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ORIGINAL PAPER

Physiological protective action of dissolved organic carbon on ion regulation and nitrogenous waste excretion of zebrafish (*Danio rerio*) exposed to low pH in ion-poor water

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Abstract

Dissolved organic carbon (DOC) represents a heterogeneous group of naturally-occurring molecules in aquatic environments, and recent studies have evidenced that optically dark DOCs can exert some positive effects on ionoregulatory homeostasis of aquatic organisms in acidic waters. We investigated the effects of Luther Marsh DOC, a dark allochthonous DOC, on ion regulation and N-waste excretion of zebrafish acutely exposed to either neutral or low pH in ion-poor water. In the first experiment, simultaneous exposure to pH 4.0 and DOC greatly attenuated the stimulation of Na^+ diffusive losses (J^{Na}_{out}), and prevented the blockade of Na^+ uptake (J^{Na}_{in}) seen in zebrafish exposed to pH 4.0 alone, resulting in much smaller disturbances in Na^+ net losses (J^{Na}_{net}). DOC also attenuated the stimulation of net Cl^- losses (J^{Cl}_{net}) and ammonia excretion (J^{Amm}_{net}) during acidic challenge. In the second experiment, zebrafish acclimated to DOC displayed similar regulation of J^{Na}_{in} and J^{Na}_{out}, and, therefore, reduced J^{Na}_{net} at pH 4.0, effects which persisted even when DOC was no longer present. Protective effects of prior acclimation to DOC on J^{Cl}_{net} and J^{Amm}_{net} at pH 4.0 also occurred, but were less marked than those on Na^+ balance. Urea fluxes were unaffected by the experimental treatments. Overall, these effects were clearly beneficial to the ionoregulatory homeostasis of zebrafish at low pH, and were quite similar to those seen in a recent parallel study using darker DOC from the upper Rio Negro. This suggests that dark allochthonous DOCs share some chemical properties that render fish tolerant to ionoregulatory disturbances during acidic challenge.

Keywords Natural organic matter · Na^+ uptake · Ammonia excretion · Paracellular Na^+ losses · Chloride net fluxes · Allochthonous DOC

Introduction

Acidic waters can cause an imbalance of ionoregulation of freshwater fishes, particularly through the inhibition of active uptake of Na^+ and Cl^-, and also through stimulation of ionic diffusive losses (reviewed by Wood 1989; Kwong et al. 2014), resulting in decreases in plasma levels of major ions and a reversal of the electrical potential across the gill epithelium (McWilliams and Potts 1978). Dissolved organic matter (DOM), measured as dissolved organic carbon (DOC), represents a group of molecules that occur naturally in freshwater environments, produced by the degradation of lignin-rich plant material and by the decay of dead organic biomass (Thurman 1985). Recent studies have indicated that DOC molecules can directly affect the physiology of aquatic organisms and in particular exert some advantageous effects on ionoregulatory homeostasis in acidic waters.

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These positive effects of DOC on ionoregulation fall into two categories: (i) reduction or prevention of increased diffusive ion losses during low pH exposure (Amazonian stingray, *Potamotrygon* sp., Wood et al. 2003; cardinal tetra, *Paracheirodon axelrodi*, Matsuo and Val 2007; cichlid *Geophagus* sp. and catfish *Pimelodes* sp., Gonzalez et al. 2002, 2005; zebrafish, *Danio rerio*; Duarte et al. 2016; Al-Reasi et al. 2016) and (ii) reduction or prevention of inhibited active uptake of ions (or even stimulation of uptake), particularly Na^+, during low pH exposure (*Potamotrygon* sp., Wood et al. 2003; rainbow trout, *Oncorhynchus mykiss*, Matsuo et al. 2004; *Paracheirodon axelrodi*; Matsuo and Val 2007; *Geophagus sp*. and *Pimelodes* sp., Gonzalez et al. 2002, 2005; *Danio rerio*; Duarte et al. 2016; Al-Reasi et al. 2016). While these patterns of DOC effects seem to be qualitatively consistent, their magnitude varies greatly among species. In addition, some of these same investigations (Wood et al. 2003; Duarte et al. 2016; Al-Reasi et al. 2016) also examined DOC effects on the excretion of nitrogenous wastes (ammonia and urea), but responses were inconsistent amongst investigations.

Against this background, the picture that emerges is that beneficial physiological effects of DOC on ionoregulation of aquatic organisms under acidic conditions may be species-specific, and also related to the specific physicochemical properties of the specific DOC source (Wood et al. 2011). In particular, these include the proton binding index (PBI, a measure of chemical reactivity) and the specific absorbance coefficient (SAC_{340}, an indicator of aromatic composition of DOC). Optically darker, aromatic DOCs of allochthonous origin with high SAC_{340} and high PBI seem to be the most effective in protecting ionoregulatory homeostasis under acidic conditions (Al-Reasi et al. 2013b, 2016; Duarte et al. 2016), though the relative importance of SAC_{340} versus PBI in this regard are unknown.

To date, the most marked protective effects were those reported by Duarte et al. (2016), who isolated DOC from the Upper Rio Negro at São Gabriel da Cachoeira (SGC) district (Brazil), a highly acidic, ion-poor "blackwater" river of the Amazon basin which nevertheless supports a great diversity and endemicity of fish species (Goulding et al. 1988). When tested at a realistic environmental concentration (8.7 mg C L^{-1}), SGC DOC completely prevented the large net losses of Na^+ and Cl^- which otherwise occurred when zebrafish were exposed to pH 4.0 in ion-poor water (i.e., very low levels of Na^+, K^+, Ca^{2+}, Mg^{2+} and Cl^-). For Na^+, this was accomplished by a marked attenuation of the otherwise elevated diffusive efflux rate, and by an actual stimulation of the active influx rate above control levels. For the latter, the normal coupling of Na^+ influx to ammonia excretion (Kumai and Perry 2011; Kwong et al. 2014) was maintained by the presence of SGC DOC during low pH exposure, whereas it broke down in the absence of this DOC, with complete inhibition of Na^+ influx despite elevated ammonia excretion. Furthermore, prior acclimation to SGC DOC (8.9 mg C L^{-1}) at neutral pH provided similar protection against acid-induced ionoregulatory disturbances, even if the DOC was no longer present. Duarte et al. (2016) speculated that these remarkable effects of SGC DOC were critical in allowing a variety of fish species to thrive in the Rio Negro, because the highly acidic, ion-poor "blackwater" would otherwise be highly toxic. They attributed these actions to the very high PBI and SAC_{340} values of the DOC (Table 1), but also noted that an unusual proteinaceous component of SGC DOC was detected by parallel factor analysis (PARAFAC) of excitation–emission (EEM) matrix scans (Stedmon and Bro 2008; Ishii and Boyer 2012) and may have been another important factor. This was suspected to be violacein, a purple pigment produced by *Chromobacterium violaceum*, a microbe which is abundant in Rio Negro waters (Guarim 1979; Caldas 1990).

In a recent study, Al-Reasi et al. (2016) reported that another highly aromatic DOC isolated from an acidic bog in Canada, Luther Marsh (LM), had rather limited effects on Na^+ balance in zebrafish exposed to pH 5.0, suggesting that the SGC effects were unique. Notably, the PBI and SAC_{340} values of LM DOC were not as high as for SGC DOC, and it appeared to lack the unusual proteinaceous component (Table 1). However, the comparison was confounded by the less acidic pH (5.0 versus 4.0), lower concentration of DOC (6 versus 8–9 mg C L^{-1}) and different water quality (much higher ion levels) used by Al-Reasi et al. (2016) in comparison to Duarte et al. (2016).

To resolve this issue, in the current study, we have evaluated the effects of LM DOC under virtually identical conditions to those used in the SGC study (Duarte et al. 2016). Specifically, we have tested the hypothesis that the protective effects of LM DOC would be far less than those of SGC DOC under these conditions, reflecting differences in the chemistries of the two DOCs (Table 1). For this purpose, we examined the effects of acute exposure to pH 4.0 on the ionoregulatory homeostasis and nitrogenous waste excretion of zebrafish, in the presence and absence of LM DOC, in two experimental series, first in fish acclimated to ion-poor water without DOC, and second in fish acclimated to high DOC concentration in the same water.

Materials and methods

Experimental animals and acclimation

All the experimental procedures with zebrafish were approved by the McMaster University Animal Research Ethics Board (AUP 12-12-45), and were performed in accordance with the guidelines on "The care and use of fish in

Table 1 Comparison between some physicochemical properties of natural dissolved organic carbon (DOC) samples isolated by reverse-osmosis from Luther Marsh (Canada) and Rio Negro (Brazil)

DOC source	Coordinates	Type	SAC_{340}	$Abs_{254/365}$	FI	HA	FA	Tryp	Tyr	Binding ligand capacities (L_T, μmol mg^{-1})			
										Acid	Intermediate	Basic	PBI
Luther Marsh (LM)	43°37′N 80°26′W	Terrigenous	39.3[a]	3.7[a]	1.2[a]	95.0–84.7[bc]	9.7–5.0[bc]	> 1.0[bc]	–	1.7[a]	0.7[a]	1.4[a]	0.4[a]
São Gabriel da Cachoeira (SGC)	0°07′S 67°05′W	Terrigenous	73.0	2.9	1.3	51.3	36.0	7.8	4.7	1.2	0.8	1.5	0.6

[a]Data from Al-Reasi et al. (2013a); [bc]Al-Reasi et al. (2012) and Al-Reasi et al. (2011)

research, teaching and testing" of the Canadian Council for Animal Care (2005).

Adult zebrafish (0.395 ± 0.012 g in Series 1 and 0.411 ± 0.015 g in Series 2) were purchased from Pets Mart (Hamilton, Canada), and acclimated in 50-L aquaria to laboratory conditions for 1 month in moderately hard Lake Ontario water (Na^+ 600 μM, Cl^- 800 μM, K^+ 50 μM, Ca^{2+} 900 μM and Mg^{2+} 300 μM, pH 8.0). During this period fish were fed daily to satiation with a commercial food (New Life Spectrum, Homestead, USA). After this first acclimation period, 50% of the water was replaced daily with reconstituted ion-poor water (IPW) until the desired final composition was reached: Na^+ 50 μM, Cl^- 80 μM, K^+ 15 μM, Ca^{2+} 10 μM and Mg^{2+} 3 μM, pH 7.0, a very similar ionic composition to that used in the previous study (Duarte et al. 2016). Fish were allowed to acclimate for at least 1 week to this IPW before the experiments. Biological filters kept total ammonia levels below 20 μM in all acclimations. All acclimations and experiments were conducted in a temperature-controlled room (23–24 °C) with a 12 h /12 h light/dark regime.

DOC isolation and experimental solutions

The DOC isolate was obtained by reverse-osmosis from Luther Marsh (LM, 43°37′N 80°26′W) in Ontario, Canada, as previously described in detail (Al-Reasi et al. 2012). Briefly, after collection, the NOM concentrates were treated with a cation exchange resin (Amberlite IR-118 (H), Sigma-Aldrich, St. Louis, USA), to avoid interferences by cations built up during reverse-osmosis (Al-Reasi et al. 2012). DOC concentrate from LM was then 0.45-μm filtered (Acrodisc™, Pall, Ann Arbour, USA), characterized for physico-chemical properties (Al-Reasi et al. 2012, 2013a, b) and stored at 4 °C prior to use. In both experimental series, LM DOC concentrate was the same batch as originally used in earlier studies (Al-Reasi et al. 2012, 2013a, b). LM DOC concentrate was diluted with reconstituted ion-poor water (IPW, see below), and test solutions were stored at dark for 24 h prior to the experiments (Glover et al. 2005b). Immediately before the start of tests, the pHs of all experimental solutions, with or without DOC, were adjusted to neutral pH 7.0 (with 0.01 N KOH) or pH 4.0 (with 0.01 N HNO_3) as appropriate.

Experimental setup

Prior to the experiments, fish ($N = 10$ per treatment) were transferred from the holding aquaria to individual 40-mL aerated chambers filled with reconstituted ion-poor water (IPW—pH 7.0, with or without DOC), for a 1-h settling period. Then 0.01 μCi mL^{-1} of $^{22}NaCl$ (Amersham, Little Chalfont, UK) was added to each experimental chamber, and following 5 min of mixing by aeration, a 3-h flux

measurement was started with 6-mL water samples taken at 0 and 3 h, representing the control period. After this first 3-h flux period, water in each chamber was replaced with a fresh reconstituted IPW solution representing one of the three experimental conditions (see below). This procedure involved two people working simultaneously with 60-mL syringes, one removing the water and the other adding it, so the fish was never air-exposed. Again, 0.01 μCi mL^{-1} of $^{22}NaCl$ was added to all chambers, and following 5 min of mixing, another 3-h flux measurement was carried out. Further, for all three groups, water in chambers was changed over again, back to fresh IPW—pH 7.0 (with or without DOC) similar to the control period, and after the addition of $^{22}NaCl$ (0.01 μCi mL^{-1}), a 3-h recovery flux measurement was made in this acclimation water. This protocol of water and radioisotope renewal every 3 h ensured that internal specific activity never exceeded 10% of external specific activity, so backflux correction was unnecessary (Kirschner 1970). Mean background ammonia concentrations ranged from 13 to 19 μmol L^{-1} and did not differ significantly among experimental treatments or series.

Throughout all experiments, pH values in all chambers were measured hourly during each flux period using a hand-held H160 portable meter and epoxy body pH probe (Hach Co., Loveland, CO, USA) which was calibrated with precision pH 4.0 and 7.0 buffers (Fisher Scientific, Toronto, Canada) prior to each round of measurements (i.e., at each hour). The pH was adjusted to the desired level (pH 7.0 or pH 4.0) with 0.001 N KOH or 0.001 N HNO_3 when necessary (see Table 2 for pH, DOC, and ionic composition of experimental solutions). In Table 2, the values of pH represent the average of pH measurements over each 3-h flux period. Water samples were kept at 4 °C prior to measurements of ^{22}Na radioactivity, and total Na^+, Cl^-, ammonia and urea concentrations. After the experiments, fish were weighed and monitored; no mortalities occurred under any of the experimental conditions tested.

Series 1: acute effects of LM DOC on ion regulation and nitrogen excretion of zebrafish at low pH

The acute effects of LM DOC on unidirectional and net Na^+ flux rates, and net flux rates of Cl^-, ammonia and urea were assessed in zebrafish exposed to pH 7.0 and then acutely exposed to pH 4.0, with recovery at pH 7.0 thereafter. For this purpose, all three groups in the control period (0–3 h) were exposed to ion-poor water at pH 7.0 with no DOC (i.e., IPW—pH 7.0) which was the water to which they were acclimated. In the acute exposure period (3–6 h), the three treatments were ion-poor water plus DOC at pH 7.0 (IPW + DOC—pH 7.0), ion-poor water at pH 4.0 (IPW—pH 4.0; i.e., no DOC), and ion-poor water plus DOC at pH 4.0 (IPW + DOC—pH 4.0). During the recovery period (6–9 h), all three groups were exposed to IPW—pH 7.0, as in control period. In treatments where LM DOC was added the concentration of DOC was kept at around 9 mg C L^{-1} (see Table 2 for more details).

Series 2: effects of acclimation to LM DOC on ion regulation and nitrogen excretion of zebrafish at low pH

The effects of prior acclimation to LM DOC on unidirectional and net Na^+ flux rates, and Cl^-, ammonia and urea net flux rates were assessed in zebrafish exposed to pH 7.0 and pH 4.0. Therefore, fish were acclimated for 2 weeks to IPW + DOC—pH 7.0 (9.1 mg C L^{-1} of LM DOC, see Table 2 for more details), prior to the experimental exposures. All experimental procedures were conducted as described above, but in the control period (0–3 h), all groups were exposed to the acclimation condition, IPW + DOC—pH 7.0. In the acute exposure period (3–6 h), treatments were either IPW—pH 7.0, IPW—pH 4.0 (i.e., no DOC) or IPW + DOC—pH 4.0, followed by a final recovery period (6–9 h) for all groups in IPW + DOC—pH 7.0. The treatments with the presence or absence of DOC during the acute exposure to pH 4.0 were designed to differentiate effects

Table 2 Mean ionic composition, pH and DOC concentrations of all experimental solutions used in experimental series (1 and 2) for flux measurements with zebrafish under two different regimes of acclimation

Acclimation regime	Exposure conditions	pH	DOC	Na^+	Cl^-	Ca^{2+}	Mg^{2+}
Ion-poor water—pH 7.0 (Series 1)	IPW + DOC—pH 7.0	7.01 ± 0.08	8.8 ± 0.1	51.9 ± 2.1	89.1 ± 6.6	10.8 ± 0.3	3.2 ± 0.3
	IPW—pH 4.0	4.08 ± 0.03	0.8 ± 0.2	49.2 ± 2.6	88.5 ± 2.2	9.8 ± 0.3	3.4 ± 0.3
	IPW + DOC—pH 4.0	4.09 ± 0.02	8.9 ± 0.1	51.5 ± 2.5	91.6 ± 7.1	10.9 ± 0.3	3.7 ± 0.4
Ion-poor water + DOC—pH 7.0 (Series 2)	IPW—pH 7.0	7.08 ± 0.02	0.7 ± 0.1	49.3 ± 2.4	91.3 ± 2.6	9.7 ± 0.4	3.4 ± 0.3
	IPW—pH 4.0	4.06 ± 0.01	0.7 ± 0.2	50.3 ± 1.3	91.8 ± 3.2	10.9 ± 0.2	3.7 ± 0.2
	IPW + DOC—pH 4.0	4.07 ± 0.1	9.1 ± 0.1	51.5 ± 1.9	88.6 ± 1.7	10.0 ± 0.3	3.2 ± 0.1

DOC concentration of dissolved organic carbon from Luther Marsh (LM) in experimental solutions (mg C L^{-1}). All major ions concentrations were expressed in μM. Means ± 1 SEM

acquired from the prior acclimation to LM DOC from those dependent on the continued presence of LM DOC.

Analytical procedures and calculations

Unidirectional and net Na^+ flux rates (in nmol g^{-1} h^{-1}) were measured following procedures described according to Wood (1992). Briefly, mean specific activity (SA) of the radioisotope (cpm $nmol^{-1}$) in water samples was determined as the mean ratio between the concentration of ^{22}Na radioactivity (cpm mL^{-1}), and the concentration of total Na^+ in the water (nmol mL^{-1}) during each flux period. Unidirectional influx rates (J_{in}^{Na}) of fish at each experimental period were calculated as:

$$J_{in}^{Na} = \left(cpm_i - cpm_f\right) \times V \left(SA \times T \times W\right)^{-1}, \quad (1)$$

where cpm_i is the radioisotope concentration (cpm mL^{-1}) at the start of flux period, cpm_f is the radioisotope (cpm mL^{-1}) at the end of flux period, V is the volume of water in the experimental chamber (mL), T is the flux period (h) and W is the wet mass of fish (g).

The net flux rates (J_{net}) of Na^+, as well as of Cl^-, ammonia and urea, at each experimental period were calculated as:

$$J_{net} = \left(X_1 - X_2\right) \times V \left(T \times W\right)^{-1}, \quad (2)$$

where X_1 and X_2 were, respectively, the initial and final Na^+, Cl^- or total ammonia concentrations (nmol mL^{-1}) in the water during the flux period. Unidirectional efflux rates (J_{out}^{Na}) of Na^+ at each experimental period were then calculated as:

$$J_{out}^{Na} = J_{net}^{Na} - J_{in}^{Na}. \quad (3)$$

Total Na^+, K^+, Ca^{2+} and Mg^{2+} concentrations in water samples were determined using an atomic absorption spectrophotometer (Varian SpectrAA 220FS, Mulgrave, Australia), while ^{22}Na radioactivity in all water samples were determined using a Wizard 1480 Auto Gamma Counter (Perkin Elmer, Waltham, USA). Total Cl^- concentrations in water samples were determined colorimetrically through the mercury thiocyanate method (Zall et al. 1956), while the concentrations of ammonia and urea in the water were measured colorimetrically according to Verdouw et al. (1978) and Rahmatullah and Boyde (1980), respectively. In all cases, the experimental values were corrected for background absorbance due to the presence of coloured DOC in the water.

Statistical analyses

All data are reported as means ± 1 S.E.M. ($N = 10$). Significant differences in Na^+ influx (J_{in}^{Na}), efflux (J_{out}^{Na}), and net flux rates (J_{net}), and also in Cl^-, ammonia, and urea J_{net} values in both experimental series, were determined through a one-way repeated measures ANOVA, followed by the a posteriori Dunnett's multiple comparison test. In the case of a failed normality test, a non-parametric Kruskal–Wallis test was performed. Statistical significance was accepted at $p < 0.05$. All statistical analyses and graphics employed Sigma Stat and Sigma Plot software (Jandel Scientific, San Jose, USA).

Results

Series 1: acute effects of LM DOC on ion regulation and nitrogen excretion of zebrafish at low pH

The mean rates of J_{in}^{Na} (ranging from 336 to 482 nmol g^{-1} h^{-1}) and J_{out}^{Na} (−571 to −768 nmol g^{-1} h^{-1}) of zebrafish during the control period (i.e., pH 7.0 with no DOC) were quite similar and not significantly different among themselves in the three groups, resulting in slightly negative values of J_{net}^{Na} (−235 to −345 nmol g^{-1} h^{-1}) (Fig. 1a). During the exposure to DOC at neutral pH (i.e., IPW + DOC—pH 7.0), J_{in}^{Na} and J_{out}^{Na} were somewhat higher, but not statistically different, from those rates seen in fish during the control period (1.4-fold and 1.2-fold greater, respectively). Fish acutely exposed to pH 4.0 with no DOC (i.e., IPW—pH 4.0) demonstrated a complete inhibition of J_{in}^{Na} and a massive stimulation in J_{out}^{Na} (5.3-fold higher relative to the respective control mean), and over 3.2-fold higher compared to fish exposed to IPW + DOC—pH 7.0. Therefore, J_{net}^{Na} of zebrafish exposed to IPW—pH 4.0 became highly negative, 12.9-fold higher than in the same fish during the control period (Fig. 1a). In contrast, zebrafish acutely exposed to pH 4.0 in the presence of LM DOC (IPW + DOC—pH 4.0) did not display the large inhibition of J_{in}^{Na} seen in fish at IPW—pH 4.0 (no DOC) (Fig. 1a) and indeed kept mean J_{in}^{Na} at a value (680 nmol g^{-1} h^{-1}) quite similar to those of fish during both the control period and during exposure to IPW + DOC—pH 7.0. In addition, zebrafish acutely exposed to IPW + DOC—pH 4.0 exhibited much smaller disturbances in J_{out}^{Na}, and therefore in J_{net}^{Na}. J_{out}^{Na} and J_{net}^{Na} were significantly attenuated by 35 and 60%, respectively, relative to fish exposed to IPW—pH 4.0 (Fig. 1a). Nevertheless, both J_{out}^{Na} and J_{net}^{Na} rates remained significantly greater (i.e., more negative) than those seen in the same fish during the control period (by 2.7-fold and 5.1-fold, respectively) (Fig. 1a). During the recovery period (i.e., IPW—pH 7.0), fish previously exposed to DOC at both neutral and low pH exhibited J_{in}^{Na} rates significantly higher by about 1.5-fold in relation to fish in the control period in IPW—pH 7.0 (Fig. 1a). In zebrafish previously exposed to IPW—pH 4.0, J_{in}^{Na}, J_{out}^{Na} and J_{net}^{Na} returned during the recovery period to values very similar to those measured in this same group of fish during the control period (Fig. 1a). In summary, LM DOC offered complete protection of J_{in}^{Na}, and

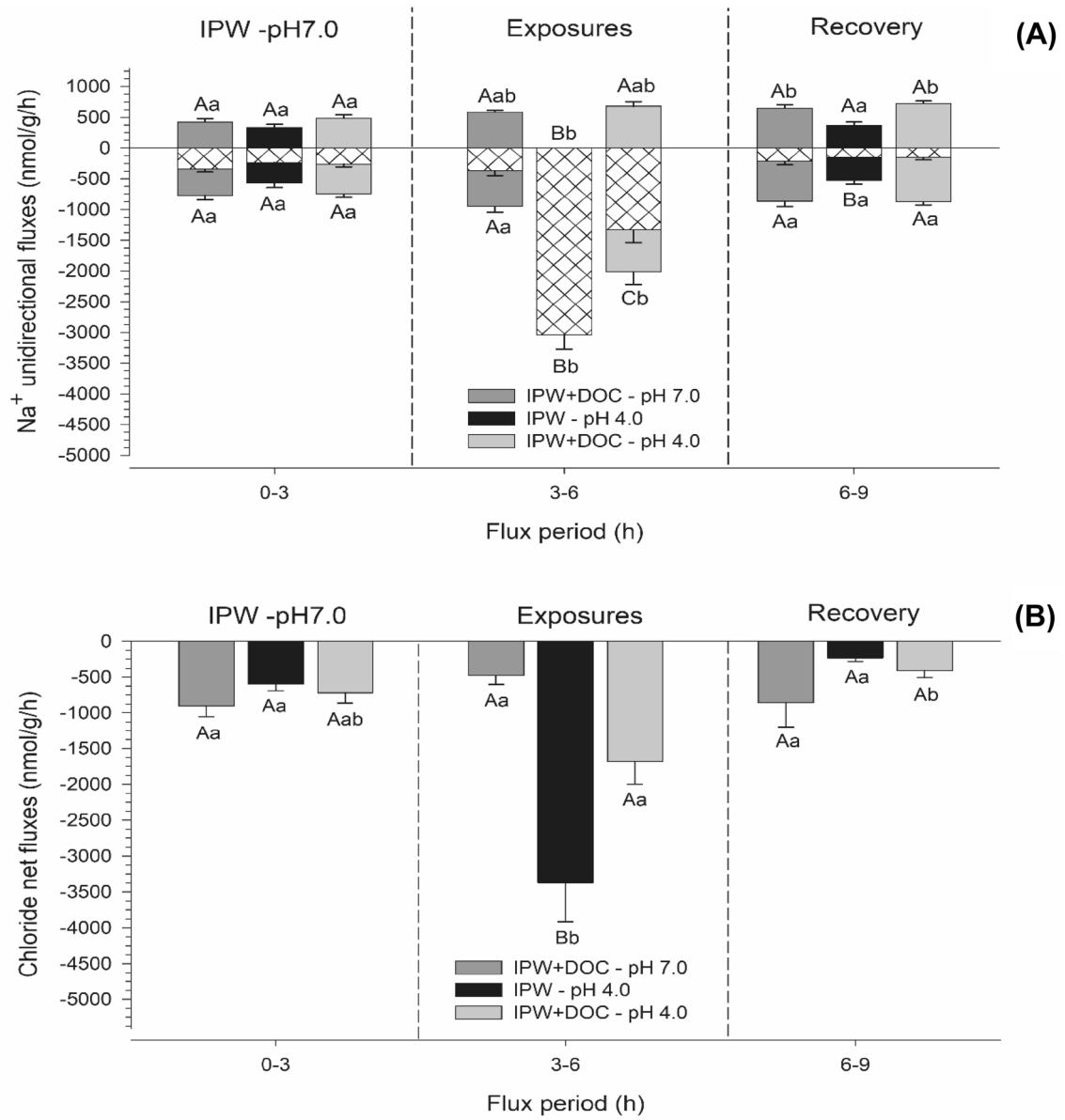

Fig. 1 Sodium unidirectional influx (J_{in}^{Na}, upward positive solid bar), sodium unidirectional efflux (J_{out}^{Na}, downward negative solid bars) and sodium net flux rates (J_{net}^{Na}, cross-hatched bars) (**a**) and chloride net fluxes (J_{net}^{Cl}) (**b**) of adult zebrafish in ion-poor water (IPW) at series 1. Means ± 1 SEM ($N = 10$ in each treatment). In the left-hand panel, the first three sets of bars represent fish initially tested (0–3 h) under the same control condition (no DOC) to which they were all acclimated (IPW—pH 7.0), and then in the middle panel acutely exposed (3–6 h) to either IPW + DOC—pH 7.0, or IPW—pH 4.0, or IPW + DOC—pH 4.0, followed in the right-hand panel by a recovery period (6–9 h) in which all fish were again exposed to the common acclimation condition (IPW—pH 7.0). Upper case letters represent significant differences ($p < 0.05$) in J_{in}^{Na} or J_{out}^{Na} and J_{net}^{Cl} among fish under different exposure regimes (different shading schemes) within the same flux period. Lower case letters represent significant differences ($p < 0.05$) in J_{in}^{Na} or J_{out}^{Na} and J_{net}^{Cl} of animals in the same regime of exposure (bars with same shading scheme), among different flux periods. Bars sharing the same letter are not significantly different

substantial protection of J_{out}^{Na} (and therefore J_{net}^{Na}) against the effects of acute low pH exposure.

Mean chloride net flux rates (J_{net}^{Cl}) exhibited by the three groups of zebrafish during the control period were not significantly different, ranging between − 600 and − 903 nmol g^{-1} h^{-1} (Fig. 1b). During the exposure to LM DOC at neutral pH (i.e., IPW + DOC—pH 7.0), J_{net}^{Cl} remained unchanged. However, acute exposure to IPW—pH 4.0 with no DOC resulted in a large significant stimulation of J_{net}^{Cl} to a more negative value (− 3371 nmol g^{-1} h^{-1}) in comparison to their respective control (i.e., 5.6-fold higher than fish at IPW—pH 7.0) (Fig. 1b). This exacerbated J_{net}^{Cl}

was also 7.1-fold higher compared to fish at IPW + DOC—pH 7.0 (Fig. 1b). In the IPW + DOC—pH 4.0 treatment, J_{net}^{Cl} was less than 50% of that in IPW–pH 4.0, a highly significant difference (Fig. 1b). Clearly, LM DOC again offered protection against the effects of low pH. During the recovery period, J_{net}^{Cl} rates were quite similar among all groups, which were not significantly different from those observed in the control period.

Ammonia excretion rates (J_{net}^{Amm}) were similar among all three groups in the control period, ranging between −973 and −1367 nmol g^{-1} h^{-1} (Fig. 2a). During the acute exposure to IPW—pH 4.0, zebrafish exhibited a marked stimulation in J_{net}^{Amm} by 2.5-fold, relative to both the respective control (i.e., IPW—pH 7.0), and to fish exposed to IPW + DOC—pH 7.0 (Fig. 2a). Although $J^{Amm}{}_{net}$ of zebrafish exposed to IPW + DOC—pH 4.0 was significantly increased by 1.7-fold in comparison with fish at IPW + DOC—pH 7.0, it remained about 35% lower than the J_{net}^{Amm} of fish exposed to pH 4.0 in the absence of LM DOC (IPW—pH 4.0), and returned to pre-acid exposure levels during the recovery period (Fig. 2a). In contrast, during the recovery period, J_{net}^{Amm} of fish previously exposed to low pH without DOC (IPW—pH 4.0) remained significantly elevated in relation to the respective control (IPW—pH 7.0) at a level similar to that during exposure to IPW—pH 4.0. Thus, the presence of LM DOC clearly attenuated the extent of J_{net}^{Amm} elevation during acid challenge and recovery.

In contrast to J_{net}^{Amm}, J_{net}^{Urea} remained the same (−246 to −280 nmol g^{-1} h^{-1} in the control period) among the three treatments groups throughout all three phases of the experiment (Fig. 2b). However, in all three groups, J_{net}^{Urea} was significantly lower by 60–70% during the exposure and recovery periods than during the initial control period.

Series 2: effects of acclimation to LM DOC on ion regulation and nitrogen excretion of zebrafish at low pH

In the control period after the acclimation to LM DOC (9.1 mg C L^{-1}), zebrafish displayed uniform mean rates of J_{in}^{Na} (249 to 363 nmol g^{-1} h^{-1}), J_{out}^{Na} (−459 to −655 nmol g^{-1} h^{-1}) and J_{net}^{Na} (−210 to −308 nmol g^{-1} h^{-1}) in Series 2 quite similar to Na^+ fluxes rates seen in fish acclimated to IPW—pH 7.0 (no DOC) in Series 1 (Fig. 3a versus Fig. 1a). In the experimental period, the acute exposure to neutral pH without DOC (i.e., IPW—pH 7.0), did not significantly alter J_{in}^{Na}, J_{out}^{Na} or J_{net}^{Na} (Fig. 3a). Interestingly, zebrafish exposed to IPW—pH 4.0 (no DOC present) after the acclimation to LM DOC did not suffer any inhibition in J_{in}^{Na}, but rather a significant 1.6-fold stimulation in J_{in}^{Na}, which was comparable to the 1.7-fold higher J_{in}^{Na} seen in fish exposed IPW + DOC—pH 4.0 with LM DOC present (Fig. 3a). Another interesting response of zebrafish after acclimation to LM DOC was that J_{out}^{Na} was far less stimulated by pH 4.0 (about 50% lower than in Series 1), regardless of the presence or absence of LM DOC, so that rates of J_{out}^{Na} remained very close between fish exposed to either IPW—pH 4.0 or IPW + DOC—pH 4.0. These were 2.7-fold and 3.0-fold higher than J_{out}^{Na} of fish during the control period, resulting in a moderate increment of J_{net}^{Na} in fish in both treatments (Fig. 3a). In the recovery period, J_{in}^{Na}, J_{out}^{Na} and J_{net}^{Na} rates were not significantly different among the three groups, but in zebrafish previously exposed to IPW—pH 7.0 and IPW + DOC—pH 4.0 the rates of J_{in}^{Na} were increased by 1.9-fold and 1.7-fold, respectively, in comparison to the control period (Fig. 3a). In summary, the protective effects of prior acclimation to LM DOC on both J_{in}^{Na} and J_{out}^{Na} (and therefore $J^{Na}{}_{net}$) persisted even when it was no longer present during the acute acid challenge.

$J^{Cl}{}_{net}$ rates of zebrafish were uniform among the three groups during the control period at IPW + DOC—pH 7.0 (Fig. 3b), and similar to those seen in fish acclimated to IPW—pH 7.0 (no DOC; Series 1), ranging between −801 and −932 nmol g^{-1} h^{-1}. Zebrafish acutely exposed to IPW—pH 4.0 exhibited a large stimulation of J_{net}^{Cl}, which was 4.9-fold higher than in fish in IPW—pH 7.0, and 2.5-fold higher than J_{net}^{Cl} seen in these same fish at the control period (Fig. 3b). However, it was not as great as in Series 1 (cf. Fig. 2b). Notably, in fish exposed IPW + DOC—pH 4.0, J_{net}^{Cl} was not stimulated and it remained close to levels seen in fish exposed to IPW—pH 7.0, or to IPW + DOC—pH 7.0 during the control period (Fig. 3b). There were no significant differences in J_{net}^{Cl} among fish exposed to all treatments during the recovery period, but rates tended to be lower than during the control period, a difference which was significant for the IPW—pH 7.0 treatment group (Fig. 3b). In summary, LM DOC clearly protected J_{net}^{Cl} against the effects of acid challenge, but in contrast to the parameters of Na^+ balance, the effect of prior acclimation was only marginally persistent when LM DOC was no longer present.

During the control period in IPW + DOC—pH 7.0, J_{net}^{Amm} rates were not significantly different among fish in all three experimental groups (−665 to −836 nmol g^{-1} h^{-1}), though they were somewhat lower than in Series 1 (Fig. 4a versus Fig. 2a). As seen for J_{out}^{Na} (cf. Fig. 3a), zebrafish exhibited quite similar rates of J_{net}^{Amm} during exposure to IPW—pH 4.0 and IPW + DOC—pH 4.0; however, in these fish J_{net}^{Amm} was significantly stimulated on average by 2.6-fold when compared to fish exposed to IPW—pH 7.0, and by about 2.2-fold relative to their respective controls (Fig. 4a). During the recovery period, J_{net}^{Amm} rates of fish previously exposed to both IPW—pH 4.0 and IPW + DOC—pH 4.0 were still significantly elevated by 2.1-fold in relation to fish exposed to IPW—pH 7.0, but were lowered by about 45% relative to their values during the pH 4.0 challenge (Fig. 4a). In summary, there were no

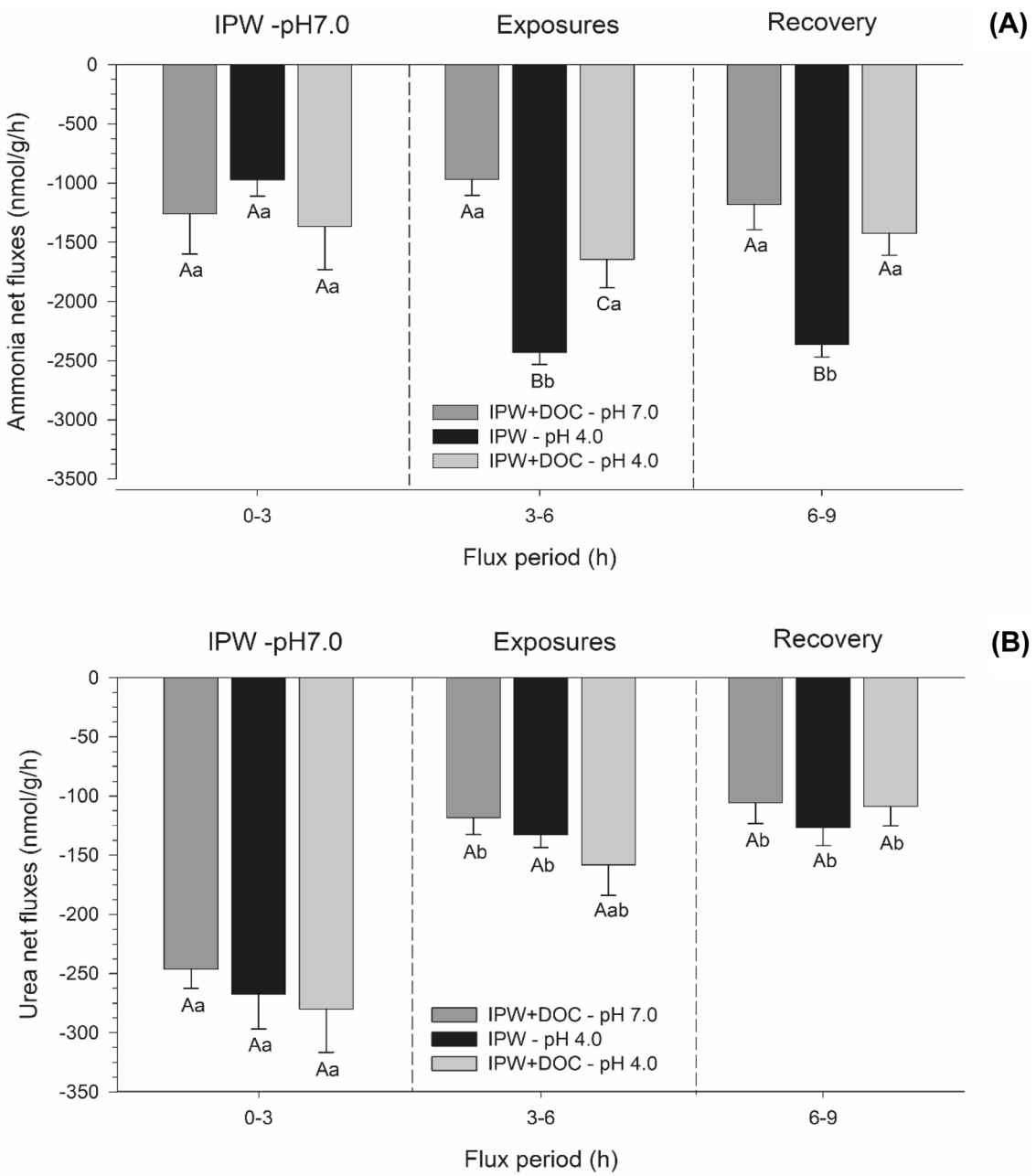

Fig. 2 Ammonia net fluxes (J_{net}^{Amm}) (**a**) and urea net fluxes (J_{net}^{Urea}) (**b**) of adult zebrafish in ion-poor water (IPW) at series 1. Means ± 1 SEM ($N = 10$ in each treatment). In the left-hand panel, the first three sets of bars represent fish initially tested (0–3 h) under the same control condition (no DOC) to which they were all acclimated (IPW—pH 7.0), and then in the middle panel acutely exposed (3–6 h) to either IPW + DOC—pH 7.0, or IPW—pH 4.0, or IPW + DOC—pH 4.0, followed in the right-hand panel by a recovery period (6–9 h) in which all fish were again exposed to the common acclimation condition (IPW—pH7.0). Upper case letters represent significant differences ($p < 0.05$) in J_{net}^{Amm} and J_{net}^{Urea} among fish under different exposure regimes (different shading schemes) within the same flux period. Lower case letters represent significant differences ($p < 0.05$) in J_{net}^{Amm} and J_{net}^{Urea} of animals in the same regime of exposure (bars with same shading scheme), among different flux periods. Bars sharing the same letter are not significantly different

clear effects of prior acclimation to LM DOC on the elevation in J_{net}^{Amm} during acid challenge, though there appeared to be some attenuation of continued elevation of ammonia excretion during recovery in contrast to that seen in non-acclimated zebrafish in Series 1.

As for J_{net}^{Amm}, absolute rates of J_{net}^{Urea} in zebrafish acclimated to LM DOC (− 136 to − 194 nmol g^{-1} h^{-1}) were slightly lower during the control period than those seen in series 1 (Fig. 4b versus Fig. 2b). However, as in series 1, these rates tended to decline over time during the experiment, such

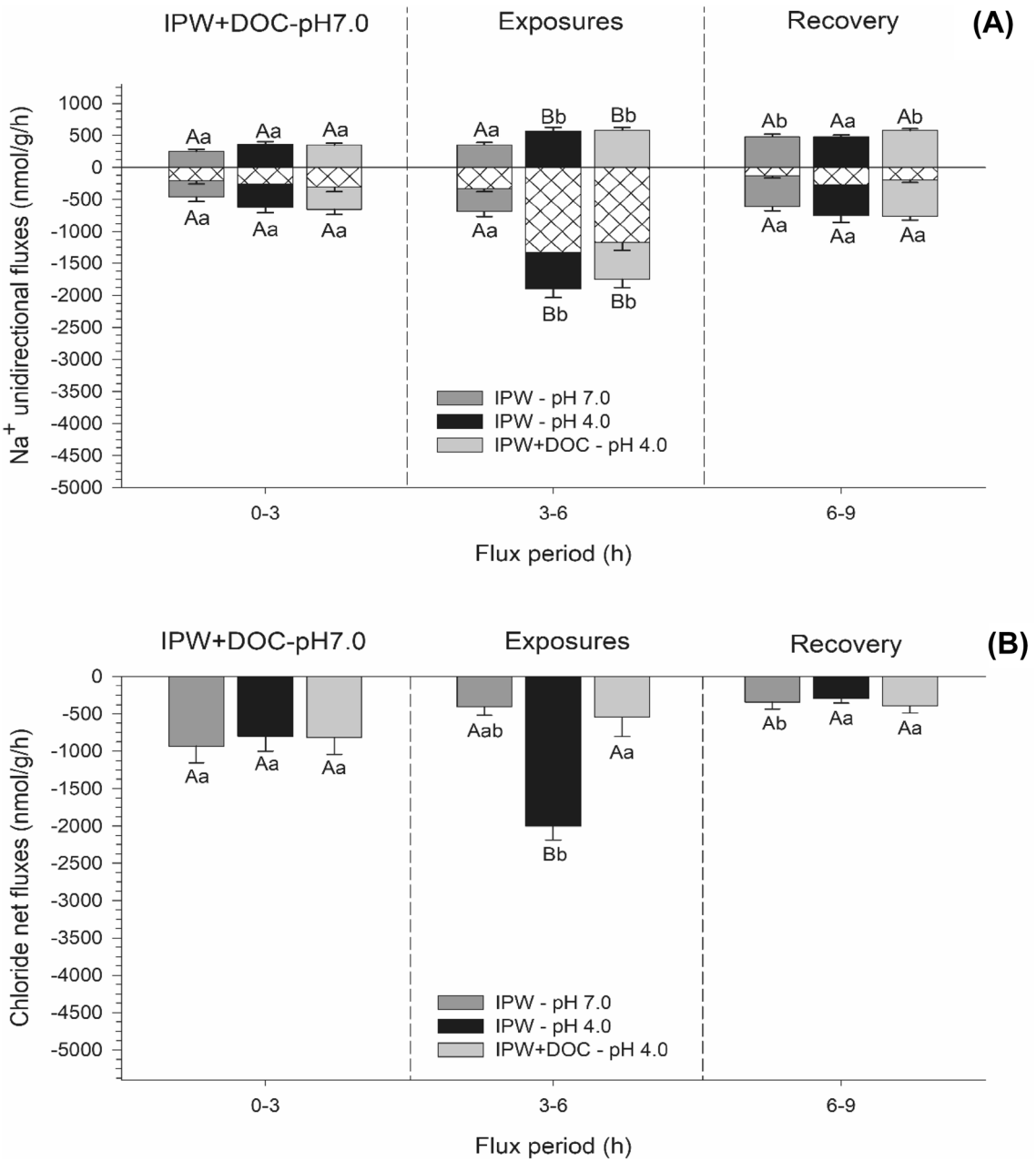

Fig. 3 Sodium unidirectional influx (J_{in}^{Na}, upward positive solid bar), unidirectional sodium unidirectional efflux (J_{out}^{Na}, downward negative solid bars) and sodium net flux rates (J_{net}^{Na}, cross-hatched bars) (**a**) and chloride net fluxes (J_{net}^{Cl}) (**b**) of adult zebrafish in ion-poor water (IPW) at series 2. Means ± 1 SEM ($N = 10$ in each treatment). In the left-hand panel, the first three sets of bars represent fish initially tested (0–3 h) under the same control condition to which they were all acclimated (IPW + DOC—pH 7.0), and then in the middle panel acutely exposed (3–6 h) to either IPW—pH 7.0, or IPW—pH 4.0, or IPW + DOC—pH 4.0, followed in the right-hand panel by a recovery period (6–9 h) in which all fish were again exposed to the common acclimation condition (IPW + DOC—pH 7.0). Upper case letters represent significant differences ($p < 0.05$) in J_{in}^{Na} or J_{out}^{Na} and J_{net}^{Cl} among fish under different exposure regimes (different shading schemes) within the same flux period. Lower case letters represent significant differences ($p < 0.05$) in J_{in}^{Na} or J_{out}^{Na} and J_{net}^{Cl} of animals in the same regime of exposure (bars with same shading scheme), among different flux periods. Bars sharing the same letter are not significantly different

that they were lower during the exposure (by about 31%) and recovery periods (by about 43%) relative to the original control rates. Again, as in series 1, there were no marked effects of the experimental treatments on J_{net}^{Urea}.

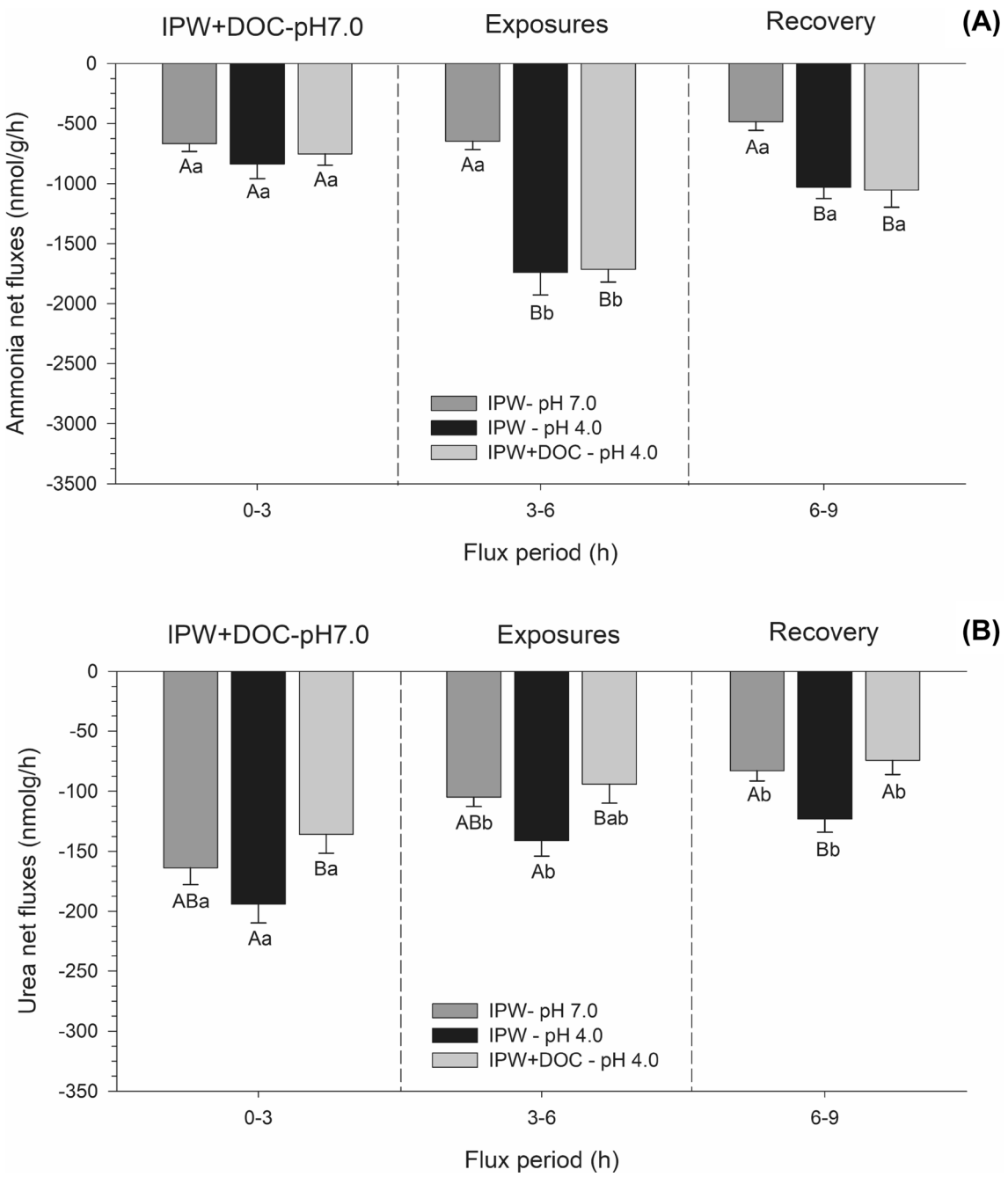

Fig. 4 Ammonia net fluxes (J_{net}^{Amm}) (**a**) and urea net fluxes (J_{net}^{Urea}) (**b**) of adult zebrafish in ion-poor water (IPW) at series 2. Means ± 1 SEM ($N = 10$ in each treatment). In the left-hand panel, the first three sets of bars represent fish initially tested (0–3 h) under the same control condition to which they were all acclimated (IPW + DOC—pH 7.0), and then in the middle panel acutely exposed (3–6 h) to either IPW—pH 7.0, or IPW—pH 4.0, or IPW + DOC—pH 4.0, followed in the right-hand panel by a recovery period (6–9 h) in which all fish were again exposed to the common acclimation condition (IPW + DOC—pH7.0). Upper case letters represent significant differences ($p < 0.05$) in J_{net}^{Amm} and J_{net}^{Urea} among fish under different exposure regimes (different shading schemes) within the same flux period. Lower case letters represent significant differences ($p < 0.05$) in J_{net}^{Amm} and J_{net}^{Urea} of animals in the same regime of exposure (bars with same shading scheme), among different flux periods. Bars sharing the same letter are not significantly different

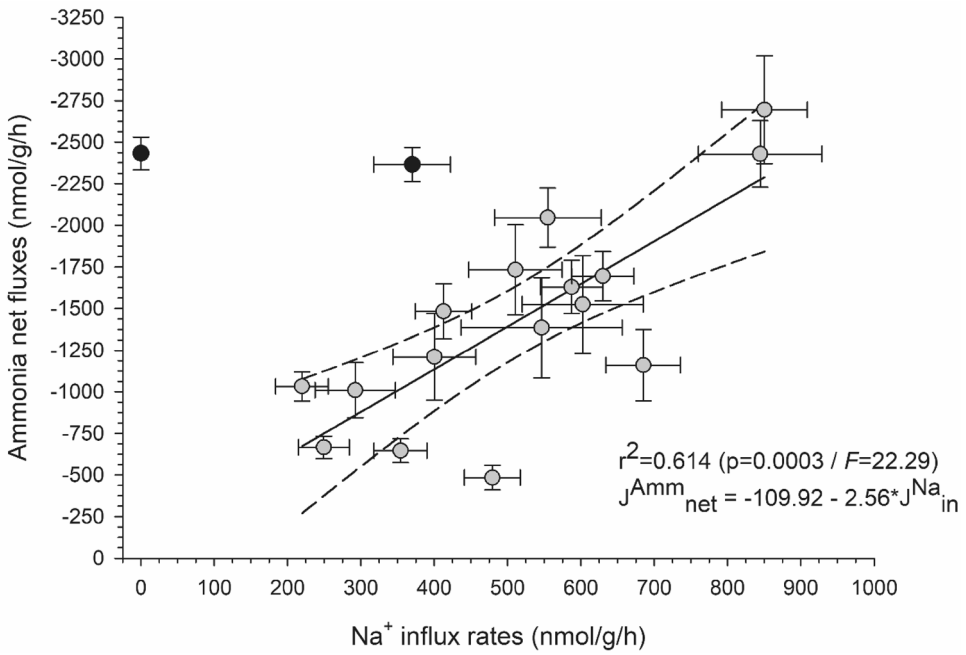

Fig. 5 The relationship between ammonia net flux rates (J_{net}^{Amm}) and Na^+ uptake (J_{in}^{Na}) of adult zebrafish in different exposure conditions in ion-poor water (IPW) at both experimental series. Means ± 1 SEM ($N = 10$ in each treatment). Gray circles represents flux rates of both J_{net}^{Amm} and J_{in}^{Na} of zebrafish under the acclimation conditions (i.e., IPW—pH 7.0 or IPW + DOC—pH 7.0; 0–3 h), and then acutely exposed to either IPW + DOC—pH 7.0, or IPW—pH 4.0, or IPW—pH 7.0 or IPW + DOC—pH 4.0 (3–6 h), followed by a recovery period in which they were again exposed to their acclimation condition (IPW—pH 7.0 or IPW + DOC—pH 7.0). Note that the two black circles (not used in the regression) represent data from fish acutely exposed to IPW—pH 4.0 (no DOC; series 01), and these same fish during the recovery period at IPW—pH 7.0, where J_{net}^{Amm} was entirely uncoupled from J_{in}^{Na}. Nonlinear regression analysis was performed using Sigma Plot v 11.0. $r^2 = 0.614$; $p = 0.0003$, $F = 22.29$

Discussion

Overview: the potency of LM versus SGC DOC

In the current study, LM DOC provided almost complete protection for the ionoregulatory homeostasis of zebrafish acutely exposed to low pH in ion-poor water. LM DOC greatly attenuated the stimulation of J_{out}^{Na} and prevented the blockade of J_{in}^{Na} (or even stimulated J_{in}^{Na}), deleterious effects which would otherwise occur in the absence of DOC at pH 4.0 (Fig. 1a). As discussed subsequently, maintenance of the linkage of J_{in}^{Na} with ammonia excretion (Fig. 5) likely played an important role in this protection. As a result, increases in the net losses of Na^+ (and also of Cl^-) at low pH were largely prevented. After acclimation of the fish to LM DOC, these effects largely persisted even when the DOC was no longer present during the period of acid exposure (Fig. 3). Indeed these ameliorative effects of LM DOC were almost as pronounced as those seen earlier in parallel experiments with Rio Negro SGC DOC (Duarte et al. 2016). These findings directly contradict our initial hypothesis that the protective effects of LM DOC would be far less than those of SGC DOC under these conditions, reflecting differences in the chemistries of the two DOCs (Table 1). Therefore, the unique chemical properties (e.g., unusual proteinaceous compounds represented by tryptophan- and tyrosine-like components; Table 1) of SGC DOC do not appear to be the important factor offering protection against the deleterious effects of low pH. Several studies have evidenced that optically dark DOCs of allochthonous origin exert a variety of marked effects on the physiology of aquatic organisms (Glover et al. 2005a; Glover and Wood 2005; Galvez et al. 2009; Wood et al. 2011; Al-Reasi et al. 2013b; Manek et al. 2014; Duarte et al. 2016), effects which may be associated with the ability of DOC molecules to bind to biological membranes (Campbell et al. 1997), particularly under low pH conditions (Vigneault et al. 2000).

LM DOC and SGC DOC share the properties of mainly allochthonous origin (indicated by relatively low FI values), optical darkness reflective of high aromatic content (indicated by relatively high SAC_{340} values), high molecular weight (indicated by relatively low $Abs_{254/365}$ values), and high chemical reactivity (indicated by relatively high PBI values) (Al-Reasi et al. 2011, 2013a). Of these, PBI and SAC_{340} are thought to have the greatest influences on biological activity (Al-Reasi et al. 2013a). These properties were not as marked in LM DOC as in SGC DOC (Table 1), but they were clearly strong enough to offer almost comparable

protection against low pH. Here, we have used the same isolate of LM DOC as in previous studies (Al-Reasi et al. 2013b, 2016) and although natural degradation can occur over time during storage, which might alter the original physicochemical properties of DOC, LM DOC clearly contributed to the maintenance of ionoregulatory homeostasis of zebrafish at low pH. Curiously, these protective effects of LM DOC were much less clearcut in zebrafish exposed to same DOC source at pH 5.0 in a recent study (Al-Reasi et al. 2016). Possibly this is because the protective mechanisms are pH-dependent. Increasing acidity will titrate the negative charge on both DOC molecules and the fish gill, thereby facilitating their interaction by hydrophobic-or hydrogen-bonding (Campbell et al. 1997). However, the comparison is confounded by the lower absolute DOC levels and the much higher ion levels in the water used by Al-Reasi et al. (2016).

Responses to low pH and the protective effects of LM DOC

It has long been known that exposure to low pH causes net ion losses in most freshwater fish, associated with inhibition of active uptake and stimulation of passive losses at the gills (Wood 1989). The zebrafish is no exception (Kwong et al. 2014), exhibiting inhibited $J^{Na}_{in,}$ elevated J^{Na}_{out}, and elevated J^{Na}_{net} and J^{Cl}_{net} in both the present (Fig. 1) and previous studies (Kumai et al. 2011; Kwong and Perry 2013b; Al-Reasi et al. 2016; Duarte et al. 2016). However, the zebrafish is unusual in showing a rapid up-regulation of J^{Na}_{in} during continued low pH exposure which helps to maintain net Na^+ balance (Kumai et al. 2011; Kumai and Perry 2011; Kwong et al. 2014). In the present study, as long as LM DOC was present during the period of low pH, or during the pre-exposure acclimation period, J^{Na}_{in} was either not inhibited at all (Fig. 1a) or actually significantly stimulated immediately (Fig. 3a), and the elevation of J^{Na}_{out} during low pH exposure was attenuated by about 35–50% (Figs. 1a, 3a). Thus, the elevation of J^{Na}_{net} was also greatly attenuated (Figs. 1a, 3a), and the elevation of J^{Cl}_{net} was reduced to a comparable extent (Figs. 1b, 3b). These effects were very similar to those seen with SGC DOC during exposure of zebrafish to pH 4.0 under identical conditions (Duarte et al. 2016). They were also consistent with, but much more pronounced than, the protective effects of DOC reported in other freshwater species, as reviewed in the Introduction.

In general, under control conditions, our zebrafish exhibited a net negative Na^+ balance of -200 to -400 nmol g^{-1} h^{-1}. This is not unusual for fasted fish, and the negative fluxes were constant over time, and similar to those reported in other studies using similar methods (Duarte et al. 2016; Al-Reasi et al. 2016). We cannot eliminate the possibility that the fish were stressed by the experimental procedures. Nevertheless, the substantial stimulations of ionic losses in zebrafish exposed to low pH were similar in magnitude to those reported in previous work under similar acidic conditions (Kumai et al. 2011; Duarte et al. 2016), and were markedly greater than in the controls, so they did not reflect a stimulation of efflux rates promoted by handling stress.

Branchial paracellular permeability is increased by low pH exposure in zebrafish (Kumai et al. 2011; Kwong and Perry 2013a, b; Al-Reasi et al. 2016), probably because H^+ displaces Ca^{2+} ions from tight junctions, thereby destabilizing them. This is thought to be the major cause of diffusive ions losses at low pH, and cortisol mobilization appears to play an important role in counteracting this effect (reviewed by Kwong et al. 2014). Thus, it is possible that LM DOC acts by increasing cortisol secretion or by increasing the availability of glucocorticoid receptors, though there is no evidence on this point. Alternately, dark, highly aromatic DOCs such as LM and SGC are rich in phenolic rings; possibly these could directly mimic the actions of cortisol, though again there is no evidence on this point. However, it is known that LM and other dark DOCs make the transepithelial potential (TEP) across the gills of trout immediately more negative (Galvez et al. 2009), and this would counteract the effect of low pH alone in making the TEP more positive (McWilliams and Potts 1978; Wood et al. 1998). Indeed, this could explain the action of LM DOC in rapidly attenuating the stimulation of J^{Na}_{out} by low pH. It would of course not explain the rapid attenuation of J^{Cl}_{net}, but our observations on Cl^- balance did not include unidirectional Cl^- flux measurements. Still another possibility is that the direct stabilization of paracellular tight junctions by DOC molecules, as seen by Ca^{2+} ions, could explain the protective effect of DOC against diffusive ionic losses at low pH. Indeed, in our previous study on SGC DOC (Duarte et al. 2016) we had suggested this very possibility—that in Ca^{2+}-poor waters, DOC molecules might rapidly modulate the tightness of the gill epithelium of zebrafish, perhaps through Ca^{2+}-like effects on tight junction integrity and/or through post-translational regulation of tight junction proteins. Nevertheless, such an action would be surprising, since Ca^{2+} is cationic, while DOC is anionic. Further studies on the mechanism of protection by DOC are needed.

As previously reported in many freshwater species (Gonzalez and Dunson 1989; Wood 1989; Gonzalez and Wilson 2001; Kwong et al. 2014), zebrafish not acclimated to DOC (series 1) revealed a typical teleost response during exposure to IPW—pH 4 with complete inhibition of J^{Na}_{in} (Fig. 1a), accompanied by massive stimulation of J^{Amm}_{net} (Fig. 2a). These effects are usually explained by H^+ blockade of Na^+ uptake and simultaneous increased passive diffusion of NH_3, facilitated by acid-trapping in the boundary layer of the gills (reviewed by Wilkie 2002). However, the inhibition of J^{Na}_{in} and stimulation of J^{Amm}_{net} were also observed in the recovery

period (i.e., IPW—pH 7.0), demonstrating that acid exposure causes persistent disturbances in the mechanisms that drive Na^+ uptake.

Because Na^+ uptake in freshwater fish is primarily coupled to H^+ efflux via direct Na^+/H^+ exchange and/or indirect V-type H^+-ATPase/Na^+ channel linkage, the inhibition of J_{in}^{Na} by environmental acidity, seen in the current study in the absence of DOC (Fig. 1a), is usually attributed to H^+ competition with Na^+ for uptake mechanisms and/or a decrease in the H^+ electrochemical gradient driving Na^+ uptake (Wood 1989; Kwong et al. 2014). In turn, ammonia excretion is known to be loosely coupled to both Na^+ uptake and H^+ efflux through the Rh-protein metabolon ("Na^+/NH_4^+ exchange complex") consisting of several membrane transporters working together (Rhcg, V-type H^+-ATPase, Na^+ channel, Na^+/H^+ exchanger, carbonic anhydrase; Weihrauch et al. 2009; Wright and Wood 2009, 2012; Shih et al. 2012; Ito et al. 2013). In most species, ammonia excretion appears to become uncoupled from Na^+ uptake during acid exposure (Wilkie 2002; Wright and Wood 2012). However, in zebrafish, the rapid recovery of J_{in}^{Na} during continued pH 4.0 exposure has been attributed to an up-regulation of the Rh-protein metabolon on the apical membranes of gill ionocytes, and cortisol mobilization is again thought to play a key role in driving this response (Kumai et al. 2012). In this scheme, elevated NH_3 excretion through the Rhcg channel removes the thermodynamic constraints on H^+ excretion and Na^+ uptake caused by low environmental pH (Kumai and Perry 2011; Shih et al. 2012; Kwong et al. 2014). In brief, NH_4^+ is deprotonated at the intracellular binding (entry) site of the Rhcg protein and NH_3 is reprotonated as it exits at extracellular sites. Thus the H^+ gradient driving the system is increased by the creation of micro-domains on both sides of the membrane. The H^+ ions may be transferred to the external water by either or both of the V-type H^+-ATPase and/or the Na^+/H^+ exchanger, thereby allowing Na^+ uptake to proceed unimpeded.

In the present study, this coupling of Na^+ uptake to ammonia excretion was evidenced by the strong correlation of J_{in}^{Na} with J_{net}^{Amm} ($r^2 = 0.614$; $P = 0.0003$; Fig. 5). Note that the two treatments that were exceptions, and not used in the regression, were for fish acutely exposed to IPW—pH 4.0 (no DOC, series 1), and for these same fish during the recovery period in IPW—pH 7.0 (Fig. 5). This J_{in}^{Na} versus J_{net}^{Amm} pattern was almost identical to that reported by Duarte et al. (2016) with SGC DOC, and the associated regression analyses exhibited comparable slopes, and the same two treatments were outliers. The presence of both LM DOC and SGC DOC reduced but did not eliminate elevation in ammonia excretion during acid challenge, and prevented it during recovery (e.g., Fig. 2a). Urea excretion was not reported by Duarte et al. (2016), but in the present study, these effects of LM DOC were specific to ammonia, as J_{net}^{Urea} was unaffected (Fig. 2b), in agreement with Al-Reasi et al. (2016). Thus, the mechanism involves only ammonia, and not urea, which is the other important nitrogenous waste. Most importantly, it would appear that the presence of LM DOC ensured that the coupling of J_{in}^{Na} with J_{net}^{Amm} was maintained during and after low pH exposure, probably due to the up-regulation of the Rh-protein metabolon as discussed earlier.

Partial protective effects of DOC against the inhibition of J_{in}^{Na} by low environmental pH have been noted in other fish species (see "Introduction"), but the complete protective effects seen in zebrafish with both LM DOC (Fig. 2a) and SGC DOC (Duarte et al. 2016), are the most pronounced ever reported. It remains to be determined whether the quantitative differences are species-specific, DOC-specific, or both, and whether the mechanisms involved are qualitatively similar. In other studies, the effects of DOC on J_{in}^{Na} have been associated with increases in maximum uptake capacity (J_{max}), and sometimes also with decreased affinity (i.e., increased K_m) of the branchial transport systems (Matsuo et al. 2004; Glover et al. 2005a; Matsuo and Val 2007; Wood et al. 2011; Al-Reasi et al. 2016), suggesting a possible direct interaction between aromatic components of DOC molecules and Na^+ transporters in the lipoprotein bilayer of the gill cells. As discussed by Wood et al. (2011), DOC binding to surface membranes can also change the fluidity of lipoprotein bilayer, altering transporter activity or the accessibility of Na^+ to transport sites. All of these, in addition to an up-regulation of the Rh-protein metabolon, are possible mechanisms of DOC action in supporting J_{in}^{Na} at low pH.

Protective effects of prior acclimation to LM DOC

Acclimation to LM DOC at pH 7.0 had negligible effects on J_{in}^{Na}, J_{out}^{Na}, J_{net}^{Na}, J_{net}^{Cl} and J_{Amm} of zebrafish, as seen by the quite similar rates compared to fish acclimated to pH 7.0 in the absence of DOC (series 1). Interestingly, although J_{out}^{Na} was still significantly increased in fish at pH 4.0, it was quantitatively reduced by 37% in relation to zebrafish not acclimated to DOC during exposure to low pH (cf. Figs. 1a, 3a). Furthermore, J_{in}^{Na} was actually stimulated by about 1.65-fold in these DOC-acclimated fish (Fig. 3a). These compensations resulted in substantial control of Na^+ net losses (about 56% lower in series 2 than in series 1; cf. Figs. 1a, 3a). Similarly, J_{net}^{Cl} was lowered by about 41% relative to animals not acclimated to DOC during exposure to low pH (cf. Figs. 1b, 3b). Overall, these findings are in excellent agreement with Duarte et al. (2016) who reported similar protective effects of prior acclimation to SGC DOC. Most importantly, in both studies, these protective effects were occurred to the same extent whether or not DOC was present during the period of low pH exposure (Fig. 3a, b).

The persistence of the protective actions of prior acclimation, even when DOC was no longer present, suggest that the binding of the amphiphilic DOC molecules to membrane surfaces (Campbell et al. 1997) is itself persistent, and/or that it exerts effects on gill physiology even hours after removal. These effects presumably include stabilizing tight junctions and supporting J_{in}^{Na} by maintaining its coupling to J_{Amm} (Fig. 5). Interestingly, acclimation to LM DOC did not attenuate to any large extent the elevated J_{Amm} of fish under acidic conditions, though it promoted quick restoration of control J_{Amm} rates during recovery (Fig. 4a). These responses suggest that the main actions of DOC in supporting high Na^+ uptake rates at low pH were via the metabolon mechanism, which requires that fish sustain increased ammonia excretion to create the electrochemical gradient favorable to drive the uptake of Na^+, as discussed earlier.

Concluding remarks

Clearly the marked protective actions against the unfavourable effects of environmental acid exposure on zebrafish, reported earlier with SGC DOC, are not unique to that Amazonian "blackwater" DOC, and are almost equally prominent in another allochthonous organic matter, Luther Marsh DOC, collected from a bog in Canada. There is a need for future studies detailing the nature of the interaction of darker DOC molecules with gill membrane proteins and its pH dependency especially in fish species native to acidic "blackwaters".

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