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RESEARCH ARTICLE

The influence of dissolved organic matter (DOM) on sodium regulation and nitrogenous waste excretion in the zebrafish (*Danio rerio*)

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ABSTRACT

Dissolved organic matter (DOM) is both ubiquitous and diverse in composition in natural waters, but its effects on the branchial physiology of aquatic organisms have received little attention relative to other variables (e.g. pH, hardness, salinity, alkalinity). Here, we investigated the effects of four chemically distinct DOM isolates (three natural, one commercial, ranging from autochthonous to highly allochthonous, all at ~6 mg C l^{-1}) on the physiology of gill ionoregulation and nitrogenous waste excretion in zebrafish acclimated to either circumneutral (7.0–8.0) or acidic pH (5.0). Overall, lower pH tended to increase net branchial ammonia excretion, net K^+ loss and [^{3}H]PEG-4000 clearance rates (indicators of transcellular and paracellular permeability, respectively). However, unidirectional Na^+ efflux, urea excretion and drinking rates were unaffected. DOM sources tended to stimulate unidirectional Na^+ influx rate and exerted subtle effects on the concentration-dependent kinetics of Na^+ uptake, increasing maximum transport capacity. All DOM sources reduced passive Na^+ efflux rates regardless of pH, but exerted negligible effects on nitrogenous waste excretion, drinking rate, net K^+ loss or [^{3}H]PEG-4000 clearance, so the mechanism of Na^+ loss reduction remains unclear. Overall, these actions appear beneficial to ionoregulatory homeostasis in zebrafish, and some may be related to physico-chemical properties of the DOM sources. They are very different from those seen in a recent parallel study on *Daphnia magna* using the same DOM isolates, indicating that DOM actions may be both species and DOM specific.

KEY WORDS: DOC, Na^+ fluxes, K^+ fluxes, Ammonia fluxes, Urea fluxes, Drinking rate

INTRODUCTION

Freshwater fish maintain constant homeostasis by active uptake of Na^+, Cl^- and other ions, thereby counterbalancing diffusive losses to the surrounding dilute water (Marshall, 2002; Evans et al., 2005). Direct excretion of nitrogenous wastes, mostly as ammonia and dominantly via gills, into the surrounding water is a well-established physiological process (Wood, 1993). The details of this process have been largely demonstrated in laboratory settings using 'tapwater'. Investigations have typically overlooked the potential influence of real-world water parameters, such as dissolved organic matter (DOM), which occur in natural freshwater. Indeed, the potential impact of DOM on ammonia excretion and Na^+ transport has received little attention compared with other natural water chemistry factors such as pH, hardness and salinity.

DOM is ubiquitous in all natural waters but varies greatly in composition and concentration. Freshwater DOM comprises heterogeneous mixtures of humic and fulvic acid molecules plus a range of other organic substances, with ≥50% of their mass as carbon, and thus are usually quantified as dissolved organic carbon (DOC) concentration (Thurman, 1985). According to McKnight et al. (2001), sources of DOM can broadly be classified into allochthonous (i.e. highly aromatic DOM that has originated from terrigenous sources) and autochthonous (i.e. only weakly aromatic, nitrogen-rich DOM that has been synthesized in the water column as a result of photosynthesis and/or decomposition of allochthonous DOM).

Studies have documented that DOM can directly influence the physiology of aquatic organisms (reviewed by Wood et al., 2011). For example, DOM molecules have been shown to accumulate on biological membranes and affect their permeability (Campbell et al., 1997; Vigneault et al., 2000), as well as alter the transepithelial potential across fish gills (Galvez et al., 2009). In the presence of DOM, aquatic organisms have been observed to survive and grow better, particularly in acidic water with low ionic content (Hargeby and Petersen, 1988; Barth and Wilson, 2010). DOM molecules also protect against ionoregulatory disturbance when organisms are exposed to acidic water or metals (Wood et al., 2003; Matsuo et al., 2004). A recent study has shown that highly allochthonous DOM from the Amazonian Rio Negro protects zebrafish against disturbances in ionoregulation and ammonia excretion at extremely low pH (Duarte et al., 2016). While some direct effects of DOM appeared to be more pronounced at low pH (Hargeby and Petersen, 1988; Campbell et al., 1997; Vigneault et al., 2000), interactions with aquatic organisms have also been observed at circumneutral pH, including beneficial effects on ionoregulation, particularly by allochthonous DOM sources (Matsuo et al., 2004; Glover et al., 2005; Glover and Wood, 2005a; Galvez et al., 2009). It therefore seems possible that DOM molecules may interact directly with epithelial ion transporters, yet it is not clear how DOM mediates the actions mechanistically.

To address this issue, the present study examined the actions of several natural DOM sources and one commercially available DOM source on Na^+ homeostasis and nitrogenous waste excretion of a model freshwater teleost, the zebrafish, under two environmentally realistic pH conditions (circumneutral pH of 7–8 and moderately acidic pH of ~5). As a follow-up, indicators of paracellular

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List of symbols and abbreviations

AHA	Aldrich humic acid
BL	Bannister Lake
DOC	dissolved organic carbon
DOM	dissolved organic matter
HRC	H^+-ATPase-rich cell
J_{in}	influx rate
J_{max}	maximal Na^+ uptake
J_{out}	efflux rate
K_m	binding affinity of transport sites
LO	Lake Ontario
LM	Luther Marsh
m	fish mass
MS-222	tricaine methanesulfonate
PBI	proton binding index
[^{3}H] PEG-4000	tritium-labelled polyethylene glycol, molecular weight 4000
SA	specific activity
t	exposure time
β	buffer capacity

permeability [branchial uptake of polyethylene glycol MW 4000 (PEG-4000); Robertson and Wood, 2014] and transcellular permeability (net K^+ loss rates; Lauren and McDonald, 1985) were evaluated to highlight possible mechanisms of DOM action on epithelial Na^+ fluxes. Our specific hypotheses were that the presence of DOMs would induce beneficial effects for Na^+ transport and ammonia excretion, that these would become more marked at low pH, and that these would differ among DOMs with different origins and physico-chemical characteristics. Table 1 summarizes these characteristics for the four DOM sources tested, showing that they varied from highly autochthonous with weak aromaticity (Lake Ontario) to highly terrigenous with strong aromaticity (Luther Marsh, Aldrich Humic Acid). Note however that Bannister Lake, with more intermediate optical characteristics, had the highest buffer capacity.

MATERIALS AND METHODS

Test organisms

Adult zebrafish [*Danio rerio* (Hamilton 1822)] from a local supplier (Mississauga, ON, Canada) were held in 40-litre aquaria in aerated, dechlorinated Hamilton tap water (moderately hard water from Lake Ontario; Table S1) at 26–27°C on a 12 h:12 h light:dark photoperiod, and fed Nutrafin Max fish flakes (Rolf C. Hagen, Montreal, QC, Canada) two times per day. Water was re-circulated through Aqua Clear 300 aquarium filters with activated carbon, foam and bio-max inserts (Rolf C. Hagen). Prior to acclimation to low pH or other experimentation, zebrafish were acclimated to this water for at least 2 weeks. All experiments were carried out during daytime and fish were fasted for 2 days before running the exposures. Procedures were approved by the McMaster University Animal Research Ethics Board (AUP 09-04-10) and conformed to the guidelines of the Canadian Council on Animal Care.

Acclimation to low pH

A week before experimentation at acidic pH (~5), zebrafish were acclimated to low pH. A 20-litre glass aquarium was used to accommodate 30 fish and water pH was kept within 4.90–5.10 using a pH meter-titrator system (PHM82 pH meter and TTT80 titrator, Radiometer, Copenhagen, Denmark), which controlled discharge of 0.25 N H_2SO_4 (ACS grade, Caledon Laboratories, Georgetown, ON, Canada) to maintain the water at or slightly below the pH endpoint set by the titrator. The pH of the aquarium water was checked and adjusted using an SP70 portable pH meter with a Ag/AgCl pH electrode (VWR sympHony, VWR International, Beverly, MA, USA), and approximately one-quarter of the water was replaced each day with fresh dechlorinated water pre-adjusted to pH 5.

DOM solutions

Three different DOM isolates (Table 1) were obtained by reverse osmosis from three sites in Ontario, Canada: Lake Ontario (LO), Bannister Lake (BL) and Luther Marsh (LM). Al-Reasi et al. (2012) provides details on collection, GPS coordinates of the sites and treatment of these DOM sources. A commercially available DOM, Aldrich Humic Acid (AHA, Sigma-Aldrich Chemical, St Louis, MO, USA), was included for comparison (Table 1). Additional physico-chemical characteristics of these DOM sources are presented elsewhere (Al-Reasi et al., 2012, 2013a,b). All DOM solutions were prepared at nominal DOC concentrations of 6 mg C l^{-1}, using dechlorinated water at the appropriate pH, which was also utilized as a control (no added DOM).

All experiments, except the kinetic trials, were conducted in Hamilton dechlorinated tap water, with or without DOM and other ion additions. For Na^+ influx kinetic experiments, Na^+-free artificial freshwater was prepared as described below. Because the addition of the LO isolate in particular augmented the levels of Na^+ and Ca^{2+} ions, all exposure solutions including the control were tested prior to experimentation for Ca^{2+} and Na^+ concentrations, and appropriate quantities of $CaCO_3$ (Sigma-Aldrich) and NaCl (Caledon Laboratories) were added to match the DOM-amended experimental solutions. Ca^{2+} in particular is known to play a role in regulating membrane permeability (McDonald and Rogano, 1986) and affecting Na^+ uptake in a concentration-dependent manner (Glover and Wood, 2005b). Because of the low solubility of

Table 1. Characteristics of dissolved organic matter (DOM) isolates and humic substances used in the experiments to examine sodium regulation and nitrogenous waste excretion in the zebrafish (*Danio rerio*)

			Buffer capacity, β (μmol pH^{-1} l^{-1})		
DOM source	SAC_{340} (cm^2 mg^{-1})	Type (FI)	pH~5.0	pH≥7	PBI
Lake Ontario	4.85±0.10	Autochthonous (2.54)	8.60±0.32	3.86±0.35	0.20±0.04
Bannister Lake	14.16±0.07	Autochthonous (1.51)	22.84±6.16	8.90±1.69	0.30±0.08
Luther Marsh	39.30±0.37	Terrigenous (1.19)	9.71±0.85	6.25±0.44	0.44±0.05
Aldrich humic acid	79.98±0.96	Coal-derived (0.83)	5.21±0.40	5.21±0.20	0.32±0.04

Specific absorbance coefficient (SAC_{340}) was used as a proxy for aromaticity of the DOM source. Designation of the DOM isolates as allochthonous (terrigenous) or autochthonous is based on the fluorescence index (FI=emission intensity of 450 nm/emission intensity of 500 nm, both taken at excitation at 370 nm) (McKnight et al., 2001) and is shown in brackets. SAC_{340} and FI values are from Al-Reasi et al. (2012). Buffer capacities and proton binding indices (PBI; unitless) were calculated from titrations of DOM isolates (Al-Reasi et al., 2013a,b).

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$CaCO_3$, all solutions were aerated overnight with pure CO_2 (Air Liquide, Burlington, ON, Canada). Excess CO_2 was removed by bubbling the solutions with air for 24 h starting the next day. Then, adjustment of solutions to the chosen pH (7–8 or ~5) was performed using dilute H_2SO_4 or/and KOH (ACS grade, Caledon Laboratories) approximately 16–20 h before exposure.

Unidirectional Na^+ influx kinetics

The concentration-dependent kinetics of the unidirectional Na^+ influx rate were determined in synthetic Na^+-free water with the average composition of other ions designed to simulate the dechlorinated tap water used for acclimation (Table S1). The Na^+-free water was prepared using $CaCO_3$ and $4MgCO_3{\cdot}Mg(OH)_2{\cdot}4H_2O$ (analytical grade, Mallinckrodt, Dublin, Ireland) added to deionized water (≥17.5 MΩ cm; Barnstead Nanopure II, Thermo Scientific, Barnstead, NH, USA) based on the recipe of Goss and Wood (1990). The solution was then aerated using CO_2, followed by air, as mentioned above. The measured chemistry of dechlorinated tap water and the artificial Na^+-free water is listed in Table S1. DOM was added to the artificial water from BL, LM and AHA concentrates at a nominal DOC concentration of 6 mg C l^{-1}. Because the LO isolate contained an elevated centration of Na^+ ions, it was excluded from the kinetics experiments because the lowest [Na^+] point of ~75 µmol l^{-1} could not be achieved when this DOM was added.

All kinetic exposures were run in shielded 60-ml plastic containers served with an aeration line. Six fish (mean weights in Table S2) were transferred individually into each of the nominal Na^+ concentrations of 75, 150, 300, 600, 1200 and 2400 µmol l^{-1} (as NaCl). The pH (7–8 or ~5) was adjusted throughout the exposure. After a recovery period (30 min) from handling, the radiotracer $^{22}Na^+$ as $^{22}NaCl$ (Eckert & Ziegler, Valencia, CA, USA) was added in constant proportion to the nominal [Na^+] in order to keep relatively constant specific activities (SA) for all solutions. In both this and the next experimental series, the external SA was kept at least 10-fold greater than internal SA throughout the exposure, so that backflux correction was not necessary (Maetz, 1956; Kirschner, 1970). In each 3-h period, Na^+ uptake was determined based on $^{22}Na^+$ incorporation by fish. One millilitre of water was sampled at the start and end of the experiment for determination of water [Na^+] and $^{22}Na^+$ radioactivity in counts per minute (cpm). At the end, fish were rinsed in a high 'cold displacement' solution (250 mmol l^{-1} NaCl) for 1 min to displace any surficially adsorbed $^{22}Na^+$, euthanized with an overdose of neutralized MS-222 (~1.0 g l^{-1} tricaine methanesulfonate, Syndel Laboratories, Vancouver, BC, Canada), rinsed in deionized water and blotted dry on Whatman No. 1 filter paper (G.E. Healthcare, Little Chalfont, UK). Fish were then transferred into pre-weighed plastic vials, weighed and assayed for gamma radioactivity. Additional water samples were obtained for ion and DOC measurements.

Unidirectional Na^+ influx rate

Steady-state measurements of unidirectional Na^+ influx were conducted in 60 ml of each solution with six individual fish for 3- or 6-h exposure periods; the water Na^+ concentration was 900–1000 µmol l^{-1} (Table S3). The pH (7–8 or ~5) was adjusted throughout the exposure. After 30-min recovery from handling, ~1.0 µCi of $^{22}Na^+$ was added, and a 10-min equilibration period followed before collection of the first 1-ml sample. Before termination of the exposure and processing of the fish as described above for Na^+ influx kinetic experiments, a second 1-ml sample was obtained. Additional water samples were collected for DOC and ionic analysis.

Unidirectional Na^+ efflux, and net K^+, ammonia and urea excretion rates

For 12–13 h, 15 fish were kept in 1.0 litre of aerated, dechlorinated water inoculated with 50–100 µCi of radioactive $^{22}Na^+$ at the appropriate pH. To remove $^{22}Na^+$ adsorbed to the surface of the organism, each fish was then rinsed in fresh dechlorinated water for 2 min and quickly rinsed in deionized water. The fish were then transferred individually into the 30-ml aerated exposure solutions (control, or with added DOM at the correct pH) containing 900–1000 µmol l^{-1} Na^+ (Table S4). The $^{22}Na^+$ efflux was monitored directly over 3 and 6 h. Throughout the exposure, pH (7–8 or ~5) was adjusted using dilute H_2SO_4 except in one treatment. Dilute KOH was used to adjust pH of the LO solutions in the circumneutral pH experiment only; therefore, K^+ fluxes were not determined in this treatment. At 10 min after introduction of the fish, four 1-ml water samples were obtained for initial measurements of $^{22}Na^+$ radioactivity, K^+, ammonia and urea concentrations; the latter two were frozen at −20°C until analysis. Subsequent water samples were taken at 3 and 6 h for the same analyses. At the end, additional samples were taken for DOC and ion concentrations. The fish were then quickly rinsed in fresh dechlorinated water to remove any surficial $^{22}Na^+$, euthanized as above and weighed individually in pre-weighed tubes.

For fish digestion, 1.8 ml of 1.0 N HNO_3 (trace metal grade, Fisher Scientific, Fairlawn, NJ, USA) was added to each fish, and then the sealed vial was heated for 48 h at ~65°C, with occasional vortexing to enhance digestion. After centrifugation (5000 ***g***, 10 min), known amounts of the supernatant were counted for $^{22}Na^+$ radioactivity, and then diluted 50-fold with 1% HNO_3 to determine the whole-body Na^+ content for calculation of the internal SA of Na^+.

Uptake of [^{3}H] PEG-4000

In parallel experiments, fish were exposed to radiolabeled polyethylene glycol (MW 4000) ([^{3}H] PEG-4000, American Radiolabeled Chemicals, St Louis, MO, USA) for 4.5 h in order to measure gill paracellular permeability and drinking rate (see Robertson and Wood, 2014). The water Na^+ concentration was 900–1000 µmol l^{-1} (Table S4). Fish were introduced into 60 ml of each solution at the appropriate pH, and allowed to settle for 30 min prior to addition of 10 µCi of [^{3}H] PEG-4000. Water samples (1 ml) were taken 10 min later and at the end of the experiment. The pH was adjusted throughout. Thereafter, fish were rinsed in fresh dechlorinated water followed by deionized water, euthanized in neutralized MS-222, and then dissected carefully to obtain the whole gastrointestinal tract or gut. The tract and the fish were placed in separate pre-weighed vials and weighed.

Each carcass was digested in 1.0 N HNO_3 as described above. Digestion of the gut was performed by adding 1 ml of 2 N HNO_3 for 48 h at ~65°C with vortexing. After centrifugation, supernatants (1.5 ml of fish digest or ~0.9 ml of tract digest) were mixed with 5× volume of Ultima Gold™ scintillation cocktail (PerkinElmer, Waltham, MA, USA) in glass scintillation vials. To measure the radioactivity in 1 ml of each water sample, 2 ml of Optiphase 'Hisafe' 3 scintillation cocktail (PerkinElmer) was added.

Chemical analyses

[^{3}H] PEG-4000 radioactivities of water samples and tissue digests (carcass or gastrointestinal tract) were quantified using a liquid scintillation analyzer (Tri–Carb 2900TR, PerkinElmer Life and

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Analytical Services, Downers Grove, IL, USA); the samples were incubated in the dark for at least 1 h prior to counting to minimize chemiluminescence. Each tissue sample was quench-corrected to the same counting efficiency as water samples by internal standardization. The $^{22}Na^{+}$ radioactivities of fish and water samples were determined by gamma counting using a Wizard 3″ 1480 automatic gamma counter (PerkinElmer, Woodbridge, ON, Canada). The concentrations of Na^{+} in zebrafish whole bodies and of Na^{+}, Ca^{2+}, Mg^{2+} and K^{+} in water were analyzed by flame atomic absorption spectrometry (SpectroAA220FS, Varian, Mulgrave, Australia). The total DOC concentrations of water samples were measured directly using a Shimadzu TOC-$V_{CPH/CPN}$ total organic carbon analyzer (Shimadzu Corporation, Kyoto, Japan). Total ammonia and urea concentrations in water were analyzed spectrophotometrically according to Verdouw et al. (1978) and Rahmatullah and Boyde (1980), respectively. Freshly prepared 6 mg l^{-1} solutions of each DOM were used to prepare blanks and standards for ammonia and urea to avoid background interference in the absorbance measurements. Microplates were scanned using a SpectraMAX 340pc microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Calculations and statistical analyses

In experiments measuring the concentration-dependent kinetics of unidirectional Na^{+} influx rate, lines were fitted to experimental data and kinetic parameters were determined graphically from the plots produced by SigmaPlot for Windows (Version 10.0, Systat, Point Richmond, CA, USA) according to the Michaelis–Menten equation (Wood, 1992):

$$J_{in} = \frac{J_{max} \times [Na^{+}]}{K_m + [Na^{+}]}, \quad (1)$$

where J_{in} is Na^{+} influx rate, J_{max} is maximum influx rate (i.e. an index of the number of sites available for Na^{+} uptake) and K_m is an index of the binding affinity of the sites for Na^{+} transport (equal to the $[Na^{+}]$ at which J_{in} is 50% J_{max}). As K_m magnitude increases, the affinity of the transporter decreases (Wood, 1992). See Fig. 1 legend for additional curve-fitting information.

The unidirectional Na^{+} influx rates (J_{in}) were calculated based on the amount of radioisotope $^{22}Na^{+}$ incorporated into the fish:

$$J_{in} = \frac{cpm}{SA_{ext} \times m \times t}, \quad (2)$$

where cpm is the counts per minute of each fish, SA_{ext} is the measured specific radioactivity of the exposure water (cpm μmol^{-1}), m is the fish mass (kg) and t is the time of exposure (h).

The unidirectional Na^{+} efflux rates (J_{out}) were calculated from the appearance of $^{22}Na^{+}$ in the exposure water:

$$J_{out} = \frac{(cpm_i - cpm_f) \times V}{SA_{int} \times m \times t}, \quad (3)$$

where cpm_i and cpm_f are initial and final counts per minute (l^{-1}) lost by the fish into the external water, V is the volume (l) of the exposure water, SA_{int} is the measured specific internal activity of the fish (cpm μmol^{-1}), and m and t are as above.

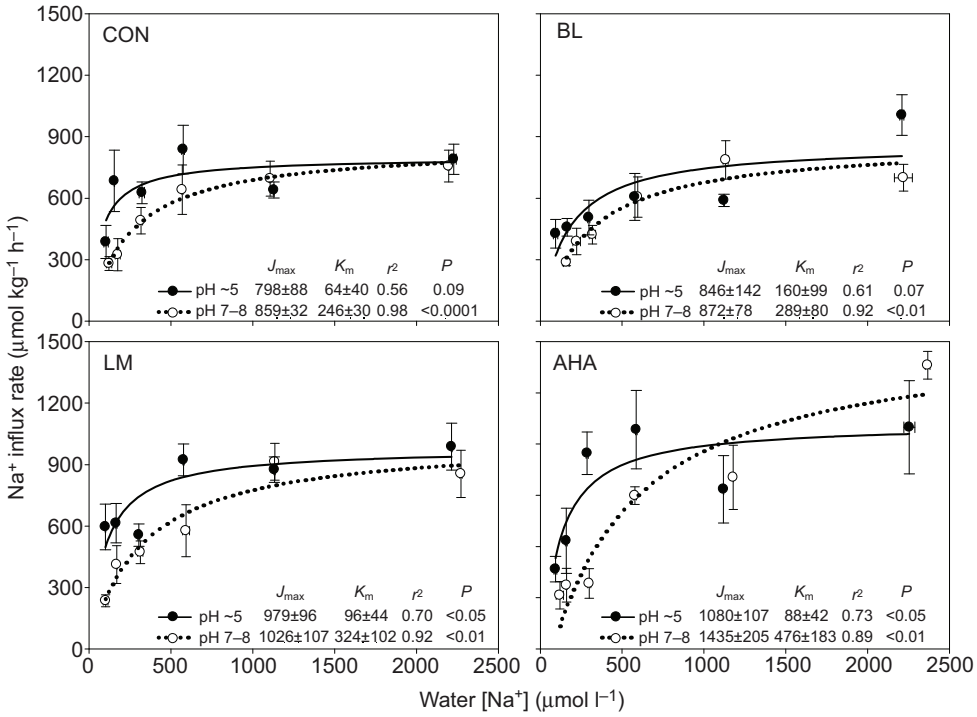

Fig. 1. The effect of three dissolved organic matter (DOM) sources added at 6 mg l^{-1} dissolved organic carbon (DOC) on the Michaelis–Menten transport kinetics parameters of Na^{+} influx in zebrafish. Experiments were carried out at circumneutral pH (7–8, dotted line with open symbols) and low pH (~5, solid line with closed symbols). Each point represents the mean±s.e. of n=6 fish (exception; n=5 fish for the BL treatment of pH 7–8 at the lowest Na^{+} concentration). The Michaelis–Menten relationships were fitted to the mean values, and the kinetics parameters [maximal Na^{+} transport rate (J_{max}, μmol kg^{-1} h^{-1}) and uptake affinity (K_m, μmol l^{-1})] were obtained directly from the output of SigmaPlot, and presented as means±s.e., together with r^2 and P-values for the relationships. Fits to the individual data points rather than the means yielded lower r^2 and P-values because of the higher sample size, but the same absolute J_{max} and K_m values. Note that for two data sets, BL at pH 5.0 and AHA at pH 7–8, a linear relationship would provide a better fit (higher r^2) than the Michaelis–Menten relationship, but as there is no theoretical reason to favour the former, the latter was applied to all data sets for consistency. CON, control; BL, Bannister Lake; LM, Luther Marsh; AHA, Aldrich humic acid.

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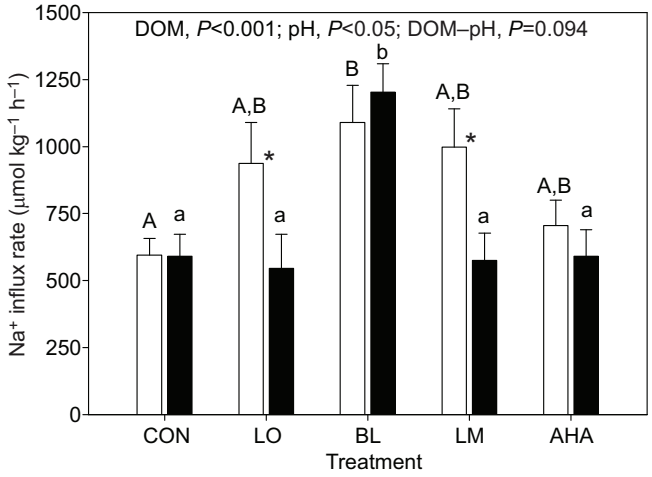

Fig. 2. Unidirectional Na^+ influx rates (μmol kg^{-1} h^{-1}) of zebrafish in the absence (CON, no added DOM) or presence of DOM sources added at 6 mg C l^{-1} at the circumneutral pH 7–8 (white bars) and low pH∼5 (black bars) in dechlorinated tap water with 900–1000 μmol l^{-1} Na^+. Plotted values represent the means±s.e. (n=6 fish, exception; n=5 fish for BL treatment of pH∼5) of Na^+ influx rates over a 6-h exposure period. The results of two-way ANOVA are shown. Within a pH, bars sharing the same letter (uppercase for pH 7–8, lowercase for pH∼5) are not significantly different. Asterisks indicate significant differences between pHs within the same DOM treatment. CON, control; LO, Lake Ontario; BL, Bannister Lake; LM, Luther Marsh; AHA, Aldrich humic acid.

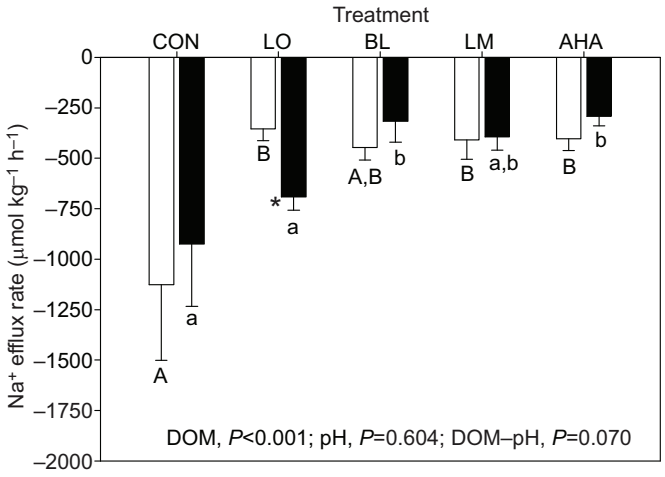

Fig. 3. Effects of DOM on the unidirectional Na^+ efflux rates (μmol kg^{-1} h^{-1}) of zebrafish at the circumneutral pH 7–8 (white bars) and low pH∼5 (black bars) over the 6-h exposure period, in dechlorinated tap water with 900–1000 μmol l^{-1} Na^+. Plotted values represent the means±s.e. of n=6 (exception: n=5 fish for LM treatment of pH∼5). The results of two-way ANOVA are shown. Within a pH, bars sharing the same letter (uppercase for pH 7–8, lowercase for pH∼5) are not significantly different. Asterisks indicate significant differences between pHs within the same DOM treatment.

The net flux rates of K^+, total ammonia and urea were calculated as the difference between the initial and final concentrations ($[solute]_i - [solute]_f$, in μmol l^{-1}), divided by the fish mass, m (kg), taking into account volume, V (l), and time, t (h), as follows:

$$J_{net} = \frac{([solute]_i - [solute]_f) \times V}{m \times t}. \quad (4)$$

All fluxes were expressed in μmol kg^{-1} h^{-1}. Although fluxes were measured over 0–3, 0–6 or 3–6 h periods, trends were similar. Therefore, for simplicity, only one set of data has been shown in Figs 2–5, though a few important differences between the different flux periods have been pointed out in the Results.

The branchial paracellular permeability was measured as the clearance rate of [^{3}H] PEG-4000 from the external water using the total radioactivity cpm in the carcass digest (i.e. excluding the gastrointestinal tract) factored by the water cpm of [^{3}H] PEG-4000 (cpm ml^{-1}), the total fish mass (kg) (i.e. the carcass plus the gastrointestinal tract) and time (h) (see Robertson and Wood, 2014). Similarly, the drinking rate was estimated from the cpm of the total tract digest divided by the total fish mass and time. The units for both clearance rate and drinking rate are ml kg^{-1} h^{-1}.

SigmaStat software for Windows (Version 3.5, Systat) was used to perform all statistical analyses. The software facilitated the use of one-way ANOVA to compare J_{max} and K_m values among treatments for the kinetics experiments based on input of the mean, sample size and standard error values for each treatment, as obtained from the output produced by SigmaPlot curve fitting (see above). Two-way ANOVA was employed to test for differences in influx and efflux rates within and between DOM treatments and pH conditions. Data were checked for normality and homogeneity of variance while performing ANOVA. In the case of violations, data were $\log_{10}$, reciprocal or rank transformed. The negative values for net efflux rates were multiplied by −1 (absolute values) before transformation. Multiple *post hoc* comparisons employed Tukey's test whenever significant differences were detected for the one-way and two-way ANOVAs. The effect of time on efflux rates was examined by paired Student's t-test. The significance of the coefficient of determination (r^2) was determined to evaluate linear regressions. All values have been reported as means±s.e. and differences were considered significant at P<0.05.

RESULTS

The influence of DOM on Na^+ influx kinetics

Under the specified water chemistry (Table S2), the concentration dependence of Na^+ uptake by zebrafish exhibited Michaelis–Menten saturation kinetics (Fig. 1; see legend for additional information). At circumneutral pH, there was a tendency for higher J_{max} (maximal Na^+ influx rate) and higher K_m values (i.e. lower Na^+ affinity) in the presence of all three DOM sources (BL, LM and AHA) relative to the control (nominally DOM-free water). One-way ANOVA revealed a significant overall effect of DOM on J_{max} ($F_{3,20}$=4.775, P<0.05) and the *post hoc* multiple comparisons showed that fish had significantly higher J_{max} in the presence of AHA relative to those in controls and BL. However, statistically similar K_m values were observed for all treatments at circumneutral pH ($F_{3,20}$=0.782, P=0.518). In the acidic pH, fish showed statistically similar J_{max} and K_m values ($F_{3,20}$=1.281, P=0.310 and $F_{3,20}$=0.445, P=0.723, respectively) in all treatments. There was a tendency for decreased J_{max} and K_m values at pH 5.0 in all treatments relative to those at circumneutral pH. Nevertheless, the only significant difference was a lower K_m value in the acidic control treatment (t=3.640, d.f.=10, P<0.01, t-test) relative to that at circumneutral pH. Note that at pH 5.0, the estimated K_m values (range=64–160 μmol l^{-1}) were close to the lowest water Na^+ level tested (75 μmol l^{-1}), so these values may be less reliable.

The influence of DOM on unidirectional Na^+ influx rates

Fig. 2 shows steady-state influx rates measured over 6 h at water Na^+ concentrations close to those in the acclimation water (i.e. ∼900–1000 μmol l^{-1}; Table S3), well above the K_m values shown in Fig. 1. Influx rates measured over 3 h exhibited similar trends (data not

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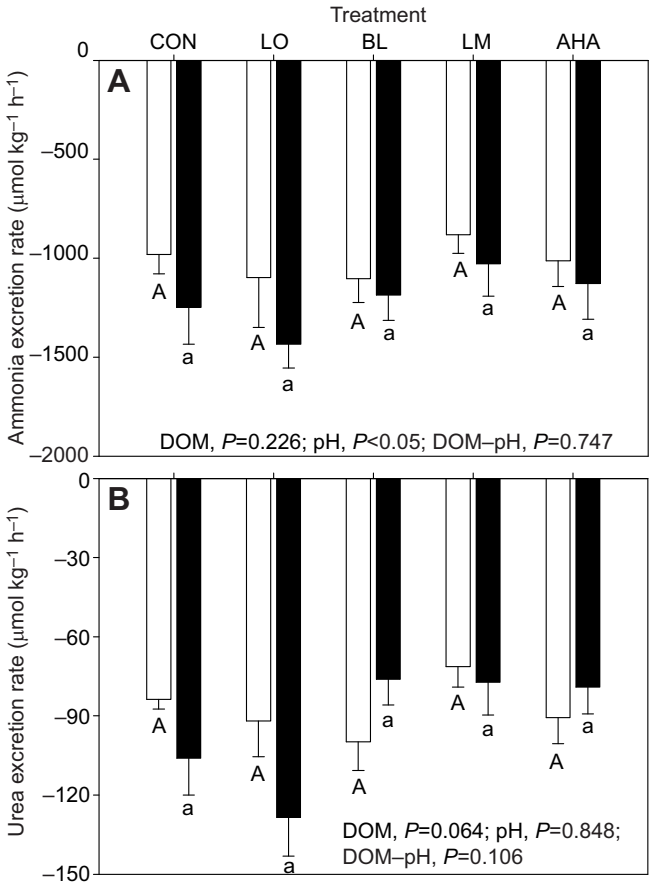

Fig. 4. The influence of different DOM sources on nitrogenous waste excretion by zebrafish. (A) Total ammonia excretion rates and (B) urea excretion rates (shown in units of urea-N) of zebrafish in the absence (CON, no added DOM) or presence of DOM sources added at 6 mg l^{-1} DOC at circumneutral pH (7–8, white bars) and low pH (~5, black bars) over the 3–6 h exposure period in dechlorinated tap water with 900–1000 μmol l^{-1} Na^+. Plotted values represent the means±s.e. of n=9 fish for exposure at pH~5 and 6 fish for exposure at pH 7–8 (exception: n=8 fish for AHA treatment of pH~5). The results of two-way ANOVA are shown. Within a pH, bars sharing the same letter (uppercase for pH 7–8, lowercase for pH~5) are not significantly different.

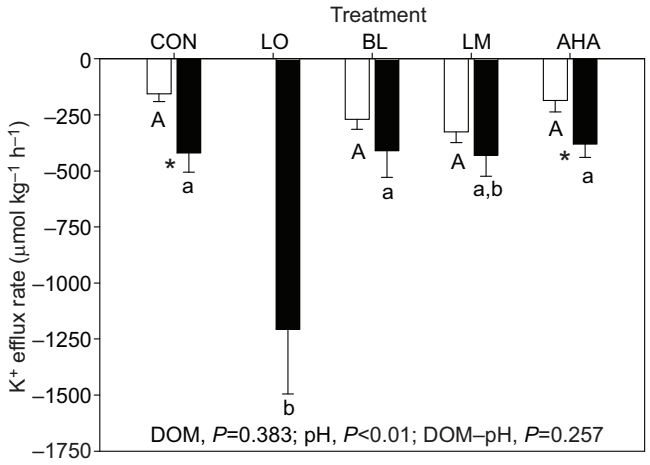

Fig. 5. Net K^+ efflux rates (μmol g^{-1} h^{-1}) of zebrafish at the circumneutral pH 7–8 (white bars) and low pH~5 (black bars) over the 3–6 h exposure period in dechlorinated tap water with 900–1000 μmol l^{-1} Na^+. K^+ efflux was measured as a marker for transcellular permeability of freshwater gills (see Results, 'The influence of DOM on net K^+ loss rates'). Plotted values represent the means±s.e. of n=6 fish (exception: n=5 fish for AHA treatment of pH 7–8). The results of two-way ANOVA are shown. Within a pH, bars sharing the same letter (uppercase for pH 7–8, lowercase for pH~5) are not significantly different. Asterisks indicate significant differences between pHs within the same DOM treatment. Because dilute KOH was used to adjust the pH of the LO solutions in the circumneutral pH experiment, K^+ flux rates were not determined.

shown). At pH 5.0, Na^+ influx rate in the control condition was the same as at circumneutral pH. A trend for greater Na^+ influx rates was seen in the presence of natural DOM sources, particularly at circumneutral pH (Fig. 2). The rates varied significantly among various DOM treatments ($F_{4,49}$=6.837, P<0.001, two-way ANOVA) and between the pHs tested ($F_{1,49}$=5.116, P<0.05). However, there was not a statistically significant interaction between treatment and pH ($F_{4,49}$=2.104, P=0.094). Fish in the BL treatment at both pH 5.0 and circumneutral pH demonstrated Na^+ influx rates significantly higher than those of their respective controls, and generally higher than in all the other DOM treatments (significant at low pH only). Na^+ influx rates were the same in the BL isolate at acidic and circumneutral pH. In contrast, rates in fish incubated in LO and LM at pH ~5 were significantly lower compared with those at pH 7–8 in the same DOM types (Fig. 2).

The influence of DOM on unidirectional Na^+ efflux rates

Fig. 3 illustrates the 3–6 h unidirectional Na^+ efflux rates, measured directly at water Na^+ concentrations (~900–1000 μmol l^{-1}; Table S4) close to those in the acclimation water. At pH 5.0, Na^+ efflux rate in the control condition was the same as at circumneutral pH (Fig. 3). However, at both pHs, fish exposed to DOM sources generally exhibited lower Na^+ efflux rates relative to their respective controls (Fig. 3). Na^+ efflux rates were significantly affected by DOM ($F_{4,49}$=6.206, P<0.001, two-way ANOVA), but neither pH ($F_{1,49}$=0.273, P=0.604, two-way ANOVA) nor their interaction ($F_{4,49}$=2.315, P=0.070, two-way ANOVA) exhibited significant effects. Relative to the controls, the *post hoc* comparisons showed that the reductions in Na^+ efflux rates were significant in LO, LM and AHA at circumneutral pH, and in BL and AHA at acidic pH. Zebrafish in the presence of LO showed Na^+ efflux rates similar to those of control fish and significantly higher rates than those in the presence of BL and AHA under the acidic condition. In addition, fish had different Na^+ efflux rates within the same treatment at the two pHs in the presence of LO only (Fig. 3). The efflux rates were generally higher at 0–3 h, although the patterns with DOM and pH were similar (data not shown); indeed all DOM sources significantly reduced Na^+ efflux at acidic pH (data not shown).

The influence of DOM on ammonia and urea excretion rates

Ammonia (Fig. 4A) and urea excretion rates (Fig. 4B) were measured simultaneously with unidirectional Na^+ efflux rates; the 3–6 h data are shown. Ammonia excretion rates were very similar for all the DOM treatments ($F_{4,64}$=1.457, P=0.226, two-way ANOVA). However, there was an overall effect of pH ($F_{1,64}$=4.857, P<0.05) with slightly higher rates in the acidic condition, though none of the individual differences were significant. There was no significant interaction between treatment and pH ($F_{4,64}$=0.484, P=0.747).

Urea excretion rates (Fig. 4B) were substantially lower than ammonia excretion rates (Fig. 4A), representing approximately 15% of the latter on a per unit N basis. Rates were similar in all treatments ($F_{4,64}$=2.342, P=0.064, two-way ANOVA) and pH conditions ($F_{1,64}$=0.037, P=0.848), and there was no interaction between the treatment and pH ($F_{4,64}$=1.992, P=0.106).

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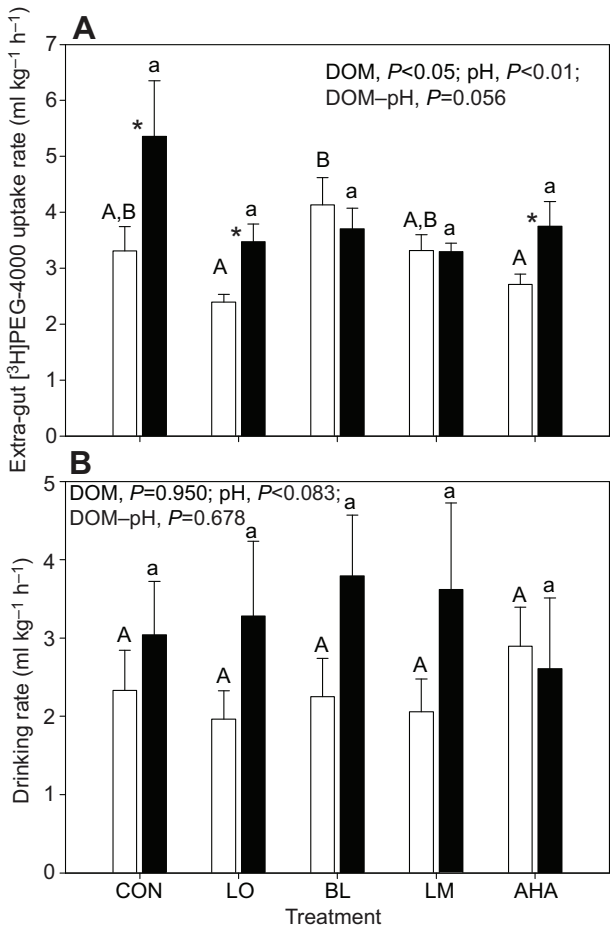

Fig. 6. The influence of different DOM sources on branchial paracellular permeability and drinking rates of zebrafish. The influence of DOM sources on (A) whole-body [^{3}H]PEG-4000 uptake (as a paracellular permeability marker) and (B) drinking rates of zebrafish at circumneutral pH (7–8, white bars) and low pH (~5, black bars) measured in 4.5-h exposures in dechlorinated tap water with 900–1000 µmol l^{-1} Na^+. Plotted values represent the means±s.e. of n=6 fish (exception: n=5 fish for BL treatment for whole-body uptake and CON and AHA treatments for drinking rates), with values expressed as clearance rates of the external medium. The results of two-way ANOVA are shown. Within a pH, bars sharing the same letter (uppercase for pH 7–8, lowercase for pH~5) are not significantly different. Asterisks indicate significant differences between pHs within the same DOM treatment.

As with Na^+ efflux rates, ammonia excretion rates tended to decrease over time (data not shown). This decline was significant for only the AHA treatment at circumneutral pH (t=−4.208, d.f.=5, P<0.01, paired t-test), but at acidic pH it was significant for all treatments except AHA (t=0.343, d.f.=8, P=0.741). Urea excretion rates were stable over time (data not shown).

The influence of DOM on net K^+ loss rates

Net K^+ loss rates were also measured during the Na^+ efflux experiments, as indicators of branchial transcellular permeability; rates were stable over time (Fig. 5; the 3–6 h data are shown). DOM had no significant effect ($F_{4,39}$=1.047, P=0.383, two-way ANOVA) but K^+ loss rates were consistently elevated at acidic pH ($F_{1,39}$=9.089, P<0.01). There was no interaction between these two factors ($F_{3,39}$=1.400, P=0.257). The pH-related differences were significant in the control and AHA treatments. Additionally, at low pH, K^+ loss rates were higher in the LO treatment than in the control and other DOM treatments, except for LM (Fig. 5).

The influence of DOM on branchial clearance rates of [^{3}H] PEG-4000 and drinking rates

Branchial clearance rates of [^{3}H] PEG-4000 from the external water (Fig. 6A) were measured as indicators of paracellular permeability, together with drinking rates (Fig. 6B). Branchial [^{3}H] PEG-4000 clearances were influenced significantly by DOM treatment ($F_{4,50}$=3.189, P<0.05, two-way ANOVA) and pH ($F_{1,50}$=10.291, P<0.01), but the interaction term narrowly avoided significance ($F_{4,50}$=2.478, P=0.056). At circumneutral pH, rates were higher in the BL than in the LO or AHA treatments, but these DOM-related differences did not occur at pH 5.0. However, significantly higher branchial [^{3}H] PEG-4000 clearance rates were observed at acidic pH in the control, LO and AHA treatments relative to their counterparts at circumneutral pH (Fig. 6A).

Drinking rates were surprisingly high, on average approximately 60% of branchial clearance rates of [^{3}H] PEG-4000 (Fig. 6B versus 6A). Zebrafish exhibited similar drinking rates regardless of treatment ($F_{4,47}$=0.175, P=0.950, two-way ANOVA) or pH ($F_{1,47}$=3.134, P=0.083) and there was no significant interaction between the two factors ($F_{4,47}$=0.581, P=0.678; Fig. 6B).

DISCUSSION

Overview

The effects of four chemically distinct DOM isolates (three natural, one commercial, ranging from autochthonous to highly allochthonous, all at ~6 mg C l^{-1}) on ionoregulation and nitrogenous waste excretion were examined in the zebrafish at circumneutral (7.0–8.0) and low pH (~5.0). In the natural habitat of zebrafish (the River Ganges drainage), reported DOC concentrations vary between 2 and 9 mg C l^{-1} (Ittekkot et al., 1985), but pH values are normally above 7.0 (Sarin et al., 1989). All DOM sources tended to stimulate Na^+ influx rate, an effect that was most prominent for a mixed autochthonous–allochthonous isolate (BL) at both pHs. DOM sources also exerted subtle effects on the concentration-dependent kinetics of Na^+ uptake, tending to increase J_{max}, an effect correlated with their aromatic content. Remarkably, all DOM sources reduced passive Na^+ efflux rates regardless of pH, but this action appeared to be independent of their physico-chemical characteristics, and was not associated with clear effects on either transcellular or paracellular permeability. Conversely, the various DOM sources exerted little or no effect on nitrogenous waste excretion, net K^+ losses, drinking rate or paracellular permeability. Overall, these DOM actions were very different from those seen in a parallel study on *Daphnia magna* using the same DOM isolates and pHs (Al-Reasi et al., 2013b).

Sodium uptake in the presence of DOM

Under control conditions at circumneutral pH, kinetic parameters (K_m, J_{max}) describing the concentration-dependence of Na^+ uptake (Fig. 1) were similar to two earlier reports on zebrafish under comparable conditions (Boisen et al., 2003; Kumai et al., 2011). All DOM sources (particularly BL) tended to increase steady-state Na^+ influx rates (Fig. 2), and this was concordant with a similar trend for increased maximal uptake rates (J_{max}) (Fig. 1). The latter was generally associated with decreased uptake affinities (i.e. higher K_m), suggesting uncompetitive stimulation (or removal of an uncompetitive inhibitor) by the DOM sources. Whereas K_m was statistically unchanged in all treatments, a significant increase in J_{max} occurred for the commercial DOM (AHA), so non-competitive stimulation could also apply (see Cornish-Bowden, 1974, for distinction). Similarly, Matsuo et al. (2004) found that AHA caused a significant increase in J_{max}, but no change in K_m for Na^+ uptake in

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rainbow trout. AHA, as well as Suwannee River DOM, also enhanced Na^+ uptake in *D. magna* by augmentation of J_{max} (Glover et al., 2005), but AHA, as well as all the natural DOM sources tested here, had no effect on Na^+ influx in our recent parallel study on *D. magna* (Al-Reasi et al., 2013b). Amazonian black water (8.6 mg C l^{-1}) increased J_{max} non-significantly but had no effect on K_m, or on Na^+ influx rates, of freshwater stingrays relative to reference water with low DOM (0.6 mg C l^{-1}; Wood et al., 2003). Overall, we conclude that DOM effects on Na^+ influx at circumneutral pH, while generally positive, are subtle and dependent upon both species and source.

After 1 week of acclimation to acidic pH 5.0, the steady-state Na^+ influx rate was identical to the rate at circumneutral pH in the control treatment (Fig. 2). Recent studies (Kumai and Perry, 2011; Kumai et al., 2011; Kwong et al., 2013, 2014; Kwong and Perry, 2013; Duarte et al., 2016) on zebrafish exposed for a similar period to even lower pH (4.0) suggest that Na^+ influx is initially inhibited but later restored, coincident with an activation of increased ammonia excretion, which drives Na^+ uptake via an Rh protein–Na^+ transporter metabolon (Wright and Wood, 2009). In accord with this interpretation, acclimation to less extreme acidity (pH 5.0 in the present study) resulted in a modest but significant overall stimulation of ammonia excretion (Fig. 4A). However, both J_{max} and K_m values tended to fall with acclimation to pH 5.0 (Fig. 1), indicative of uncompetitive inhibition (Cornish-Bowden, 1974), whereas Kumai et al. (2011) reported that both parameters increased significantly after acclimation to pH 4.0. The latter workers also reported increased Na^+ efflux and elevated Na^+ influx rates in acid-acclimated zebrafish, whereas these did not change under control conditions in the present study. It seems likely that the less severe acid exposure (pH 5.0 versus pH 4.0) resulted in less overall disturbance of ionoregulatory parameters in the present study, though other factors such as differences in water ions and/or background DOC quality and quantity (none of which were reported by Kumai et al., 2011) could also be involved.

Nevertheless, for the Na^+ influxes, BL continued to be stimulatory and thereby protective, whereas LO, LM and AHA were not protective (Fig. 2). The enhanced Na^+ influx in the presence of BL was not in response to an exacerbated efflux, because in fact, Na^+ efflux was significantly reduced (Fig. 3), lending further support to a direct effect of the BL isolate on influx (Fig. 2). This heterogeneity may relate to differences in the physico-chemistry of the various DOM isolates (Table 1).

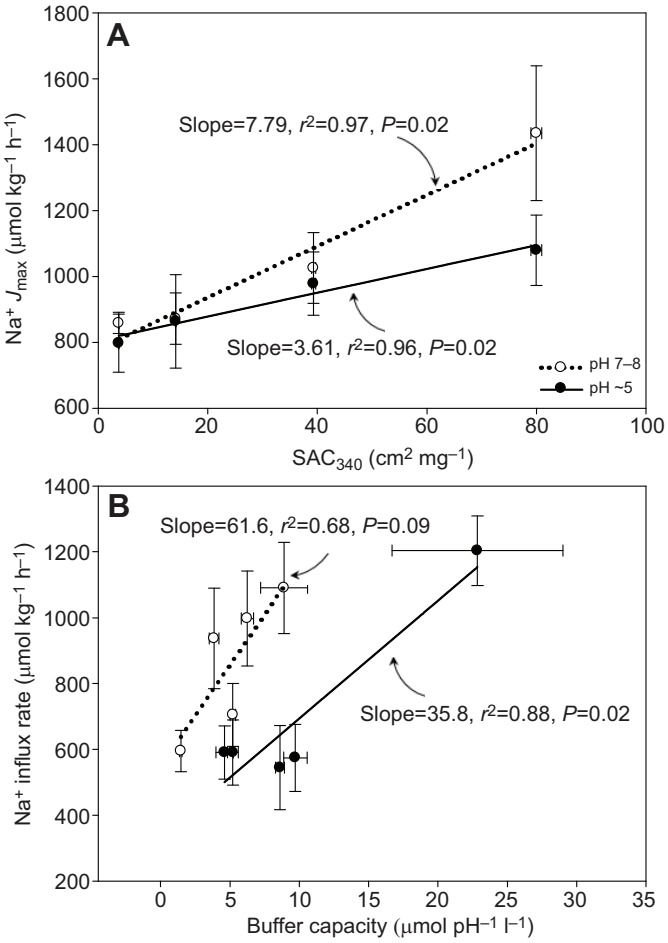

Fig. 7. Aromaticity and chemical reactivity of DOM sources, as estimated by specific absorbance coefficient (SAC_{340}) and buffer capacity, respectively, correlated significantly with Na^+ regulation of zebrafish. Linear regressions of (A) maximal Na^+ transport rates or J_{max} of Na^+ influx in zebrafish against specific absorption coefficients (SAC_{340}) of DOM sources and (B) the unidirectional Na^+ influx rates against buffer capacities of DOM sources at circumneutral pH (7–8, dotted line with open symbols) and low pH (~5, solid line with closed symbols). Each point represents the mean±s.e. (for J_{max} and Na influx rate) or ±s.d. (for SAC_{340} and buffer capacity). SAC_{340} values and buffer capacities of the DOM sources (Table 1) were taken from Al-Reasi et al. (2012) and Al-Reasi et al. (2013b), respectively.

Sodium efflux in the presence of DOM: paracellular versus transcellular effects

In the present study, unidirectional Na^+ efflux was measured directly (see Wood et al., 2009) to avoid any possible artefact (e.g. exchange diffusion) associated with the more commonly used technique of subtracting measured influx from measured net flux (Wood, 1992). All DOM sources tended to reduce Na^+ efflux rates in all treatments relative to the control at both pH conditions; however, the reductions were statistically significant only in LO, LM and AHA at circumneutral pH and in BL and AHA at acidic pH (Fig. 3). Notably, the protective effects of all DOM sources were also seen in the 0–3 h measurements, where efflux rates were higher, probably because of handling stress. These results are in agreement with the findings of Wood et al. (2003) and Duarte et al. (2016) that the natural DOM in Amazonian black water reduced both Na^+ and Cl^- efflux rates across the gills of freshwater stingrays and zebrafish, respectively, at extremely low pH (4.0). However, Wood et al. (2003) reported that AHA had the opposite effect. The present results differ completely from those of our recent study on *D. magna*, where these same DOM sources had no effects on Na^+ efflux rates at either circumneutral or low pH (Al-Reasi et al., 2013b).

Net K^+ loss rates and ^{3}[H]PEG-4000 clearance rates were measured to diagnose whether the effects of DOM were exerted on transcellular or paracellular routes of Na^+ loss. ^{3}[H]PEG-4000 clearance is a well-established paracellular permeability marker (Wood et al., 2009; Kwong and Perry, 2013; Robertson and Wood, 2014). K^+ loss was used by Lauren and McDonald (1985) as an indicator of transcellular permeability because K^+ ions are approximately 100-fold more concentrated inside gill epithelial cells than in blood plasma. However, neither of these indicators explained the protective actions of DOM sources against Na^+ efflux. At circumneutral pH, none of the DOM sources significantly altered either ^{3}[H]PEG-4000 clearance (Fig. 6A) or K^+ loss (Fig. 5). Furthermore, there were overall effects of low pH in increasing both ^{3}[H]PEG-4000 clearance rates and net K^+ loss rates, again in contrast to the patterns seen with Na^+ efflux rates (Fig. 3). Notably,

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in both cases, BL protected against these effects of low pH, whereas AHA did not. Increased ^{3}[H]PEG-4000 permeability in zebrafish exposed to more extreme low pH (4.0) has been reported previously, and there is evidence that at least part of the elevated Na^+ loss under this condition occurs by a paracellular route (Kwong and Perry, 2013). Elevated net K^+ loss rates have been similarly noted in salmonids exposed to pH 4.0–4.5 (McDonald and Wood, 1981; McDonald, 1983). Interpretation of the present [^{3}H]PEG-4000 results seems straightforward: the DOM effects on Na^+ effluxes are not paracellular. However, the K^+ loss rate results are more problematic; for example, cellular K^+ channels may respond differently from cellular Na^+ channels, and recent findings of active K^+ excretion by specific gill ionocytes in certain situations (Furukawa et al., 2012) further confound interpretation. Clearly, more work is needed to determine whether DOM reduces transcellular Na^+ efflux rates in zebrafish.

The influence of DOM on nitrogenous waste excretion

Ammonia excretion rates were slightly elevated by low pH, whereas urea excretion rates were unaffected, and neither were influenced by the various DOM sources (Fig. 4A,B). We now know that ammonia (Rh proteins; Wright and Wood, 2009) and urea transporters (McDonald et al., 2012) help move these nitrogenous wastes across the gills of fish, including zebrafish (e.g. Nakada et al., 2007; Braun et al., 2009). As noted earlier, the increase in ammonia excretion at low pH may help sustain Na^+ uptake via the metabolon under these conditions (Kumai and Perry, 2011; Kumai et al., 2011; Kwong et al., 2013, 2014; Kwong and Perry, 2013; Duarte et al., 2016). The complete absence of DOM effects suggests that these compounds do not directly interact with either Rh or urea transporter mechanisms in the zebrafish gill. Notably, these results again differ completely from findings on *D. magna* (Al-Reasi et al., 2013b), where some of these same DOM sources greatly reduced both ammonia (at circumneutral and low pH) and urea excretion (at low pH only).

The influence of DOM on drinking rates

The tendency for higher drinking rates at low pH was not significant, and there was no effect of any of the DOM sources (Fig. 6B). We are aware of no previous studies of DOM or pH effects on drinking. In freshwater fish, drinking rates are traditionally thought to be very low, but the higher values in the present study (approximately 2.0 and 3.0 ml kg^{-1} h^{-1} at circumneutral and acidic pH, respectively) are similar to those measured in freshwater trout (Pyle et al., 2003; Robertson and Wood, 2014) and killifish acclimated to 10% seawater (Scott et al., 2006). The much lower drinking rates reported by Kwong et al. (2013) for zebrafish acclimated to ion-poor water may reflect the use of a much smaller marker molecule (^{3}[H]PEG-400) that can be absorbed across the intestinal tract (Wood and Grosell, 2012), thereby underestimating the true rate.

Are DOM effects on ionoregulation related to their physico-chemical properties?

In recent investigations (Galvez et al., 2009; Al-Reasi et al., 2011, 2012, 2013a,b), we have found that three physico-chemical properties of DOM are particularly useful in explaining their biological effects: the specific absorbance coefficient at 340 nm (SAC_{340}), which is an index of their aromaticity (i.e. their phenolic content), the buffer capacity (β) and the proton binding index (PBI), a measure of their chemical reactivity. When each of the parameters measured in this study were regressed against each of these properties, most relationships were insignificant, but several stood out prominently (Table S5). The J_{max} for Na^+ uptake increased linearly with SAC_{340} uptake (significant at both pHs; Fig. 7A), and the unidirectional Na^+ influx rate increased linearly with buffer capacity (significant only at acidic pH; Fig. 7B). The former relationship (Fig. 7A) is in accord with findings that SAC_{340} correlates directly with a more negative transepithelial potential across the gills, which will favour Na^+ uptake (Galvez et al., 2009), and that SAC_{340} correlates directly with protection against toxicants that inhibit Na^+ transport (Al-Reasi et al., 2011, 2012, 2013a). The allochthonous, highly coloured DOM sources are more effective in this regard in contrast to paler, more autochthonous sources. As discussed by Wood et al. (2011), some aspect of the phenolic ring structure of DOM clearly promotes Na^+ uptake capacity. The relationship of Na^+ influx rate with β of the DOM has not been seen previously, but makes sense in that a higher β will stabilize the pH of the branchial micro-environment to allow the Rh protein–Na^+ transporter metabolon to function (Wright and Wood, 2009), especially at low pH (Duarte et al., 2016). The lack of relationship of Na^+ efflux with any of these parameters suggests that all natural DOM sources reduce Na^+ efflux in a non-specific manner, associated with binding of these amphiphilic molecules to the gill surface (Campbell et al., 1997), exerting a Ca^{2+}-like effect (Wood et al., 2003, 2011).

Conclusions

The present results add to the mounting evidence that natural DOM sources profoundly influence the physiology of aquatic organisms (Wood et al., 2011). In the zebrafish, these included stimulation of unidirectional Na^+ uptake rate and reduction of unidirectional efflux rate at both circumneutral and low pH, effects that are likely beneficial to ionoregulatory homeostasis, especially in the latter circumstance. Nitrogenous waste excretion was not affected. Clearly, such effects are both species and DOM specific, because parallel experiments with the same DOM sources on *D. magna* revealed no actions on Na^+ homeostasis, but pronounced actions in reducing ammonia and urea efflux rates, especially at low pH (Al-Reasi et al., 2013b). The interaction of DOM molecules with different membrane transporters in zebrafish and *D. magna* may account for the different ionoregulatory responses of the two species. Although little is known about nitrogenous waste transport in daphnids, there is evidence that adults may utilize the $Na^+/K^+/2Cl^-$ cotransporter for Na^+ uptake (e.g. Glover and Wood, 2005b). In zebrafish, the HRC (H^+-ATPase-rich cell), one of four types of ionocyte on the branchial epithelium, bears H^+-ATPase, Na^+/H^+ exchangers and Rh (ammonia) transporters on its apical membrane, and appears to be responsible for both Na^+ uptake and ammonia excretion (Hwang et al., 2011; Kwong et al., 2014). One commonality, however, was that BL, the mixed autochthonous–allochthonous DOM isolate with a relatively high SAC_{340} and the highest β (Table 1), was the most effective in both *D. magna* and zebrafish.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

H.A.A.-R. performed all experiments, analyzed the data, and wrote the paper. C.M.W. and S.D.S. jointly contributed to conceptual design, and edited the paper.

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Supplementary information
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