise slightly for LO, such that the NQ in the latter was significantly greater by 109% than for LM (Fig. 6A). However,

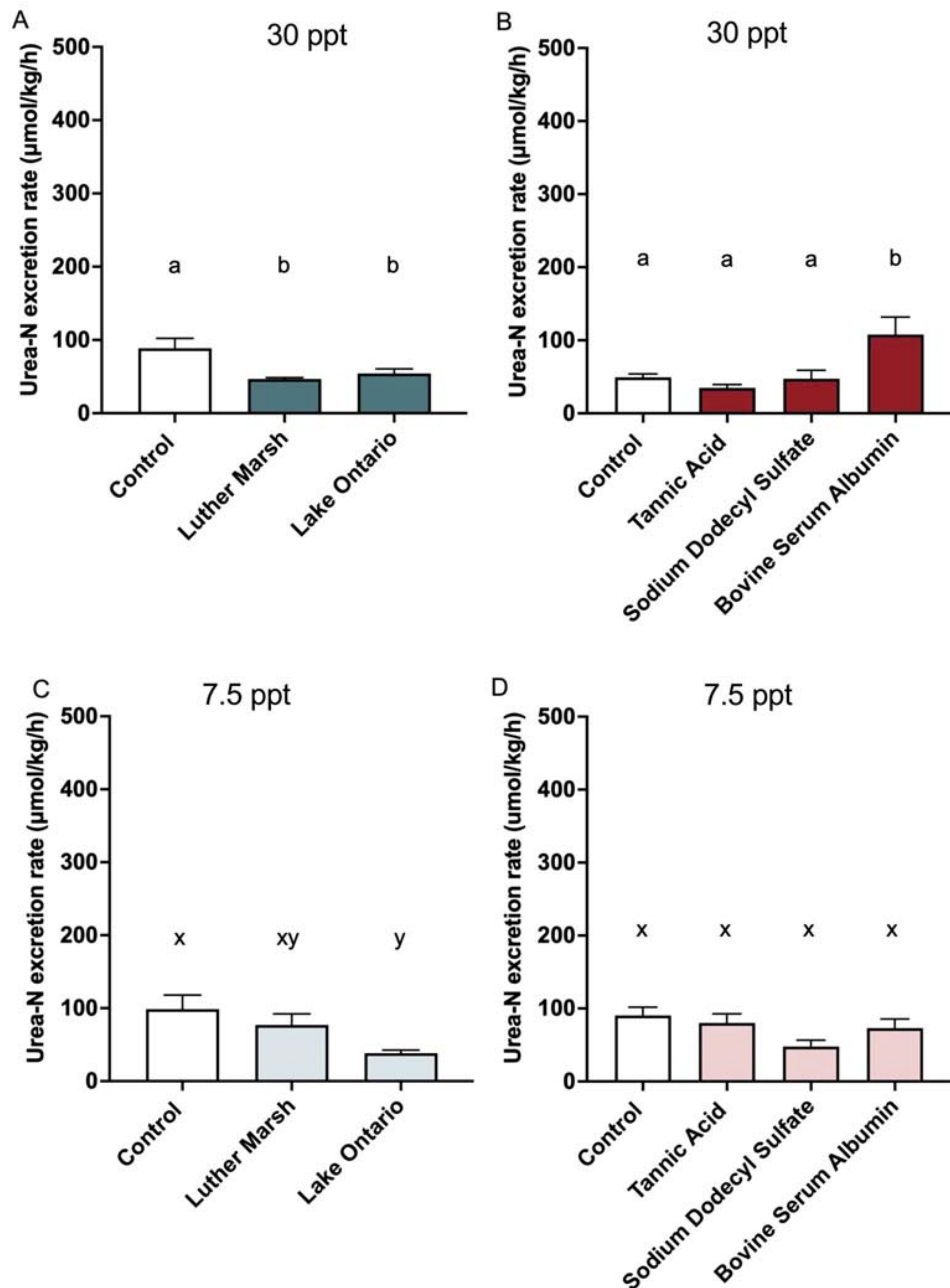

Fig. 5 The interactive effects on urea-N excretion rate (µmol/kg/h) of (**A**) 30 ppt and natural DOCs (Luther Marsh and Lake Ontario), $n = 6$ (*Series 1*), (**B**) 30 ppt and model compounds (tannic acid, sodium dodecyl sulfate and bovine serum albumin), $n = 6$ (*Series 3*), (**C**) 7.5 ppt natural DOCs (Luther Marsh and Lake Ontario), $n = 6$ (*Series 2*), and (**D**) 7.5 ppt and model compounds (tannic acid, sodium dodecyl sulfate and bovine serum albumin), $n = 5–6$, (*Series 4*) of Pacific sanddab (*Citharichthys sordidus*). Data are means ± SEM. Bars not sharing the same letters are significantly different ($p < 0.05$) (analysis of variance with Tukey's test)

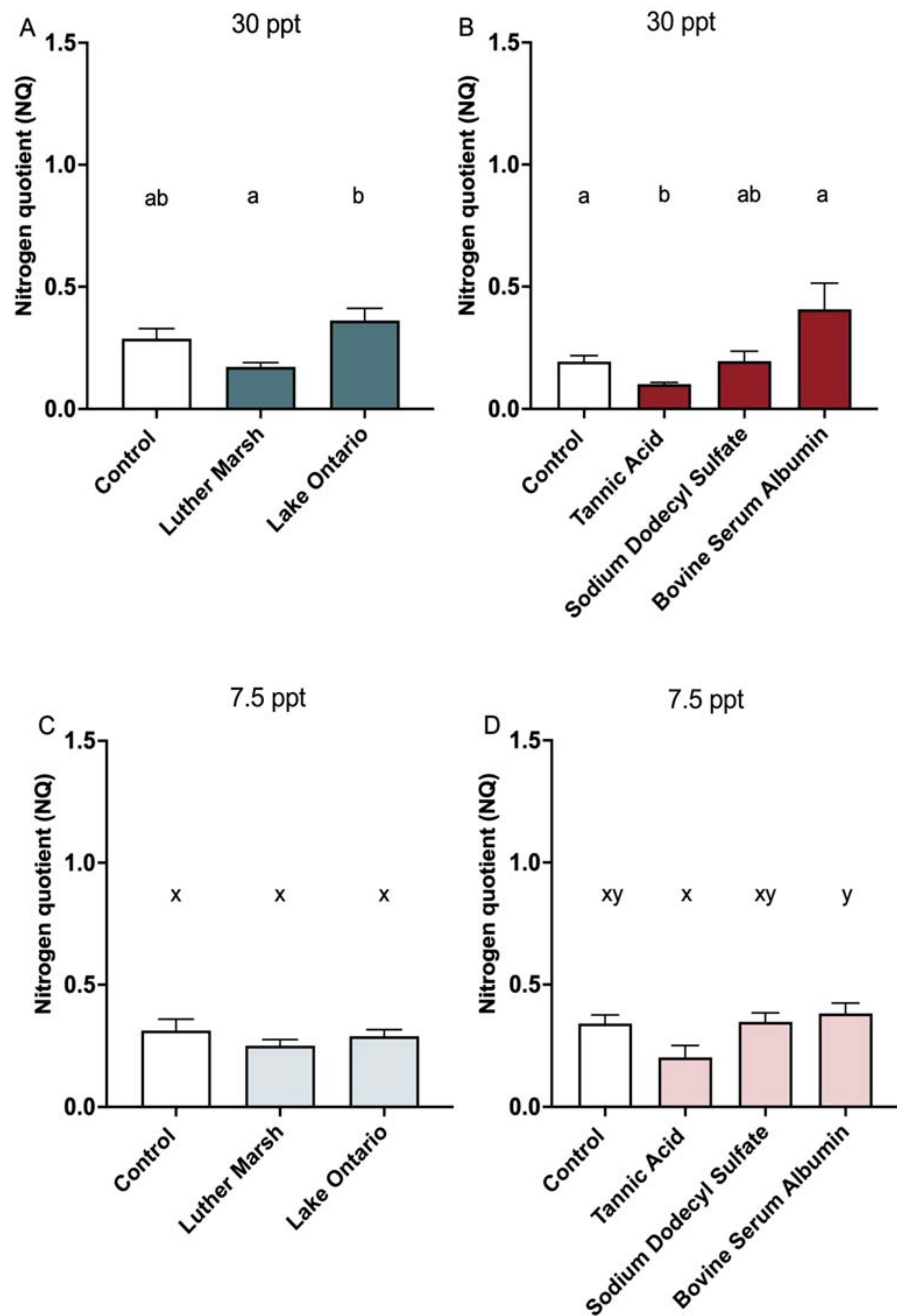

Fig. 6 The interactive effects on the nitrogen quotient (NQ) of (**A**) 30 ppt and natural DOCs (Luther Marsh and Lake Ontario), $n=6$ (*Series 1*), (**B**) 30 ppt and model compounds (tannic acid, sodium dodecyl sulfate and bovine serum albumin), $n=6$ (*Series 3*), (**C**) 7.5 ppt natural DOCs (Luther Marsh and Lake Ontario), $n=6$ (*Series 2*), and (**D**) 7.5 ppt and model compounds (tannic acid, sodium dodecyl sulfate and bovine serum albumin), $n=5$–6, (*Series 4*) of Pacific sanddab (*Citharichthys sordidus*). Data are means + SEM. Bars not sharing the same letters are significantly different ($p<0.05$) (analysis of variance with Tukey's test)

there was a significant fall (48%) with TA and a non-significant increase with BSA resulting in a significant difference (109%) between the lower value in TA and the 109% higher value in BSA (Fig. 6B). SDS had no effect.

At 7.5 ppt, the nitrogen quotient (NQ) was unaffected by exposure to either of the natural DOCs (Fig. 7C). However, it decreased by 48% in response to TA when compared to the 30 ppt control (Fig. 6B). At 7.5 ppt there were no significant changes in the NQ relative to the control value, although again there was a significant difference between the lower value in TA and the 87% higher value in BSA (Fig. 6D).

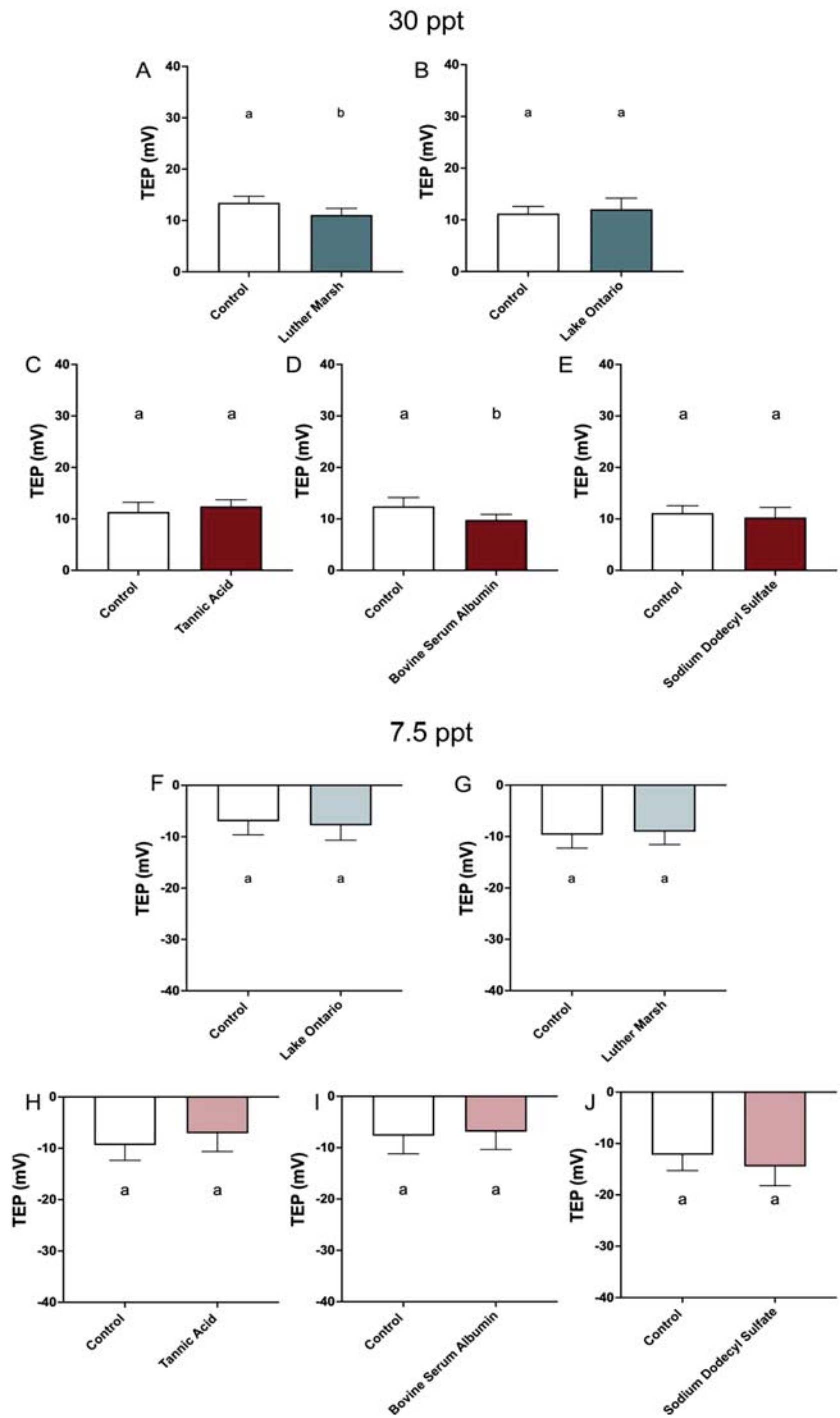

Fig. 7 The interactive effects on transepithelial potential (TEP) of (**A**, **B**) 30 ppt and natural DOCs (Luther Marsh and Lake Ontario), $n=9$ (**C**, **D**, **E**) 30 ppt and model compounds (tannic acid, sodium dodecyl sulfate and bovine serum albumin), $n=9$ (*Series 5*) (**F**, **G**) 7.5 ppt and natural DOCs (Luther Marsh and Lake Ontario), $n=5$, and (**H**, **I**, **J**) 7.5 ppt and model compounds (tannic acid, sodium dodecyl sulfate and bovine serum albumin), $n=5$, (*Series 6*) of Pacific sanddab (*Citharichthys sordidus*). Data are means ± SEM. Bars not sharing the same letters are significantly different ($p<0.05$) (Student's paired t-test)

The effects of DOCs and model compounds on TEPs at two different salinities

At 30 ppt, where TEP was positive, there were significant decreases in TEP in response to acute exposure to LM DOC and to BSA (Fig. 7A and D), whereas LO DOC (Fig. 7B), TA (Fig. 7C) and SDS (Fig. 7E) had no effects. At 7.5 ppt, where the TEP was negative, there were no significant changes in TEP in response to any of the experimental solutions (Fig. 7F, G, H, I and J).

Discussion

Overview

In accord with previous studies on this species (Onukwufor and Wood 2022; Morris and Wood 2023), an acute decrease in salinity resulted in increases in diffusive water flux rate, ammonia excretion rate, and NQ in the Pacific sanddab, responses that were not associated with changes in $\dot{M}O_2$ (Table 3). Furthermore, the TEP changed from a positive value in seawater to a negative value at low salinity, in accord with observations on many other teleost species reflecting the transition from a predominantly electrogenic potential in seawater to a diffusion potential in freshwater (Potts 1984; Wood and Grosell 2008). As explained in the Introduction, our goal was to examine the rapid physiological response that might occur when fish first enter dilute estuaries; in future studies it will be of interest to see whether these responses persist when sanddabs are resident for long periods in low salinity waters.

The fundamental physicochemical differences between LO and LM DOCs, as established in earlier freshwater studies (i.e. greater aromaticity, molecular weight, humic acid-like composition, and allochthonous character in the latter; Al-Reasi et al. 2012, 2013a, b, 2016; Duarte et al. 2018; Morris et al. 2024) were still seen when these sources were investigated in seawater. Our present results demonstrate that these DOCs, as well as model compounds, interact with the gill transport responses in a complex manner as a function of both salinity and source.

Originally, we hypothesized that (i) the effects of DOC would be greater at lower salinity; however, our data often showed greater effects at higher salinity, therefore, our first hypothesis was not supported. For example, there was a significant change in diffusive water flux rate in response to LM DOC at 30 ppt that was not present at 7.5 ppt (Fig. 2A and C). This was also the case for urea-N excretion rate for LM DOC and BSA (Fig. 5). Additionally, there were DOC effects on TEP at 30 ppt but not at 7.5 ppt (Fig. 7). (ii) The effects of LM DOC were indeed greater than those of LO DOC, supporting our second hypothesis. LM significantly affected diffusive water flux rate, urea-N excretion rate and TEP at 30 ppt (Figs. 2A, 5A and 7A). There were no significant effects of LM at 7.5 ppt, whereas LO only significantly affected urea-N excretion rate which was observed at both salinities (Fig. 5A and C). (iii) In accord with our third hypothesis, the TEP responses to LM and BSA were different at 30 ppt compared to 7.5 ppt with significant decreases at 30 ppt only (Fig. 7A, C, F and I). (iv) In support of our final hypothesis, the effects of the model compounds did help illuminate which specific properties of the natural DOCS were contributing to the observed responses, as outlined subsequently.

Optical indices of natural DOCs are a function of salinity

Although the DOC concentration for the control solutions and concentrations within the same DOC source were quite similar between salinities, there were differences in SAC_{340} and MWI (Table 1). Previous studies have shown that ionic strength and pH play important roles in the molecular conformation of DOCs (Chin and Gschwend 1991; Gennings et al. 2001; Morris et al. 2024) where for example, protonation results in condensation that limits light exposure, lowering absorption (Pace et al. 2012). It is likely that the change in absorption with salinity, reflected in the SAC_{340} and MWI values (Table 1), is due to the effects of ionic strength and pH combined on the molecular conformation of the DOCs. Additionally, high ionic strength could potentially alter the solubility of the DOC, thereby precipitating certain fractions (Tipping and Hurley, 1998; Kalbitz et al. 2000) in turn, altering the physicochemical characterization parameters. The characteristics of LO DOC and LM DOC are quite different, with the former being less aromatic, more autochthonous, and smaller in molecular size (Table 1). Therefore, it is perhaps not surprising that their optical responses to changes in salinity differ. This suggests that key chemical moieties of LO DOC or LM DOC that may interact at the gill at 30 ppt may no longer be accessible for interaction at 7.5 ppt due to changes in molecular conformation (e.g. condensation) which may result in an altered physiological response.

Diffusive water flux and ammonia excretion can be uncoupled from oxygen consumption, and independently regulated across salinities, in the presence and absence of DOC

Diffusive water flux is dominated by the transcellular pathway and aquaporin mediated flux and occurs through the entire gill surface area (Wood et al. 2019). The significant

increase in diffusive water rate with a decrease in salinity (Table 3) is in accord with previous studies on this (Onukwufor and Wood 2022; Morris and Wood 2023) and other species (Motais et al. 1969; Evans 1969). As with the earlier investigations on the Pacific sanddab, $\dot{M}O_2$ did not change with salinity, confirming that diffusive water flux can be uncoupled from O_2 uptake. To our knowledge, no previous studies have considered how this uncoupling may be influenced by DOC in marine fish. Given that there were significant effects of DOCs on diffusive water flux rate that differed with salinity (Fig. 2) but there were no DOC effects on $\dot{M}O_2$ (Fig. 3), we can conclude that DOCs are acting only on diffusive water flux, and therefore the uncoupling is not influenced by DOC.

Seawater fish have lower expression of aquaporins in the gill compared to freshwater fish which has been correlated with lower diffusive water flux rate, lower mRNA abundance, and lower protein abundance (Lignot et al. 2002; Cutler et al. 2007; Tipsmark et al. 2010; Jung et al. 2012; Madsen et al. 2015; Breves et al. 2016; Ruhr et al. 2020). Perhaps DOC increases diffusive water flux rate, particularly LM DOC at 30 ppt (Fig. 2), by direct effects on the function of aquaporin proteins. Altered mRNA expression associated with new protein synthesis seems less likely as we investigated acute (within 1 h) effects of DOC on diffusive water flux rate. Detectable changes in AQP3 mRNA expression and cellular protein distribution have been seen within 3 h of an experimental treatment (acute hypoxia), but were evaluated at a higher temperature (Ruhr et al. 2020). Decreasing the salinity from 30 ppt to 7.5 ppt results in a large decrease or even slight reversal of the trans-gill osmotic gradient. Another feasible explanation is remodeling mechanisms of the gill. For example, rapid changes in the morphological relationships between ionocytes and adjacent pavement cells (reviewed by Wood and Eom 2021) may cause immediate changes in exposure of gill ionocytes (Marshall and Nishioka 1980; Wood and Marshall 1994) which could be a response to low salinity. Further investigation is required.

Similarly, ammonia excretion can be uncoupled from MO_2, as ammonia excretion rates become greater at low salinity without a corresponding change in MO_2 (Table 3). By metabolic theory (Van Den Thillart and Kesbeke 1978), an NQ = 0.27 represents aerobic respiration being 100% fueled by protein. The first presented NQ values for wild caught marine flatfish indicated that 83–86% of aerobic respiration is fueled by protein at 30 ppt under steady-state conditions (Morris et al. 2023). In agreement, our combined controls at 30 ppt exhibited an NQ of 0.241, suggesting that 89% of aerobic respiration is fueled by protein (Fig. 6; Table 3). However, the increase in ammonia excretion at unchanged MO_2 in fish acutely transferred to 7.5 ppt resulted in a significant rise in NQ to 0.327 (Table 3). This was not quite significantly above the theoretical maximum of 0.27 ($p = 0.067$, by 1-sample-t-test). While classically this would be representative of an increase in aerobic protein metabolism (Van Den Thillart and Kesbeke 1978), in our acute exposure study this was more likely explained by an improved gradient for ammonia excretion due to the lower water pH at lower salinity (Table 1), creating a non-steady state condition to transiently increase ammonia efflux across the gills.

The shared physiological responses between natural DOCs and model compounds as a function of salinity are likely due to key chemical moieties

Natural DOCs are heterogenous, complex molecules that have several key chemical groups or moieties. Therefore, we used three model compounds that are rich in particular moieties and/or have the DOC characteristic of interest to illuminate the mechanisms of DOC effects. At 30 ppt, exposure to both LM DOC and SDS resulted in an increase in diffusive water flux rate (Fig. 2A and B). This may be due to the shared aliphatic chains, contributing to the surfactant property of both molecules, and possibly altering aquaporin function. Salinity may alter the surfactant property of natural DOC as the elevated diffusive water flux rate was not present with LM DOC at 7.5 ppt yet persisted with SDS. At 7.5 ppt, LM DOC may have undergone a change in molecular conformation. For example, molecular condensation with the change in ionic strength may have rendered the aliphatic chain no longer accessible to interact with the gill surface, thereby altering the physiological response.

The consistent, though not always significant reductions in ammonia excretion in response to both LM DOC and TA (Fig. 4) could be physiologically important. LM DOC, compared to LO DOC is more aromatic (higher SAC_{340} value, Table 1) and thereby similar to the poly phenolic, aromatic model compound, TA. The ammonia-transporting function of the Rhesus (Rh) glycoproteins organized into a metabolon has been described in both freshwater and marine fish (Nakada et al. 2007; Nawata et al. 2007, 2010; Wright and Wood 2009). The already complex mechanism(s) of ammonia excretion in freshwater fish is certainly influenced by low ambient pH and elevated DOC concentrations (Morris et al. 2021). It is possible that here, the phenolic groups of both LM DOC and TA are influencing Rh proteins, so as to reduce ammonia excretion rates.

LO is an autochthonous DOC with generally higher amino acid and peptide content when compared to allochthonous DOCs such as LM (Thurman 1985). This is reflected in the higher relative % protein concentration in LO DOC than LM DOC seen in our PARAFAC data (Fig. 1E). BSA

is a relatively large tyrosine and peptide rich molecule, so it is more similar to LO DOC. At 30 ppt, there were significant decreases in urea-N excretion rate in response to both natural sources of DOCs, but at 7.5 ppt the effect remained significant for LO only (Fig. 5A and C). At 30 ppt, there was a significant increase in urea-N excretion rate in response to BSA (Fig. 5B). Possibly, amino acid-rich DOCs interact with UT, the facilitated diffusion transport responsible for branchial urea excretion in fish (McDonald et al. 2006).

The reductions in TEP in response to both LM DOC and BSA at 30 ppt were the only significant DOC effects; none occurred at 7.5 ppt (Fig. 7). The origin and polarity of TEP are different at the two tested salinities, as discussed earlier. Therefore, the significant responses could be an influence on the inwardly directed Na^+ diffusion potential and/or the electrogenic extrusion of Cl^-, both of which contribute to the positive (inside) TEP that is present in marine fish at 30 ppt (Potts 1984; Wood and Grosell 2008). Given that effects were only observed at 30 ppt and not at 7.5 ppt where the Cl^- extrusion pump would be inactive, it was likely an effect on the latter. The chemistry of BSA is more similar to LO DOC than LM DOC with the shared key chemical moiety being peptides, however, LM DOC is more aromatic than LO DOC and BSA does have many cyclic amino acid groups (i.e. tyrosine). Perhaps it is the common aromatic rings between BSA and LM DOC that are influencing TEP.

Additionally, DOC could indirectly affect the physicochemical environment leading to global changes in physiology. For example, DOC may act as a pH buffer in the external microenvironment of the gills (Wood et al. 2003; Al-Reasi et al. 2016; Morris et al., 2021), and possibly a change in salinity likewise influences the gill microenvironment. Given the charged chemical moieties on DOC molecules, DOC could also possibly influence the stability of the tight junctions or even the electrical properties of the entire gill epithelium (Campbell et al. 1997; Galvez et al. 2008), as seen with the changes of TEP discussed above.

Concluding remarks

Overall, our results show that the effect of natural DOCs on the osmoregulatory responses of the gill of marine flatfish are salinity-dependent. These maybe due to changes in both the physicochemical properties of the DOC, and changes in the gill physiology, as a function of salinity. Rapidly reduced salinity with increased DOC concentrations is an environmentally realistic challenge for Pacific sanddab, as outlined in the Introduction. Indeed, the experimental exposures used in this study can certainly occur in the natural environment (McNicol et al. 2023). Estuaries that receive inflow from a DOC-rich forested catchment or river would have a reduced salinity in the presence of elevated DOC concentrations (Maxey et al. 2020). To complete our mechanistic study, exposures of DOC in the presence of full-strength seawater was integral, although perhaps less common in the natural environment. Therefore, it is important to determine not only the effects of DOC as a function of salinity but also to identify which key chemical moieties of DOC could be playing a role to extrapolate our findings to the great diversities of DOCs that exist in different systems. We have described shared physiological responses between natural DOCs and model compounds and can conclude that key chemical groups of DOCs, including aliphatic chains, phenolic groups and peptides likely play a role in gill function in response to DOCs across salinities in the Pacific sanddab.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00360-024-01580-2.

Acknowledgements We thank the Research Coordinator, Tao Eastham, and support staff at Bamfield Marine Sciences Centre for their help that facilitated this research. We also thank two anonymous reviewers whose comments improved the paper. CM was supported by an NSERC CGS-D scholarship. Supported by an NSERC Discovery Grants (RGPIN-2023-03714) to CMW and (RGPIN-2020-04826) to DSS.

Data availability Data are available upon request.

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