Comparative Biochemistry and Physiology, Part C 158 (2013) 207–215

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part C

journal homepage: www.elsevier.com/locate/cbpc

The effect of dissolved organic matter (DOM) on sodium transport and nitrogenous waste excretion of the freshwater cladoceran (*Daphnia magna*) at circumneutral and low pH

Hassan A. Al-Reasi [a,*], Usman Yusuf [a], D. Scott Smith [b], Chris M. Wood [a]

[a] Department of Biology, McMaster University, Hamilton, ON, Canada L8S 4K1
[b] Department of Chemistry, Wilfrid Laurier University, Waterloo, ON, Canada N2L 3C5

ARTICLE INFO

Article history:
Received 26 June 2013
Received in revised form 29 August 2013
Accepted 29 August 2013
Available online 10 September 2013

Keywords:
Dissolved organic matter
Daphnia magna
Na^+ metabolism
Ammonia and urea excretion

ABSTRACT

Dissolved organic matter (DOM), a heterogeneous substance found in all natural waters, has many documented abiotic roles, but recently, several possible direct influences of DOM on organism physiology have been reported. However, most studies have been carried out with a limited number of natural DOM isolates or were restricted to the use of commercial or artificial humic substances. We therefore employed three previously characterized, chemically-distinct natural DOMs, as well as a commercially available humic acid (Aldrich, AHA), at circumneutral (7–8) and acidic pH (~5), to examine DOM effects on whole-body Na^+ concentration, unidirectional influx and efflux rates of Na^+, and ammonia and urea excretion rates in *Daphnia magna*. Whole-body Na^+ concentration, Na^+ influx, and Na^+ efflux rates were all unaffected regardless of pH, suggesting no influence of the various natural DOMs on active uptake and passive diffusion of Na^+ in this organism. Ammonia and urea excretion rates were both increased by low pH. Ammonia excretion rates were reduced at circumneutral pH by the most highly colored, allochthonous DOM, and at low pH by all three natural DOMs, as well as by the commercial AHA. Urea excretion rates were not influenced by the presence of the various DOMs in circumneutral solutions, but were attenuated by the presence of two allochthonous DOM sources (isolated from Bannister Lake and Luther Marsh) at acidic pH. The observed reductions may be attributed partially to the higher buffering capacities of natural DOM sources, as well as their ability to interact with biological membranes as estimated by a new measure calculated from their acid–base titration characteristics, the Proton Binding Index (PBI).

1. Introduction

Dissolved organic matter (DOM) is a complex group of molecules produced during the decomposition of lignin-rich plant materials and the decay of dead organic biomass in a poorly-understood process known as humification (Ertel et al., 1984; Hatcher and Spiker, 1988). In freshwater ecosystems, DOM molecules are ubiquitous and their mass (usually ≥50% dissolved organic carbon or DOC as a heterogeneous mixture of humic and fulvic acids) exceeds that of living organisms (Thurman, 1985; Thomas, 1997). The source of DOM in the ecosystem can be allochthonous (i.e. terrigenous - organic matter produced on land and then washed into the water body), autochthonous (organic matter generated within the water column by microrganisms such as algae and bacteria), or of mixed autochthonous and allochthonous origin (McKnight et al., 2001). Depending on their concentrations and origins, DOM molecules are responsible for the yellow to brown color of surface water; allochthonous DOMs tend to be darker in color (Schwartz et al., 2004). The heterogeneous nature of various DOM sources also reflects their variability in chemical structure and composition, characteristics which can be probed using the absorbance and fluorescence spectroscopy and titration (e.g. Al-Reasi et al., 2013).

Several direct interactions of DOM with freshwater organisms have been documented recently (Steinberg et al., 2006). For example, DOM molecules have been shown to accumulate on cell membranes (Campbell et al., 1997) with impacts on their permeability (Vigneault et al., 2000), especially under conditions of low water pH coupled with higher DOC concentrations. Even at circumneutral pH, the presence of added DOM induced a more negative transepithelial potential in trout gills, and the effects were greater with darker, more allochthonous DOMs (Galvez et al., 2009). Some studies have indicated that DOM molecules have the potential to induce toxicity (e.g. Meems et al., 2004; Timofeyev et al., 2004; Matsuo et al., 2006; Steinberg et al., 2006). However, other investigations have found that organisms in soft acidic waters experience higher survival (Hargeby and Petersen, 1988) and improved growth (Barth and Wilson, 2010) in the presence of DOMs, and are protected against negative changes in ionoregulation (Gonzalez et al., 1998, 2002; Wood et al., 2003; Matsuo et al., 2004). DOMs may also facilitate increased ammonia excretion at low pH, so

* Corresponding author at: Department of Biology, Sultan Qaboos University, P.O. Box: 36 Al-Khod, Postal Code: 123 Muscat, Oman. Tel.: +968 24141448; fax: +968 24413415.
E-mail address: alreasi@squ.edu.om (H.A. Al-Reasi).

1532-0456/$ – see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.cbpc.2013.08.004

as to alkalinize the gill surface (Wood et al., 2003). Similar to low pH conditions (e.g. Havas et al., 1984; Wood et al., 1998), metals such as copper (e.g. Grosell and Wood, 2002; Alsop and Wood, 2011; Zimmer et al., 2012) have been reported to disrupt Na^+ regulation and ammonia excretion in freshwater organisms. From metal toxicity studies, DOM molecules are well-known to offer protection by sequestering metal ions as complexes in the water, thereby reducing bioavailability and uptake, and these protective actions can be related to the physicochemical properties of the various DOMs (Al-Reasi et al., 2011, 2012). However, Wood et al. (2011) have postulated that part of the protective effect of DOM may also be a direct interaction with the processes of Na^+ transport. Indeed, Matsuo et al. (2004), Glover et al. (2005), and Glover and Wood (2005a) reported that the presence of DOM may stimulate active Na^+ uptake in the rainbow trout (*Oncorhynchus mykiss*) and the water flea (*Daphnia magna*), respectively.

Generally, it is apparent that DOMs may interact in a positive or negative fashion with the functioning of gills or other transporting epithelia of freshwater animals (Wood et al., 2003; Matsuo et al., 2004; Glover et al., 2005; Galvez et al., 2009). Furthermore, there is some evidence that commercially prepared DOMs may have different direct actions than natural DOMs (e.g. Wood et al., 2003; Glover et al., 2005; Glover and Wood, 2005a). Recently, through titration and spectroscopic studies, we developed a new Proton Binding Index (PBI) to summarize the chemical reactivity of various DOMs (Al-Reasi et al., 2013). The present study investigated the influence of various natural DOMs on two fundamental physiological functions, Na^+ regulation and nitrogenous waste excretion, of the freshwater cladoceran, *D. magna*, and evaluated whether effects could be related to their buffering capacities or PBI values. We hypothesized that DOMs would affect the processes of ionoregulation and nitrogenous waste excretion positively, especially at lower environmental pH. We predicted enhancement of Na^+ uptake and/or reduction of Na^+ efflux to counteract the accelerated net Na^+ loss commonly seen in acidic water. Overall, the results do not support our hypotheses with respect to the effects of natural DOM on Na^+ homeostasis, but revealed some marked effects of both low pH and various DOMs on nitrogenous waste excretion in *D. magna*. The current study differs from previous studies in this area on daphnids (Glover and Wood, 2005a; Glover et al., 2005) in exploring a wide range of natural DOMs, and in relating the observed effects to the chemical properties of the various DOMs as revealed by their buffering capacities and PBI values (Al-Reasi et al., 2013).

2. Materials and methods

2.1. Test organisms

Daphnia magna, a widely used freshwater crustacean in ecotoxicological and physiological research, was employed as a model organism in the present study. The original *D. magna* adults were acquired from Aquatic Research Organisms (ARO, Hampton, NH, USA) and cultured for several generations under laboratory conditions (at ~23 °C with a 12 h light: 12 h dark photoperiod) in dechlorinated Lake Ontario water (city of Hamilton tap water). The water has the following chemistries; $[Na^+] = \sim 0.7$ mM, $[Ca^{2+}] = \sim 1.0$ mM, $[Mg^{2+}] = \sim 0.3$ mM, $[DOC] = 2.5 \pm 0.4$ mg C L^{-1} and pH ~7.5–8.0 (Al-Reasi et al., 2012). Thirty two to 35 organisms were reared in 650 mL of dechlorinated water which was renewed by 500 mL replacement with fresh water twice per week. The daphnids were fed once per day with unicellular green algae (*Selenastrum capricornutum*) and YCT (Yeast, CEROPHYLL®, and Trout chow). All experiments were performed on adult daphnids (5 to 6 days old); animals were starved for 24 h prior to experimentation and were not fed during exposures.

2.2. Dissolved organic matter (DOM) solutions

Four different DOM sources were tested, 3 of which were natural isolates (ranging from autochthonous to allochthonous) which were collected by reverse-osmosis from Lake Ontario (LO), Bannister Lake (BL) and Luther Marsh (LM). Details of collection and treatment of these DOMs are provided in Al-Reasi et al. (2012). A commercially available Aldrich humic acid (AHA, Sigma–Aldrich Chemical, St. Louis, MO, USA), which has been extensively used as a DOM analogue in earlier studies (e.g. Glover et al., 2005; Glover and Wood, 2005a) was included for comparison. Absorbance and fluorescence properties of DOMs are provided in detail elsewhere (Al-Reasi et al., 2012). In brief, of the three natural DOMs, LO is the most lightly colored and autochthonous, whereas LM is the darkest and allochthonous. All DOM solutions (at DOC concentrations of 6 and 12 mg C L^{-1}) were prepared using dechlorinated city of Hamilton tap water which was employed as a control (no external DOM added). These concentrations lie within the range (1–15 mg C L^{-1}) of DOM levels commonly reported for the natural surface waters (Thurman, 1985). Since the addition of reverse-osmosis collected DOM isolate has the potential to change concentrations of ions, especially sodium (Na^+) and calcium (Ca^{2+}), all exposure solutions including control were checked and balanced for Ca^{2+} and Na^+ levels. Maintaining similar ion levels for all treatments was essential, as for example Ca^{2+} 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). Therefore, appropriate amounts of calcium carbonate ($CaCO_3$ salt, Sigma–Aldrich) and sodium chloride (NaCl salt, Caledon Laboratories LTD, Georgetown, ON, Canada) were added to each solution. Because of the low solubility of $CaCO_3$, all solutions were bubbled overnight with pure carbon dioxide (CO_2, Air Liquide Canada Inc., Burlington, ON, Canada). The next day, the solutions were vigorously bubbled with air for 24 h to remove excess CO_2. About 16–20 h before exposure, each solution was initially adjusted to the desired pH (7–8 or ~5) by addition of diluted H_2SO_4 solution (made from 95–98% H_2SO_4, ACS specification, Caledon Laboratories LTD, Georgetown, ON, Canada) or/and KOH solution (made from KOH crystal, ACS specification, Caledon Laboratories). An SP70 portable pH meter with Ag/AgCl pH electrode (VWR sympHony, VWR International, Beverly, MA, USA) was employed throughout. In all these steps, solutions were stored in foil-wrapped plastic bottles to minimize the degradation of DOM due to light exposure. The Na^+ transport and whole body Na^+ concentration were evaluated at both DOC concentrations (6 and 12 mg C L^{-1}), but nitrogenous waste excretion rates were examined only at 6 mg C L^{-1}.

2.3. Whole body sodium concentrations

Ten *D. magna* adults were transferred individually into 100 mL of each exposure solution. The duration of the exposure was 24 h. The pH was checked and adjusted before the introduction of the organisms. Two pH readings for each solution were taken at the start and the end of the exposure at pH ≥7 and approximately every 2–3 h for experiments at pH ~5 during the light period. The pH was not adjusted in the dark period but the change in pH was within 0.2–0.3 units. Tables 1 and 2 summarize chemistry of the exposure water. No mortality was recorded over the 24 h exposure in all tested DOM sources. At the end of the exposure, individuals were counted, removed and rinsed in deionized water (≥17.5 MΩ cm; Barnstead Nanopure II, Thermo Scientific Barnstead, NH, USA) for 30 s. Then, organisms were blotted dry on Whatman® No. 1 filter paper. All daphnids were then placed individually into pre-weighed micro-centrifuge tubes, which were weighed again using an UMT2 electronic microbalance (Mettler-Toledo AG, Laboratory and Weighing Technologies, Greifensee, Switzerland). The mass of *D. magna* was expressed as mg wet weight. Each individual was then digested by the addition of 15 μL of concentrated trace metal grade HNO_3 (67–70% HNO_3, Fisher Scientific, Fairlawn, NJ, USA) and placed in an oven for 4 h at ~65 °C in a sealed micro-centrifuge tube. Then, 450 μL of deionized water was added to the digested individual and the micro-centrifuge tube was mixed on a Vortex Genie 2 Shaker (Scientific Industries, Bohemia, NY, USA). The solution was then

Table 1
Water chemistry of the exposure water and mass of the organisms (mean ± standard error, *n*) used for examining the influence of dissolved organic matter (DOM) on sodium metabolism and excretion of nitrogenous wastes by *D. magna* at 6 mg C L^{-1} DOC.

Treatment	Series	pH		Mass (mg wet wt.)		Na^+ (mM)	Ca^{2+} (mM)	DOC (mg C L^{-1})
		Acidic	Circumneutral	Acidic	Circumneutral			
CON	Whole body-Na^+	5.23 ± 0.13 (8)	7.80 ± 0.03 (5)	0.99 ± 0.08 (8)	1.23 ± 0.12 (14)	0.93 ± 0.01 (6)	1.45 ± 0.02 (7)	2.26 ± 0.03 (8)
	Na^+-efflux rate	5.08 ± 0.09 (8)	7.79 ± 0.01 (6)	1.22 ± 0.14 (6)	1.29 ± 0.09 (17)			
	Na^+-influx rate	5.26 ± 0.13 (8)	7.67 ± 0.10 (5)	0.94 ± 0.06 (9)	0.96 ± 0.06 (20)			
	Nitrogen excretion	5.15 ± 0.06 (30)	7.80 ± 0.01 (16)	0.52 ± 0.04 (5)	0.71 ± 0.01 (5)			
LO	Whole body-Na^+	5.17 ± 0.14 (8)	7.68 ± 0.06 (6)	1.20 ± 0.16 (10)	1.05 ± 0.07 (9)	0.94 ± 0.02 (9)	1.40 ± 0.01 (6)	6.83 ± 0.08 (6)
	Na^+-efflux rate	5.18 ± 0.13 (9)	7.70 ± 0.07 (6)	1.43 ± 0.15 (8)	1.34 ± 0.10 (8)			
	Na^+-influx rate	5.11 ± 0.11 (8)	7.53 ± 0.12 (6)	0.87 ± 0.07 (10)	1.11 ± 0.09 (9)			
	Nitrogen excretion	5.13 ± 0.07 (27)	7.68 ± 0.03 (20)	0.53 ± 0.07 (5)	0.69 ± 0.02 (5)			
BL	Whole body-Na^+	5.02 ± 0.07 (7)	7.75 ± 0.01 (4)	1.15 ± 0.05 (10)	1.08 ± 0.09 (8)	0.91 ± 0.01 (6)	1.44 ± 0.10 (6)	6.61 ± 0.15 (4)
	Na^+-efflux rate	5.16 ± 0.12 (8)	7.73 ± 0.01 (5)	1.25 ± 0.06 (8)	1.11 ± 0.04 (9)			
	Na^+-influx rate	5.07 ± 0.06 (7)	7.52 ± 0.12 (4)	0.91 ± 0.08 (8)	0.75 ± 0.07 (10)			
	Nitrogen excretion	5.06 ± 0.05 (23)	7.74 ± 0.01 (15)	0.71 ± 0.08 (4)	0.60 ± 0.04 (5)			
LM	Whole body-Na^+	5.06 ± 0.07 (9)	7.76 ± 0.01 (4)	0.90 ± 0.07 (10)	0.95 ± 0.10 (10)	0.93 ± 0.00 (6)	1.43 ± 0.01 (6)	5.73 ± 0.06 (6)
	Na^+-efflux rate	5.16 ± 0.10 (10)	7.73 ± 0.01 (5)	1.41 ± 0.12 (7)	1.01 ± 0.06 (9)			
	Na^+-influx rate	5.13 ± 0.07 (9)	7.52 ± 0.12 (4)	1.01 ± 0.06 (9)	0.88 ± 0.12 (8)			
	Nitrogen excretion	5.08 ± 0.04 (30)	7.73 ± 0.00 (15)	0.41 ± 0.02 (5)	0.61 ± 0.02 (5)			
AHA	Whole body-Na^+	5.16 ± 0.12 (7)	7.77 ± 0.01 (4)	1.01 ± 0.11 (10)	0.90 ± 0.06 (7)	0.94 ± 0.01 (6)	1.54 ± 0.05 (6)	5.11 ± 0.71 (4)
	Na^+-efflux rate	5.08 ± 0.07 (8)	7.78 ± 0.01 (5)	1.23 ± 0.15 (7)	1.51 ± 0.19 (8)			
	Na^+-influx rate	5.15 ± 0.08 (8)	7.58 ± 0.10 (4)	0.87 ± 0.10 (10)	1.15 ± 0.10 (10)			
	Nitrogen excretion	5.10 ± 0.05 (24)	7.76 ± 0.01 (15)	0.54 ± 0.08 (4)	0.67 ± 0.04 (5)			

CON-control (dechlorinated tap water with no added DOM from exogenous source); DOM isolates from Lake Ontario (LO), Bannister Lake (BL), Luther Marsh (LM) and Aldrich humic acid (AHA) were added to dechlorinated water for the other treatments.

transferred to a pre-weighed 2.0 mL centrifuge tube where it was diluted to a total volume of ~1.5 mL using the deionized water. All tubes were weighed again in order to determine the exact volume of the solution, and then assayed for Na^+ concentration (see below). The factor 1.25 was used to correct the final whole body concentration for water trapped by the carapace as suggested by Stobbart et al. (1977). The whole-body Na^+ concentration of *D. magna* was expressed as μmol mg^{-1} wet mass.

2.4. Sodium influx rate

Ten organisms were exposed to 1.25 μCi of radioactive $^{22}Na^+$ as NaCl (Eckert and Ziegler isotope products, Valencia, CA, USA) in 10 mL of solution for 1.0 h. The pH of the exposure solutions was checked and adjusted as necessary according to the target pH (7–8 or ~5). Ion concentrations and pH of the exposure water are presented in Tables 1 and 2. After 1 h, individuals were rinsed individually in a high Na^+ "cold displacement" solution (1.0 M NaCl) for 10 s to displace any $^{22}Na^+$ ions adsorbed to the surface of the organism, followed by a rinse in deionised water for 30 s as suggested by Stobbart et al. (1977) and Glover et al. (2005). The exposure solutions were also sampled. Organisms and water samples were analysed directly for $^{22}Na^+$ gamma radioactivity, as counts per minute (cpm) using a "Wizard 3" 1480 automatic gamma counter (Perkin-Elmer, Woodbridge, ON, Canada). The unidirectional Na^+ influx rate (J_{in}) was calculated based on the amount of radioactivity incorporated into the organism (Glover et al., 2005):

$$J_{in} = \frac{cpm}{SA \times m \times t}$$

where cpm is the counts per minute of each individual, SA is the specific radioactivity of the exposure water (cpm/μmol), *m* is the mass of the organism (g) and *t* is the time of exposure (h). In this calculation, the mass of each individual was divided by 1.25 to correct for water trapped by the carapace (Stobbart et al., 1977).

Table 2
Water chemistry of the exposure water and mass of the organisms (mean ± standard error, *n*) used for examining the influence of dissolved organic matter (DOM) on sodium metabolism of *D. magna* at 12 mg C L^{-1} DOC.

Treatment	Series	pH		Mass (mg wet wt.)		Na^+ (mM)	Ca^{2+} (mM)	DOC (mg C L^{-1})
		Acidic	Circumneutral	Acidic	Circumneutral			
CON	Whole body-Na^+	5.18 ± 0.08 (13)	7.49 ± 0.22 (6)	1.23 ± 0.05 (9)	1.40 ± 0.05 (19)	1.23 ± 0.02 (9)	1.85 ± 0.05 (9)	2.12 ± 0.11 (11)
	Na^+-efflux rate	5.16 ± 0.16 (5)	7.74 ± 0.13 (10)	0.96 ± 0.16 (6)	1.20 ± 0.07 (19)			
	Na^+-influx rate	5.16 ± 0.10 (8)	7.72 ± 0.17 (8)	0.98 ± 0.12 (9)	1.08 ± 0.11 (20)			
LO	Whole body-Na^+	5.04 ± 0.04 (13)	7.04 ± 0.11 (4)	1.09 ± 0.09 (9)	1.36 ± 0.08 (10)	1.18 ± 0.03 (8)	1.81 ± 0.03 (8)	12.85 ± 0.16 (6)
	Na^+-efflux rate	5.14 ± 0.08 (6)	7.42 ± 0.15 (4)	0.92 ± 0.05 (6)	1.39 ± 0.07 (8)			
	Na^+-influx rate	5.06 ± 0.07 (9)	7.24 ± 0.03 (3)	1.13 ± 0.16 (9)	0.91 ± 0.12 (9)			
BL	Whole body-Na^+	5.06 ± 0.03 (13)	7.10 ± 0.15 (4)	1.33 ± 0.09 (10)	1.28 ± 0.04 (10)	1.20 ± 0.02 (7)	1.90 ± 0.04 (8)	12.77 ± 0.30 (6)
	Na^+-efflux rate	5.07 ± 0.08 (6)	7.40 ± 0.16 (4)	0.98 ± 0.07 (7)	1.41 ± 0.09 (9)			
	Na^+-influx rate	5.10 ± 0.05 (9)	7.33 ± 0.10 (3)	1.04 ± 0.13 (9)	0.90 ± 0.11 (10)			
LM	Whole body-Na^+	5.06 ± 0.03 (13)	7.07 ± 0.10 (4)	1.26 ± 0.08 (11)	1.40 ± 0.10 (10)	1.24 ± 0.02 (8)	1.90 ± 0.04 (8)	11.18 ± 0.09 (6)
	Na^+-efflux rate	5.16 ± 0.10 (5)	7.45 ± 0.16 (4)	0.93 ± 0.07 (8)	1.52 ± 0.08 (10)			
	Na^+-influx rate	5.08 ± 0.07 (8)	7.21 ± 0.05 (3)	1.12 ± 0.08 (9)	0.94 ± 0.17 (9)			
AHA	Whole body-Na^+	5.08 ± 0.04 (13)	7.16 ± 0.15 (4)	1.20 ± 0.10 (10)	1.36 ± 0.11 (10)	1.23 ± 0.02 (8)	1.87 ± 0.04 (8)	12.38 ± 0.25 (7)
	Na^+-efflux rate	5.12 ± 0.09 (6)	7.41 ± 0.17 (4)	0.93 ± 0.07 (6)	1.59 ± 0.08 (9)			
	Na^+-influx rate	5.02 ± 0.04 (9)	7.36 ± 0.14 (3)	1.07 ± 0.10 (10)	0.84 ± 0.17 (8)			

CON-control (dechlorinated tap water with no added DOM from exogenous source); DOM isolates from Lake Ontario (LO), Bannister Lake (BL), Luther Marsh (LM) and Aldrich humic acid (AHA) were added to dechlorinated water for the other treatments.

2.5. Sodium efflux rate

Daphnids were incubated in 1.0 L of dechlorinated water inoculated with 50–100 μCi of radioactive $^{22}Na^+$ for 24 h before experimentation. Preliminary experiments demonstrated that this was sufficient time for complete equilibration of $^{22}Na^+$ with the internal Na^+ pool. Thereafter, each individual was first rinsed in fresh dechlorinated water and immediately in deionized water for 1 min. This ensured the removal of any excess $^{22}Na^+$ adsorbed to the surface of the organism or in the water trapped by the carapace. Then each daphnid was individually placed in 1.5 mL of each exposure solution (see Tables 1 and 2 for chemistry of the exposure water) and allowed to undergo efflux for 2 h. In preliminary experiments, it was found that this time period ensured a relatively abundant amount of radioactivity appearing in the external water (i.e. each individual had effluxed ≥100 cpm, whereas the background was ≤10 cpm), while avoiding significant recycling of the radioisotope. At the end of the exposure, 1 mL of the water was sampled and *D. magna* individuals were processed as above for whole-body Na^+ concentration and their weights were corrected as described above for the influx experiments. Radioactivity of individuals and exposure water samples were measured and the unidirectional Na^+ efflux rate (J_{out}) was calculated based on the appearance of $^{22}Na^+$ radioactivity in the exposure water (Glover et al., 2005):

$$J_{out} = \frac{\text{cpm}}{SA \times m \times t}$$

where cpm is the total counts per minute of the 1.5 mL exposure water for each individual, SA is the specific radioactivity of the organism (cpm/μmol), m is the corrected mass of the organism (g) and t is the time of exposure (h).

2.6. Nitrogenous waste excretion rates

This series required higher volumes of solution so experiments were performed at only one concentration of DOC (6 mg C L^{-1}) because there was not enough of some of the DOM isolates available to run the exposures at the higher DOC concentration (12 mg C L^{-1}). Five *D. magna* individuals were transferred into 12 mL of each exposure solution, representing $n = 1$. The experiment was repeated 4–5 times ($n = 4$–5). As detailed above for whole body Na^+ experiments, pH was maintained at the desired levels (7–8 or ~5) during the course of the exposure. Two mL water samples were obtained 10 min after introduction of the organisms and after 24 h. The samples were frozen immediately after collection and kept at −20 °C until chemical analysis. The excretion rates (J_x) of ammonia or urea were calculated as follow:

$$J_X = \frac{\left(Tx_f - Tx_i\right) \times V}{m \times t}$$

where Tx_f and Tx_i are final and initial concentrations of ammonia or urea, respectively, V is the volume of the exposure chamber, m is the corrected mass of the organism (g) and t is the time of exposure (h). The basal ammonia levels in the 6 mg C L^{-1} were determined before the start of the experiments. Background ammonia concentrations were at or below control levels (1.27 μM) and in almost all DOM treatments were not detected. In addition, preliminary no-organism control exposures were carried out in solutions of LO (the most autochthonous) and LM (the most allochthonous) as well as control for 24 h to account for possible ammonia production due to bacterial activity or abiotic DOM degradation. The concentrations were similar to the background reported above indicating negligible contribution to the measured production due to the presence of the test organism. Note that in all these experiments, assays for ammonia and urea employed blanks and standards made up in the respective solutions so as to account for absorbance originating from DOM itself.

2.7. Chemical analyses

Since each of the exposure solutions was prepared in one batch for all experimental series, water samples for measurements of Na^+, Ca^{2+}, and DOC concentrations were averaged for all experiments. Water samples for DOC analysis were filtered through 0.45-μm Acrodisc® syringe filter (Pall Corporation, Ann Arbor, MI, USA). The total DOC concentration in water samples was measured directly using a Shimadzu TOC-$V_{CPH/CPN}$ total organic carbon analyzer (Shimadzu Corporation, Kyoto, Japan). Whole body Na^+ concentration of *D. magna* and Na^+ and Ca^{2+} concentrations of the water samples were determined using flame atomic absorption spectrometry (SpectroAA220FS, Varian, Mulgrave, Australia). The reproducibility of the flame spectrometer and TOC analyzer was assured using certified standards diluted according to the manufacturers' manuals. Total ammonia and urea concentrations in water were determined in triplicate via spectrophotometry according to methods of Verdouw et al. (1978) and Rahmatullah and Boyde (1980), respectively, using a SpectraMAX 340pc microplate reader (Molecular Devices, Sunnyvale, CA, USA). For these assays, blanks and standards were prepared in 6 mg C L^{-1} solutions of each DOM isolate to account for any color due to the presence of DOM.

2.8. Buffering capacity and Proton Binding Index

Buffering capacity refers to quantification of the capacity, based on chemical behavior, of an aqueous solution to maintain stable pH and thereby minimize changes in H^+ and OH^- concentrations when acids or bases are added. The greater the stabilizing capacity, the greater the buffering capacity. Acid–base titrations of these DOM isolates have been presented recently (Al-Reasi et al., 2013). These acid–base titration data were utilized to estimate proton binding capacities (pK_a) and their site densities (L_T) as described in detail by Smith and Ferris (2001). The buffer capacity (β) of the organic acid was determined at specific pH (and corresponding $[H^+]$) values using the following equation for monoprotic acids (Stumm and Morgan, 1996):

$$\beta = 2.303 \sum_{i=1}^{n} \left(\frac{L_{Ti} K_{ai} [H^+]}{(K_{ai} + [H^+])^2} \right)$$

using the summation across all n K_{ai} values (dissociation constants) with associated L_{Ti} values (site densities in μmol mg^{-1}) where $[H^+]$ is the proton concentration. The calculated β is in units of μmol pH^{-1} mg C^{-1}. To determine β (μmol pH^{-1} L^{-1}) for specific samples, this value was multiplied by the measured DOC concentration (mg C L^{-1}).

The Proton Binding Index (PBI) for each sample was calculated from the acid–base titration data, as described in detail by Al-Reasi et al. (2013). PBI is unitless, and is a function of the measured acid, base and intermediate proton binding capacities of a DOM isolate. In general PBI values should be less than 1.0 and high values would represent a stronger potential for chemical reactivity, such as binding to membranes.

2.9. Statistical analyses

Statistical analyses were conducted using SigmaStat for Windows (Version 3.5, Systat Software, Inc., Point Richmond, CA, USA). Before applying the appropriate statistical techniques, normality and homogeneity of variance of data were checked by the Kolmogorov–

Smirnov test and Levene median test, respectively. Two-way analysis of variance (ANOVA) was employed to check for the contribution of DOM sources, pH or the interaction between DOM treatment and pH on the each measured endpoint (i.e. whole body Na^+ concentration, Na^+ influx and efflux rates and ammonia and urea excretion rates). Student's two-tailed t-test was used to check for differences in responses between

6 and 12 mg C L^{-1} at each pH condition for Na^+ data. When the assumptions of the normal distribution and homogeneity of variance were violated, data were first transformed on base 10 logarithmic scale (log_{10}) and then the ANOVA and/or *t*-test were performed. When significant differences were detected, the ANOVA was followed by a multiple *post hoc* comparison test (Tukey's test). Values have been reported as means ± 1 standard error (*n*) and significance was established at the 0.05 level. At this significance level, the effect size (correlation coefficient, *r* for *t*-tests, and omega, ω for ANOVAs), as a measure of the observed biological effect, was calculated for each statistical test.

3. Results

3.1. The influence of DOM on whole-body Na^+ concentration

Daphnids exposed to 6 mg C L^{-1} DOC (Fig. 1A) had similar whole-body Na^+ concentrations to that of controls (i.e. no external DOM added) regardless of treatment (i.e. DOM sources) ($F_{4,\ 86} = 0.385$, $p = 0.819$, $\omega = 0.000$, two-way ANOVA) or pH conditions ($F_{1,\ 86} = 1.973$, $p = 0.164$, $\omega = 0.000$, two-way ANOVA). Similarly, no main effect was found for treatment or pH in the presence of 12 mg L^{-1} ($F_{4,\ 98} = 1.258$, $p = 0.292$, $\omega = 0.221$; $F_{1,\ 98} = 0.001$, $p = 0.977$, $\omega = 0.000$, two-way ANOVA, respectively, Fig. 1B). At both DOC levels (6 and 12 mg C L^{-1}), any interaction between the two factors on whole body Na^+ concentration was ruled out ($F_{4,\ 86} = 2.054$, $p = 0.094$, $\omega = 0.329$; $F_{4,\ 98} = 0.230$, $p = 0.921$, $\omega = 0.000$, two-way ANOVA, respectively). Comparing the effect of DOC concentrations at the same pH conditions, daphnids maintained similar whole body Na^+ concentrations irrespective of the treatment. The only exception was at the higher AHA concentration where daphnids at circumneutral pH at 12 mg C L^{-1} (Fig. 1B) had a lower Na^+ concentration relative to those in 6 mg C L^{-1} (Fig. 1A) ($t = -3.400$, $df = 15$, $p < 0.01$, $r = 0.660$, Student's *t*-test).

3.2. The influence of DOM on Na^+ influx

Similar to the controls, the influx rates of *D. magna* adults were not statistically different among DOM sources ($F_{4,\ 93} = 1.827$, $p = 0.130$, $\omega = 0.000$, two-way ANOVA) or pH's tested ($F_{1,\ 93} = 1.810$, $p = 0.182$, $\omega = 0.000$, two-way ANOVA) in the presence of 6 mg C L^{-1} (Fig. 2A). Moreover, no interaction was detected between treatments and pH conditions to influence the Na^+ influx rates of the organisms ($F_{4,\ 93} = 1.890$, $p = 0.119$, $\omega = 0.276$, two-way ANOVA). In exposure chambers containing 12 mg C L^{-1}, while no influence was observed for treatment alone ($F_{4,\ 92} = 1.193$, $p = 0.319$, $\omega = 0.000$, two-way ANOVA) or for the interaction between treatment and pH ($F_{4,\ 92} = 1.476$, $p = 0.216$, $\omega = 0.195$, two-way ANOVA), there was a significant effect of pH alone on the Na^+ influx rate ($F_{1,\ 92} = 6.946$, $p < 0.050$, $\omega = 0.293$, two-way ANOVA). The *post hoc* multiple comparisons (Tukey's test) revealed that control *D. magna* individuals under acidic conditions had a significantly higher average Na^+ influx rate than

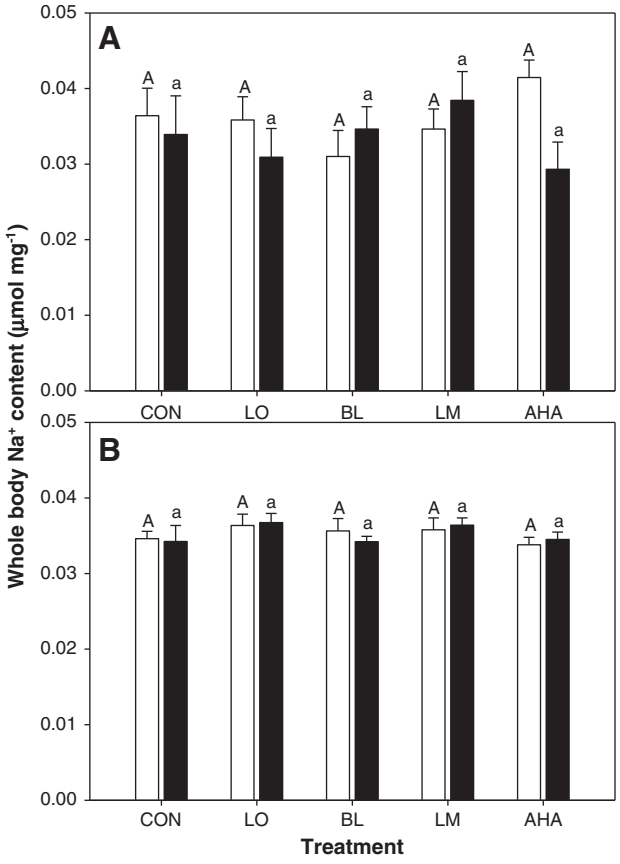

Fig. 1. Whole body Na^+ concentration (µmol mg^{-1} wet weight) of *D. magna* in the absence (CON, no added DOM) or presence of DOMs added at (A) 6 mg C L^{-1} and (B) 12 mg C L^{-1} DOC at circumneutral pH 7–8 (white bars) and low pH ~5 (black bars). Plotted values represent the mean ± standard errors for $n = 7$ – 14 of 5–6 day old adults. Within a pH, bars sharing the same letter (upper case for pH 7–8, lower case for pH ~5) are not significantly different. Asterisks indicate significant differences between pH's within the same DOM treatment.

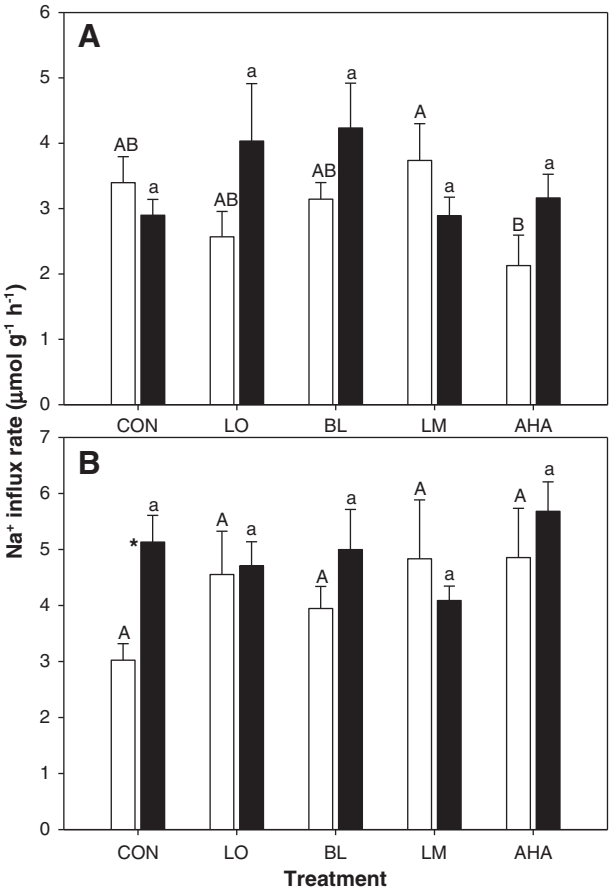

Fig. 2. Na^+ influx rates (µmol g^{-1} h^{-1}) of *D. magna* in the absence (CON, no added DOM) or presence of DOMs added at (A) 6 mg C L^{-1} and (B) 12 mg C L^{-1} DOC at circumneutral pH 7–8 (white bars) and low pH ~5 (black bars). Plotted values represent the mean ± standard errors for Na^+ influx rates over 1 h of $n = 9$ – 20 of 5–6 day old adults. Within a pH, bars sharing the same letter (upper case for pH 7–8, lower case for pH ~5) are not significantly different. Asterisks indicate significant differences between pH's within the same DOM treatment.

those in the circumneutral conditions (Fig. 2B). All other comparisons were not significant. When the two DOM concentrations (6 versus 12 mg C L^{-1}) were compared, higher uptake rates were found for *D. magna* individuals at 12 mg C L^{-1} in LM ($t = -3.564$, $df = 16$, $p < 0.01$, $r = 0.665$, Student's *t*-test) and AHA ($t = -3.943$, $df = 18$, $p < 0.01$, $r = 0.681$, Student's *t*-test) at low pH and in LO ($t = -2.293$, $df = 16$, $p < 0.05$, $r = 0.497$, Student's *t*-test) and AHA ($t = -3.414$, $df = 16$, $p < 0.01$, $r = 0.649$, Student's *t*-test) at circumneutral pH.

3.3. The influence of DOM on Na^+ efflux

There were no significant differences in Na^+ efflux rates recorded among various DOM treatments ($F_{4, 77} = 0.815$, $p = 0.520$, $\omega = 0.000$, two-way ANOVA), pH circumstances ($F_{1, 77} = 0.551$, $p = 0.460$, $\omega = 0.000$, two-way ANOVA) or even for the interaction between the two ($F_{4, 77} = 1.819$, $p = 0.134$, $\omega = 0.303$, two-way ANOVA) when the organisms were exposed to 6 mg C L^{-1} (Fig. 3A). In the 12 mg C L^{-1} treatments, Na^+ efflux rates tended to be higher, generally in acidic waters, but this was also true for the controls, so the differences could not be attributed to the effects of DOM (Fig. 3B). As at 6 mg C L^{-1}, no significant differences among the different treatments were observed in the presence of 12 mg C L^{-1} ($F_{4, 78} = 0.961$, $p = 0.434$, $\omega = 0.199$, two-way ANOVA). Although there was not a statistically significant interaction between the treatment and pH ($F_{4, 78} = 0.098$, $p = 0.983$, $\omega = 0.000$, two-way ANOVA), there were statistically significant differences ($F_{1, 78} = 12.022$, $p < 0.001$, $\omega = 0.467$, two-way ANOVA) within the DOM treatments at the two pH's (Fig. 3B). Specifically, daphnids present in LO at low pH had significantly higher Na^+ efflux rates than those at circumneutral pH (Fig. 3B). Comparing the two DOC concentrations, organisms in the presence of LO ($t = -5.153$, $df = 12$, $p < 0.01$, $r = 0.830$, Student's *t*-test) and LM ($t = -2.376$, $df = 13$, $p < 0.05$, $r = 0.550$, Student's *t*-test) experienced higher Na^+ efflux rates in 12 mg C L^{-1} relative to 6 mg C L^{-1} at pH ~5. Similarly, at pH 7–8, higher efflux rate was observed in the presence of AHA at 12 relative to 6 mg C L^{-1} ($t = -2.485$, $df = 15$, $p < 0.01$, $r = 0.540$, Student's *t*-test).

3.4. The influence of DOM on ammonia and urea excretion

In controls (i.e. no added DOM), *D. magna* had a higher average ammonia excretion rate of 4.21 ± 0.24 μmol g^{-1} h^{-1} at low pH compared to that of 2.20 ± 0.16 μmol g^{-1} h^{-1} at circumneutral pH ($t = -6.954$, $df = 8$, $p < 0.001$, $r = 0.926$, Student's *t*-test; Fig. 4A). Likewise, urea excretion rates were 0.51 ± 0.05 and 0.24 ± 0.00 μmol g^{-1} h^{-1} for acidic and circumneutral conditions, respectively (Fig. 4B). Overall, these order of magnitude differences between ammonia and urea excretion rates were consistent across treatments. Therefore, on a unit N basis (i.e. 2 N per urea molecule), urea-N excretion rates were about 20% of ammonia-N excretion rates.

The two-way ANOVA demonstrated that ammonia excretion rates of *D. magna* were significantly affected by both DOM source and pH condition and their interaction ($F_{4, 38} = 23.236$, $p < 0.001$, $\omega = 0.555$; $F_{1, 38} = 48.891$, $p < 0.001$, $\omega = 0.704$; $F_{4, 38} = 2.846$, $p < 0.05$, $\omega =$

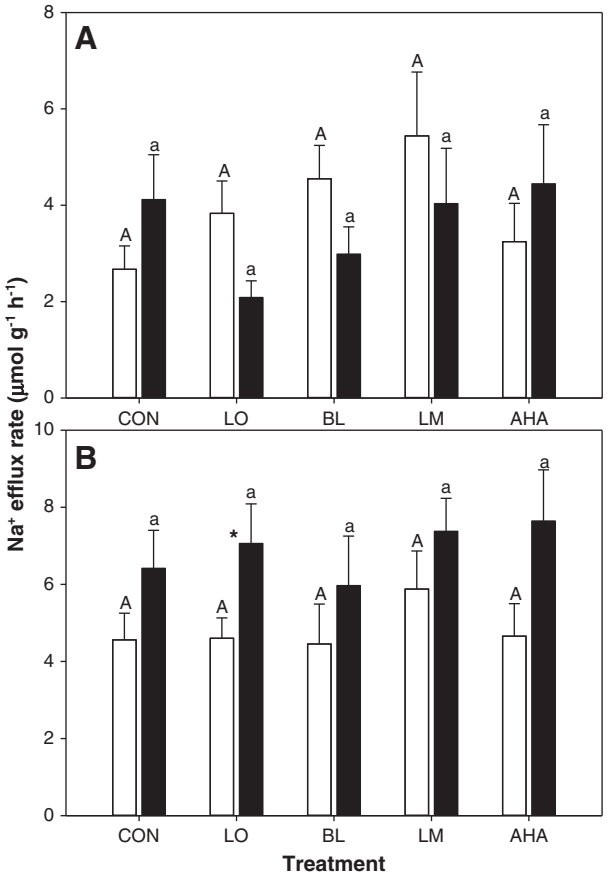

Fig. 3. Na^+ efflux rates (μmol g^{-1} h^{-1}) of *D. magna* in the absence (CON, no added DOM) or presence of DOMs added at (A) 6 mg C $L^{-1}$and (B) 12 mg C L^{-1}DOC at circumneutral pH 7–8 (white bars) and low pH ~5 (black bars). Plotted values represent the mean ± standard errors for Na^+ efflux rates over 2 h of $n = 6$ – 19 of 5–6 day old adults. Within a pH, bars sharing the same letter (upper case for pH 7–8, lower case for pH ~5) are not significantly different. Asterisks indicate significant differences between pH's within the same DOM treatment.

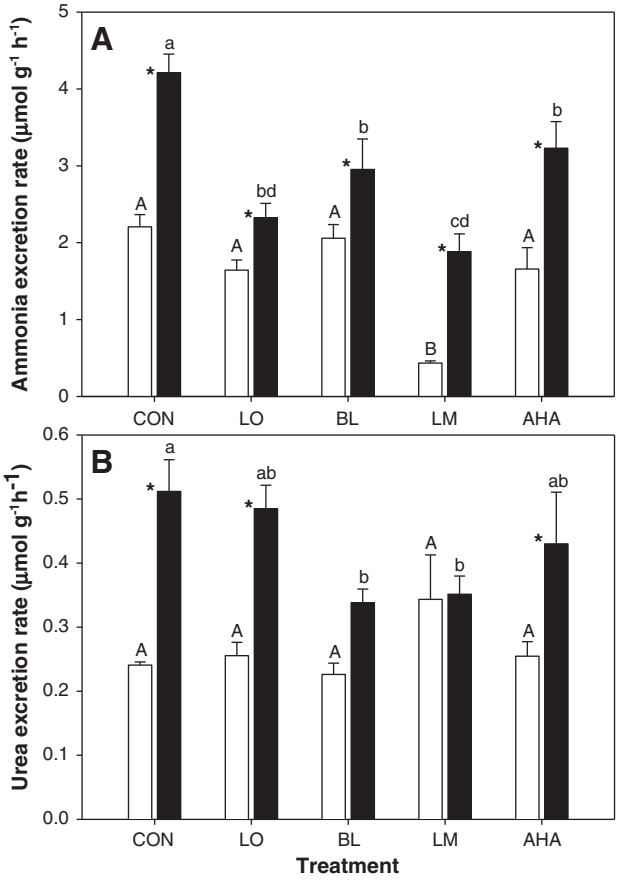

Fig. 4. Ammonia excretion rates (A) and urea excretion rates (B) of *D. magna* in the absence (CON, no added DOM) or presence of DOMs added at 6 mg C L^{-1} DOC at circumneutral pH 7–8, white bars) and low pH (~5, black bars). Plotted values represent the mean ± standard errors for rates over 24 h of $n = 4$ – 5 determinations with 5 individuals for each determination, using 5–6 day old adults. Within a pH, bars sharing the same letter (upper case for pH 7–8, lower case for pH ~5) are not significantly different. Asterisks indicate significant differences between pH's within the same DOM treatment.

0.236, respectively, Fig. 4A). For example, at circumneutral pH, organisms demonstrated similar excretion rates in all treatments except those in LM where the average rate was substantially lower (19–23% of rates in other treatments, p-values < 0.005 by Tukey's test, Fig. 4A). At pH ~5, ammonia excretion rates were significantly increased relative to pH 7–8 in all treatments, including the controls; the differences were significant in all groups (Fig. 4A). At pH ~5, lower ammonia excretion rates were observed in the presence of every DOM source relative to the control treatment (p-values < 0.005 by Tukey's test, Fig. 4A). Interestingly, daphnids in the presence of LM experienced a drastic reduction in the ammonia excretion relative to control, BL and AHA treatments (Fig. 4A).

No effect was found for various DOM treatments on urea excretion at circumneutral pH ($F_{4,\ 38} = 1.643$, $p = 0.184$, $\omega = 0.000$, two-way ANOVA). However, effects in some treatments seemed to be dependent on the pH condition as illustrated by the statistical significance of the interaction between the treatment and pH ($F_{4,\ 38} = 3.397$, $p < 0.05$, $\omega = 0.421$, two-way ANOVA). Interestingly, daphnids in BL and LM had significantly lower urea excretion rates than control at the acidic pH (p-values ≤ 0.05, Student's t-test, Fig. 4B). Moreover, pH alone appeared to influence the excretion significantly ($F_{1,\ 38} = 38.540$, $p < 0.001$, $\omega = 0.722$, two-way ANOVA). As with ammonia excretion rates, *D. magna* individuals exhibited substantially higher urea excretion rates in acidic media of CON, LO and AHA (p-values ≤ 0.05, Student's t-test, Fig. 4B) compared to the excretion rates in the corresponding circumneutral pH solutions.

3.5. The influence of DOM on water buffering capacities

Buffering capacities (β values) were higher in the presence of DOM isolates than the controls (Fig. 5). Overall, pH ~5 samples demonstrated consistently higher buffering capacity than pH 7–8 samples, though this was not true for AHA. Significant differences were observed for β between treatments ($F_{4,\ 74} = 67.348$, $p < 0.001$, $\omega = 0.665$, two-way ANOVA) and pH ($F_{1,\ 74} = 96.305$, $p < 0.001$, $\omega = 0.517$, two-way ANOVA). In addition, the buffering capacity was significantly impacted by the interaction between the treatment and pH ($F_{4,\ 74} = 10.978$, $p < 0.001$, $\omega = 0.395$, two-way ANOVA). In general, β values were greatest in BL, followed by LM.

4. Discussion

4.1. Sodium metabolism

The whole-body Na^+ concentrations, as well as the unidirectional Na^+ influx and efflux rates reported here either in control treatments (no added DOM) or in the presence of the DOMs were comparable to those found by Glover et al. (2005) and Glover and Wood (2005a,b) for the same species. In our study, *D. magna* in both circumneutral and low pH's had very similar whole-body Na^+ concentrations (Fig. 1), and generally similar unidirectional influx (Fig. 2) and efflux rates (Fig. 3). Nevertheless, there was a tendency for higher rates of both influx and efflux at the lower pH (i.e. an increase in Na^+ turnover rates), though this was not significant in most treatments. This cladoceran species has been generally reported to experience reduction in whole body Na^+ concentrations only under extreme low pH's of ≤4.5 and/or in very soft water (Potts and Fryer, 1979; Havas et al., 1984; Havas, 1985; Havas and Likens, 1985). For example, severe inhibition of Na^+ uptake was observed at pH 4.0 in water of comparable Ca^{2+} concentration (1.0 mM) to that of the present study (Glover and Wood, 2005b). In laboratory tests, *D. magna* appears to successfully regulate Na^+ metabolism over a pH range of 4.6 – 9.0, similar to the range where several *Daphnia* species are reported to be abundant in northern hemisphere lakes (Salonen and Hammer, 1986).

Overall, the three natural DOMs had negligible impact on Na^+ homeostasis. In agreement with the present study, Glover et al. (2005) similarly found no effect of another DOM isolate from Luther Marsh, one of the sources (LM) tested in the present study. However, Glover and Wood (2005a) and Glover et al. (2005) reported that AHA stimulated Na^+ influx and tended to raise whole body Na^+ concentration at circumneutral pH in *D. magna*, whereas our results (albeit under slightly different water chemistry) showed no effect on these parameters, and insignificant trends of reduced whole body Na^+ concentration (Fig. 1A) and increased Na^+ influx (Fig. 2A) at low pH. In the present study, the dechlorinated Lake Ontario water had relatively higher concentrations of Na^+ and Ca^{2+} ions (Tables 1 and 2) than the reconstituted water in Glover and Wood (2005a) and Glover et al. (2005). Consistently, no influence was seen on Na^+ efflux in the presence of AHA in this study or in Glover et al. (2005). Furthermore, at low pH, the presence of AHA was observed to cause severe exacerbation of the passive Na^+ loss of stenohaline freshwater stingrays (*Potamotrygon* spp.), while natural DOM protected against this loss (Wood et al., 2003). At least under the present water chemistry conditions (Tables 1 and 2), the unaltered Na^+ uptake (Fig. 2A) may imply no interference by DOM with the mechanisms responsible for the active Na^+ uptake in *D. magna* such as the electrogenic $2Na^+/1H^+$ exchanger (Glover and Wood, 2005b; Bianchini and Wood, 2008). Acidic pH simulated Na^+ influx of *D. magna* only in the absence of added DOMs (control, Fig. 2B), implying possible subtle beneficial effects of DOMs for *D. magna* in low pH environments since organisms would not need to accelerate Na^+ uptake.

A notable finding of the current study was the confirmation (in agreement with Glover and Wood, 2005a) that Na^+ metabolism of *D. magna* adults remained generally unaffected in the presence of the natural DOMs, regardless of their very different sources and chemistries (Al-Reasi et al., 2013). Our natural DOMs were obtained by reverse-osmosis, a method which isolates the organic matter directly from the natural source water (i.e. Lake Ontario, Bannister Lake or Luther Marsh) by differential membrane filtration. This method has been proven to yield representative organic matter from natural waters (De Schamphelaere et al., 2005). In contrast, commercial AHA and other commercially available NOMs (e.g. Suwanee River humic acid) are lyophilized. They have distinct aliphatic and aromatic molecular composition but similar elemental composition to natural water DOMs (Malcolm and MacCarthy, 1986). Absorbance and fluorescence spectroscopy has demonstrated that AHA deviates substantially from the natural DOMs utilized in this study in terms of aromatic composition and pure humic nature compared to natural DOMs which have mixed humic and fulvic molecular compositions (Al-Reasi et al., 2012).

4.2. Ammonia and urea excretion

The ranges of ammonia and urea excretion rates in this study (Fig. 4) were higher than the ranges of 0.00–2.94 and 0.10–0.17 μmol g^{-1} h^{-1}, respectively, reported for starved *D. magna* (Wiltshire and Lampert, 1999). This zooplanker (*D. magna*) liberates ammonia as the dominant excretory nitrogenous waste followed by urea (Wiltshire and Lampert, 1999) similar to other crustaceans and fish (Weihrauch et al., 2009). While Na^+ homeostasis was not affected by the acidic condition, lower environmental pH resulted in higher ammonia and urea excretion rates by daphnids relative to circumneutral pH (Fig. 4). One of the proposed mechanisms of ammonia excretion is passive diffusion of gaseous NH_3 facilitated by acid trapping (i.e. combining H^+ with NH_3 to form ammonium ion, NH_4^+) in the water next to ion-transporting epithelia (i.e. boundary layer) (Wilkie, 2002). The pH of this layer is usually lower than that of the bulk surrounding water (Wright et al., 1988) and further acidification would result in more ammonia being excreted as an adaptive response by daphnids to raise boundary layer pH. Alternatively, acidic water may be a stressful situation where protein catabolism of *D. magna* is accelerated, resulting in higher metabolic production rates of both ammonia and urea, and therefore higher excretion rates of both N-products.

The most allochthonous DOM (LM) markedly depressed ammonia excretion in both acidic and circumneutral pH conditions (Fig. 4). At the lower water pH, ammonia excretion rates were significantly attenuated by the presence of all three natural DOMs and the commercial AHA (Fig. 4A). On the other hand, *D. magna* had reduced urea excretion rates only when exposed to the two allochthonous DOM isolates (BL and LM) in lower pH media (Fig. 4B). At least in part, the reduction in ammonia excretion in the presence of the natural DOMs may be attributable to their ability to raise the buffering capacity of the water, an effect which is greater at pH ~5 than at circumneutral pH (Fig. 5). The presence of DOM molecules with higher buffer capacity may ensure that the pH of the boundary layer (one possible mechanism for ammonia excretion as explained above) can be kept relatively constant. Published humic acid titrations demonstrate buffering capacity values consistent with those calculated here. For various humic acids, buffering capacities at pH 5 calculated for 6 mg C L^{-1} are in the range of 15 to 30 μmol pH^{-1} L^{-1} and for pH 7 in the range 7.5 to 15 μmol pH^{-1} L^{-1} (Boguta and Sokolowska, 2012). The trend of higher pH samples having lower buffering makes sense because buffer capacity maxima occur when the pH = pK_a. DOM tends to have more acidic (pK_a 4–5) values than neutral pK_a values.

The most highly colored allochthonous source (LM) was intermediate in its ability to raise the buffering capacity of the water (Fig. 5), yet was very effective in reducing ammonia excretion at both circumneutral and acidic pH (Fig. 4A). Daphnids had similar urea excretion rates under both pH conditions in the presence of BL and LM. This prevention of increase in urea excretion at low pH was not observed with the other DOMs (Fig. 4B). Among the DOM sources tested, both BL and LM have high abilities to form strong tridentate complexes as estimated by their higher Proton Binding Indices (PBI; Al-Reasi et al., 2013). Fig. 6 illustrates the clear correlations between ammonia excretion rates of *D. magna* and PBI. The high aromatic content which contributes to the darker color will also allow closer spacing of functional groups, and therefore greater multidendate complexation capacity (Al-Reasi et al., 2013). These characteristics contribute to higher chemical reactivities of the molecules. Indeed, we have predicted that DOMs with higher PBI values should interact more strongly with functional groups on the external physiological membranes of aquatic animals (Al-Reasi et al., 2013). In particular, the potency of LM in attenuating nitrogenous waste excretion in daphnids supports this prediction. One possibility is the direct interaction of DOM molecules with ammonia transporters

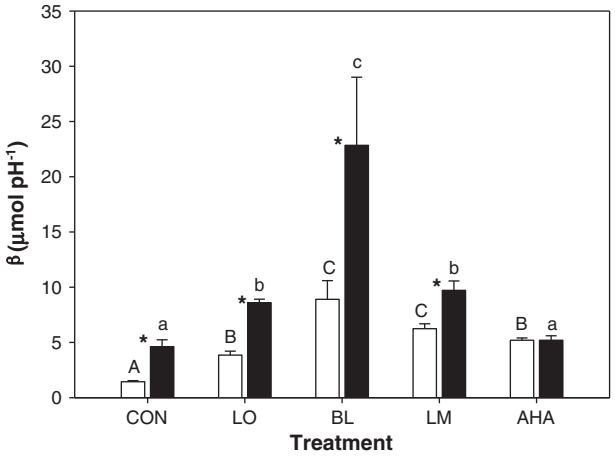

Fig. 5. Buffer capacities of control (CON, no added DOM) and treatments with DOMs added at 6 mg C L^{-1} DOC at circumneutral pH (7–8, white bars) and low pH (~5, black bars). Plotted values represent the mean ± standard errors of the capacities calculated from n = 4 – 12 titrations. Within a pH, bars sharing the same letter (upper case for pH 7–8, lower case for pH ~5) are not significantly different. Asterisks indicate significant differences between pH's within the same DOM treatment. Details of titrations are provided in Al-Reasi et al. (2013).

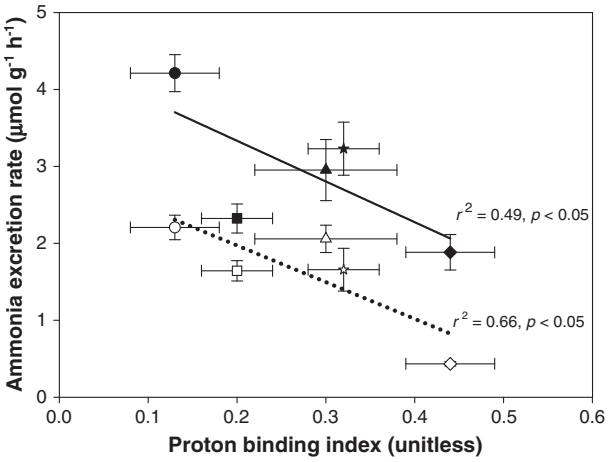

Fig. 6. The correlations between ammonia excretion rates of *D. magna* and Proton Binding Index (PBI, an index of chemical reactivity) in control (no added DOM, circle symbols) and treatments with DOMs added at 6 mg C L^{-1} DOC at circumneutral pH (7–8, dotted line, open symbols) and low pH (~5, solid line, closed symbols). Symbols of DOMs are squares (LO), triangles (BL), diamonds (LM) and stars (AHA). Plotted values represent the mean ± standard errors. The regression lines and r^2 values have been calculated separately for data at circumneutral pH versus acidic pH. The Proton Binding Index values are taken from Al-Reasi et al. (2013).

(e.g. Rhesus proteins as transporters for NH_3/NH_4^+) proposed for ammonia excretion in aquatic organisms including crustaceans (Weihrauch et al., 2009).

In the presence of various DOM sources, the highly variable physiological responses (e.g. unaffected Na^+ regulation and depressed ammonia excretion in acidic media) of *D. magna* may suggest that this organism has developed adjustments to deal with the changes in the structure and composition of aquatic DOM over time. Such adaptations have been observed for *D. magna* for other characteristics, such as the development of toxicity resistance to metals (Ward and Robinson, 2005). Cellular components responsible for chemical traffic in this crustacean would have evolved in concert with exposure to DOM sources of certain quality, similar to co-evolution with other important water quality components such as hardness, alkalinity, major ions, and pH. Indeed, DOM concentration and quality (i.e. sources) and pH are vital water chemistry factors influencing several phenomena of freshwater animals including *D. magna*. Both factors act as toxicity modifying factors for several metals (e.g. Heijerick et al., 2003; Al-Reasi et al., 2011) and impact the ammonia excretion as illustrated in the current study, particularly in the combined condition of low pH and in the presence of allochthonous DOM sources. Possible variation in response to DOMs as a result of co-evolution of organisms with specific DOMs in specific habitats is worthy of future study.

4.3. Future perspectives

It is noteworthy that the effects of natural DOMs in depressing ammonia excretion at low pH in *D. magna* are exactly opposite those reported in one study on a freshwater stingray species where natural, highly allochthonous DOM greatly raised ammonia excretion rate at low pH (Wood et al., 2003). Furthermore, natural DOMs had marked positive effects on ionoregulation in several species of fish at low pH (Gonzalez et al., 1998, 2002; Wood et al., 2003; Matsuo et al., 2004), while such effects were not generally seen in daphnids. At present it is unclear why these responses differ, and whether the different response patterns in *D. magna* are adaptive, as they are thought to be in fish. Clearly, there is a need for more work in this area on both crustaceans and fish, using natural DOMs with detailed physico-chemical characterization, rather than commercially prepared surrogates.

Acknowledgments

Hassan A. Al-Reasi was supported by a doctoral scholarship from the Government of Oman. This work was funded by Discovery grants from the Natural Sciences and Engineering Research Council of Canada (NSERC) to CMW and DSS. CMW is supported by the Canada Research Chair program.

References

Al-Reasi, H.A., Wood, C.M., Smith, D.S., 2011. Physicochemical and spectroscopic properties of natural organic matter (NOM) from various sources and implications for ameliorative effects on metal toxicity to aquatic biota. Aquat. Toxicol. 103, 179–190.

Al-Reasi, H.A., Scott, D.S., Wood, C.M., 2012. Evaluating the ameliorative effect of natural dissolved organic matter (DOM) quality on copper toxicity to *Daphnia magna*: improving the BLM. Ecotoxicology 21, 524–537.

Al-Reasi, H.A., Wood, C.M., Scott, D.S., 2013. Characterization of freshwater natural dissolved organic matter (DOM): mechanistic explanations for protective effects against metal toxicity and direct effects on organisms. Environ. Int. 59, 201–207.

Alsop, D., Wood, C.M., 2011. Metal uptake and acute toxicity in zebrafish: common mechanisms across multiple metals. Aquat. Toxicol. 105, 385–393.

Barth, B.J., Wilson, R.S., 2010. Life in acid: interactive effects of pH and natural organic acids on growth, development and locomotor performance of larval striped marsh frogs (*Limnodynastes peronei*). J. Exp. Biol. 213, 1293–1300.

Bianchini, A., Wood, C.M., 2008. Sodium uptake in different stages of crustaceans: the water flea *Daphnia magna* Strauss. J. Exp. Biol. 211, 539–547.

Boguta, P., Sokolowska, Z., 2012. Influence of phosphate ions on buffer capacity of soil humic acids. Int. Agrophys. 26, 7–14.

Campbell, P.G.C., Twiss, M.R., Wilkinson, K.J., 1997. Accumulation of natural organic matter on the surfaces of living cells: implications for the interaction of toxic solutes with aquatic biota. Can. J. Fish. Aquat. Sci. 54, 2543–2554.

De Schamphelaere, K.A.C., Unamuno, V.I.R., Tack, F.M.G., Vanderdeelen, J., Janssen, C.R., 2005. Reverse osmosis sampling does not affect the protective effect of dissolved organic matter on copper and zinc toxicity to freshwater organisms. Chemosphere 58, 653–658.

Ertel, J.R., Hedges, J.I., Perdue, E.M., 1984. Lignin signature of aquatic humic substances. Science 223, 485–487.

Galvez, F., Donini, A., Playle, R.C., Smith, S., O'Donnell, M., Wood, C.M., 2009. A matter of potential concern: natural organic matter alters the electrical properties of fish gills. Environ. Sci. Technol. 42, 9385–9390.

Glover, C.N., Wood, C.M., 2005a. The disruption of *Daphnia magna* sodium metabolism by humic substances: mechanism of action and effect of humic substance source. Physiol. Biochem. Zool. 78, 1005–1016.

Glover, C.N., Wood, C.M., 2005b. Physiological characterization of a pH- and calcium-dependent sodium uptake mechanism in the freshwater crustacean, *Daphnia magna*. J. Exp. Biol. 208, 951–959.

Glover, C., Pane, E.F., Wood, C.M., 2005. Humic substances influence sodium metabolism in the freshwater crustacean *Daphnia magna*. Physiol. Biochem. Zool. 78, 405–416.

Gonzalez, R.J., Wood, C.M., Wilson, R.W., Patrick, M.L., Bergman, H.L., Narahara, A., Val, A.L., 1998. Effects of water pH and calcium concentration on ion balance in fish of the Rio Negro, Amazon. Physiol. Zool. 71, 15–22.

Gonzalez, R.J., Wilson, R.W., Wood, C.M., Patrick, M.L., Val, A.L., 2002. Diverse strategies for ion regulation in fish collected from the ion-poor, acidic Rio Negro. Physiol. Biochem. Zool. 75, 37–47.

Grosell, M., Wood, C.M., 2002. Copper uptake across rainbow trout gills: mechanisms of apical entry. J. Exp. Biol. 205, 1179–1188.

Hargeby, A., Petersen, R.C., 1988. Effects of low pH and humus on the survivorship, growth and feeding of *Gammarus pulex* (L.) (Amphipoda). Freshw. Biol. 19, 235–247.

Hatcher, P.G., Spiker, E.C., 1988. Selective degradation of plant biomolecules. In: Frimmel, F.H., Christman, R.F. (Eds.), Humic Substances and their Role in the Environment. John Wiley and Sons, New York, pp. 59–74.

Havas, M., 1985. Aluminum bioaccumulation and toxicity to *Daphnia magna* in soft water at low pH. Can. J. Fish. Aquat. Sci. 42, 1741–1748.

Havas, M., Likens, G.E., 1985. Changes in ^{22}Na influx and outflux in *Daphnia magna* (Straus) as a function of elevated Al concentrations in soft water at low pH. Proc. Natl. Acad. Sci. U. S. A. 82, 7345–7349.

Havas, M., Huthinson, T.C., Likens, G.E., 1984. Effect of low pH on sodium regulation in two species of Daphnia. Can. J. Zool. 62, 1965–1970.

Heijerick, D.G., Janssen, C.R., De Coen, W.M., 2003. The combined effects of hardness, pH, and dissolved organic carbon on the chronic toxicity of Zn to *D. magna*: development of a surface response model. Arch. Environ. Contam. Toxicol. 44, 210–217.

Malcolm, R.L., MacCarthy, P., 1986. Limitations in the use of commercial humic acids in water and soil research. Environ. Sci. Technol. 20, 904–911.

Matsuo, A.Y.O., Playle, R.C., Val, A.L., Wood, C.M., 2004. Physiological action of dissolved organic matter in rainbow trout in the presence and absence of copper: sodium uptake kinetics and unidirectional flux rates in hard and soft water. Aquat. Toxicol. 70, 63–81.

Matsuo, A.Y.O., Woodin, B.R., Reddy, C.M., Val, A.L., Stegeman, J.J., 2006. Humic substances and crude oil induce CYP1A expression in the Amazonian fish species *Colossoma macropomum* (tambaqui). Environ. Sci. Technol. 40, 2851–2858.

McDonald, D.G., Rogano, M.S., 1986. Ion regulation by the rainbow trout, *Salmo gairdneri*, in ion-poor water. Physiol. Zool. 59, 318–331.

McKnight, D.M., Boyer, E.W., Westerhoff, P.K., Doran, P.T., Kulbe, T., Andersen, D.T., 2001. Spectrofluorometric characterization of dissolved organic matter for indication of precursor organic material and aromaticity. Limnol. Oceanogr. 46, 38–48.

Meems, N., Steinberg, C.E.W., Wiegand, C., 2004. Direct and interacting toxicological effects on the waterflea (*Daphnia magna*) by natural organic matter, synthetic humic substances and cypermethrin. Sci. Total. Environ. 319, 123–136.

Potts, T.W., Fryer, G., 1979. The effects of pH and salt content on sodium balance in Daphnia magna and Acantholeberis curvirostris (Crustacea: Cladocera). J. Comp. Physiol. B 129, 289–294.

Rahmatullah, M., Boyde, T.R.C., 1980. Improvements in the determination of urea using diacetyl monoxime; methods with and without deproteinisation. Clin. Chim. Acta 107, 3–9.

Salonen, K., Hammar, T., 1986. On the importance of the dissolved organic matter in the nutrition of zooplankton in some lake waters. Oecologia 68, 246–253.

Schwartz, M.L., Curtis, P.J., Playle, R.C., 2004. Influence of natural organic matter on acute copper, lead, and cadmium toxicity to rainbow trout (*Oncorhynchus mykiss*). Environ. Toxicol. Chem. 23, 2889–2899.

Smith, D.S., Ferris, F.G., 2001. Proton binding by hydrous ferric oxide and aluminum oxide surfaces interpreted using fully optimized continuous pK_a spectra. Environ. Sci. Technol. 35, 4637–4642.

Steinberg, C.E.W., Kamara, S., Prokhotskaya, V.Y., Manusadžianas, L., Karasyova, T., Timofeyev, M.A., Zhang, J., Paul, A., Meinelt, T., Farjalla, V.F., Matsuo, A.Y.O., Burnison, B.K., Menzel, R., 2006. Dissolved humic substances-ecological driving forces from the individual to the ecosystem level? Freshw. Biol. 51, 1189–1210.

Stobbart, R.H., Keating, J., Earl, R., 1977. A study of sodium uptake by the water flea *Daphnia magna*. Comp. Biochem. Physiol. A Physiol. 58, 299–309.

Stumm, W., Morgan, J.J., 1996. Aquatic Chemistry, Chemical Equilibria and Rates in Natural Waters, 3rd ed. John Wiley and Sons Inc., New York.

Thomas, J.D., 1997. The role of dissolved organic matter, particularly free amino acids and humic substances, in freshwater ecosystems. Freshw. Biol. 38, 1–36.

Thurman, E.M., 1985. Organic Geochemistry of Natural Waters. Dr W. Junk Publishers, Dordrecht.

Timofeyev, M.A., Wiegand, C., Burnison, B.K., Shatilina, Z.M., Pflugmacher, S., Steinberg, C.E.W., 2004. Direct impact of natural organic matter (NOM) on freshwater amphipods. Sci. Total. Environ. 319, 115–121.

Verdouw, H., Vanechteld, C.J.A., Deckkers, E.M.J., 1978. Ammonia determinations based on indophenol formation with sodium salicylate. Water Resour. 12, 399–402.

Vigneault, B., Percot, A., Lafleur, M., Campbell, P.G.C., 2000. Permeability changes in model and phytoplankton membranes in the presence of aquatic humic substances. Environ. Sci. Technol. 34, 3907–3913.

Ward, T.J., Robinson, W.E., 2005. Evolution of cadmium resistance in *Daphnia magna*. Environ. Toxicol. Chem. 24, 2341–2349.

Weihrauch, D., Wilkie, M.P., Walsh, P.J., 2009. Ammonia and urea transporters in gills of fish and aquatic crustaceans. J. Exp. Biol. 212, 1716–1730.

Wilkie, M.P., 2002. Ammonia excretion and urea handling by fish gills: present understanding and future research challenges. J. Exp. Zool. 293, 284–301.

Wiltshire, K.H., Lampert, W., 1999. Urea excretion by Daphnia: a colony-inducing factor in *Scenedesmus*? Limnol. Oceanogr. 44, 1894–1903.

Wood, C.M., Wilson, J.R., Gonzalez, R.J., Patrick, M.L., Bergman, H.L., Narahara, A., Val, A.L., 1998. Responses of an Amazonian teleost, the Tambaqui (*Colossoma macropomum*), to low pH and extremely soft water. Physiol. Biochem. Zool. 17, 658–670.

Wood, C.M., Matsuo, A.Y.O., Wilson, R.W., Gonzalez, R.J., Patrick, M.L., Playle, R.C., Val, A.L., 2003. Protection by natural blackwater against disturbances in ion fluxes caused by low pH exposure in freshwater stingrays endemic to the Rio Negro. Physiol. Biochem. Zool. 76, 12–27.

Wood, C.M., Al-Reasi, H.A., Scott, D.S., 2011. The two faces of DOC. Aquat. Toxicol. 105, 3–8.

Wright, P.A., Randall, D.J., Perry, S.F., 1988. Fish gill water boundary layer: a site of linkage between carbon dioxide and ammonia excretion. J. Comp. Physiol. B 158, 627–635.

Zimmer, A.M., Barcarolli, I.F., Wood, C.M., Bianchini, A., 2012. Waterborne copper exposure inhibits ammonia excretion and branchial carbonic anhydrase activity in euryhaline guppies acclimated to both fresh water and sea water. Aquat. Toxicol. 122–123, 172–180.