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REGULAR PAPER

Does dissolved organic carbon from Amazon black water (Brazil) help a native species, the *tambaqui Colossoma macropomum* to maintain ionic homeostasis in acidic water?

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To assess how the quality and properties of the natural dissolved organic carbon (DOC) could drive different effects on gill physiology, we analysed the ionoregulatory responses of a native Amazonian fish species, the *tambaqui Colossoma macropomum*, to the presence of dissolved organic carbon (DOC; 10 mg l^{-1}) at both pH 7.0 and pH 4.0 in ion-poor water. The DOC was isolated from black water from São Gabriel da Cachoeira (SGC) in the upper Rio Negro of the Amazon (Brazil) that earlier been shown to protect a non-native species, zebrafish *Danio rerio* against low pH under similar conditions. Transepithelial potential (TEP), net flux rates of Na^+, Cl^- and ammonia and their concentrations in plasma and Na^+, K^+ ATPase; v-type H^+ ATPase and carbonic anhydrase activities in gills were measured. The presence of DOC had negligible effects at pH 7.0 apart from lowering the TEP, but it prevented the depolarization of TEP that occurred at pH 4.0 in the absence of DOC. However, contrary to our initial hypothesis, SGC DOC was not protective against the effects of low pH. *Colossoma macropomum* exposed to SGC DOC at pH 4.0 experienced greater net Na^+ and Cl^- losses, decreases of Na^+ and Cl^- concentrations in plasma and elevated plasma ammonia levels and excretion rates, relative to those exposed in the absence of DOC. Species-specific differences and changes in DOC properties during storage are discussed as possible factors influencing the effectiveness of SGC DOC in ameliorating the effects of the acid exposure.

KEYWORDS
acidic water, Amazon black water, ionoregulation, net fluxes, Rio Negro, transepithelial potential

1 | INTRODUCTION

When exposed to acidic freshwaters, most fishes experience disturbances in their ionic homeostasis, caused by inhibition of active Na^+ and Cl^- uptake, stimulation of passive diffusive ionic losses and reversal of the transepithelial potential (TEP) at the gills (Gonzalez *et al*., 2005; Nelson, 2015; Wood, 1989). These responses can lead to ionic imbalances and consequent decreases in plasma concentrations of major ions, which in turn may lead to death (Milligan & Wood, 1982). However, several Amazonian fish species seem to have developed mechanisms to avoid these ionic disturbances at low pH conditions (Gonzalez *et al*., 2005). Among these species, the *tambaqui Colossoma macropomum* (Cuvier 1816) (Teleostei, Characiformes, Serrasalmidae), is considered one of the most tolerant species to low pH. In aquaculture, it grows well at pH 4.0 (Aride *et al*., 2007) and in the wild, it migrates in the high-water period from basic (pH *c*. 7.0) white water into acidic (pH *c*. 4.0) black water in order to feed in flooded forests (Araujo-Lima & Goulding, 1998; Val & Almeida-Val, 1995). The ability

of the acidophilic *C. macropomum* to tolerate acidic environments is related to avoiding ionic imbalance, which has been described in at least four previous studies (Gonzalez *et al*., 1998; Wilson *et al*., 1999; Wood *et al*., 1998, 2017). For example, other Amazonian fishes less resistant to low pH, such as *Brycon hilarii* (Valenciennes 1850) and *Hoplosternum littorale* (Hancock 1828), as well as temperate salmonid species that have been studied extensively (Nelson, 2015; Wood, 1989), presented increases in net loss rates of Na^+ and Cl^-, reduced levels of Na^+ and Cl^- in plasma and enhanced plasma protein and red blood cell concentrations (indicative of ionic disturbance (Milligan & Wood, 1982) during gradual exposure to pH 4.0 and pH 3.5, disturbances which were quantitatively much greater than in the acidophilic *C. macropomum* (Wilson *et al*., 1999).

The Amazon acid black waters have unique physical and chemical characteristics, including low dissolved ion concentrations (particularly, Na^+ *c.* 16 μM, Cl^- *c.* 19 μM and Ca^{2+} *c.* 10 μM) and low pH (3–6) which is attributed to the high amount of humic substances (HS; Furch, 1984). These comprise a heterogeneous combination of higher molecular weight humic acids (HA) and lower molecular weight fulvic acids (FA; Al-Reasi *et al*., 2013), accounting for 50%–90% of the total dissolved organic carbon (DOC) content (Hedges *et al*., 1994; Leenheer, 1980). There is mounting evidence that the DOC can play an important role in ameliorating the effects of the low pH on the osmoregulatory systems of aquatic organisms (Duarte *et al*., 2016; Wood *et al*., 2003, 2011). This was initially proposed by Gonzalez *et al*. (1998, 2002) and first demonstrated by Wood *et al*. (2003) though in an indirect manner, in endemic freshwater stingrays *Potamotrygon* spp.. In comparison with water of very similar ionic composition but lacking in DOC, the natural Rio Negro black water was able to ameliorate the effects of the acid exposure by decreasing the passive efflux rates of Na^+ and Cl^- ions across the gills and by reducing the inhibition of the active influx rates of both Na^+ and Cl^-. However, the first direct evidence that the DOC isolated from Amazonian black water can exert some protection against the effects of acidic pH was recently published by Duarte *et al*. (2016). For zebrafish *Danio rerio* (Hamilton 1822), a non-native Amazonian species used as a model organism, the DOC isolated from the São Gabriel da Cachoeira (SGC) region of the upper Rio Negro reduced the diffusive losses of Na^+ and Cl^- and provided a stimulation of Na^+ uptake, demonstrating an almost full protection against the ionoregulatory failure imposed by pH 4.0 in waters of low ionic strength (*i.e*., low Na^+, Cl^- and Ca^{2+}concentrations; Duarte *et al*., 2016). Until now, it remains unknown whether these protective effects for *D. rerio* exerted by the native SGC DOC will be extended to a native fishes. Moreover, there is evidence that the capacity of the DOC to interact with biological membranes, which may alter their permeability (Galvez *et al*., 2008; Vigneault *et al*., 2000) as well as other basic physiological functions (Duarte *et al*., 2016; Galvez *et al*., 2008; Wood *et al*., 2003), is associated with the spectroscopic characteristics of the DOC molecules. Properties of DOC that promote these biological activities include its aromaticity, as indicated by its specific absorbance coefficient at wavelength 340 nm (SAC^{340}) and the proton binding capacities (PBI (Al-Reasi *et al*., 2013, 2016; Duarte *et al*., 2016; Galvez *et al*., 2008; Holland *et al*., 2017).

With this background in mind, in the present study we characterised the spectroscopic properties of natural SGC DOC that had been isolated from black water. We tested the hypothesis that this natural SGC DOC would have protective actions against the unfavourable effects of acid water (pH 4.0) exposure on the ionoregulatory homeostasis of native *C. macropomum*. Therefore, to address this issue, we assessed the TEP, Na^+ and Cl^- net losses, ammonia excretion rates, concentration of major ions (Na^+, Cl^-), ammonia and urea in plasma and the specific activity of gill ionoregulatory enzymes (Na^+, K^+ ATPase; v-type H^+ ATPase, carbonic anhydrase) of *C. macropomum* under two pH conditions (pH 7.0 and 4.0), in the presence and absence of SGC DOC.

2 | MATERIALS AND METHODS

All experimental and holding procedures followed Brazilian animal care guidelines and were previously approved by the National Institute for Research of the Amazon (INPA) animal care committee (registration number 047/2012).

2.1 | Water collection and DOC concentration and characterisation

Water samples for DOC collection and concentration were obtained from SGC black water (0° 07′ S, 67° 05′ W; dissolved oxygen 6.7 mg l^{-1}; temperature 29.5°C; conductivity 13.7 μS cm^{-1}; DOC 9.9 mg l^{-1}; pH 4.4; Na^+ 14.3; Cl^- 19.2; K^+ 9.5; Ca^{2+} 20.5; Mg 2.4 μM), on the upper Rio Negro. The black water was collected and concentrated in 2013, as described by Duarte *et al*. (2016). After that, the concentrate was treated with a cation exchange resin (Amberlite IR – 118 (H), Sigma-Aldrich; www.sigma-aldrich.com) to remove the influence of the cations, which were concentrated together with the DOC by reverses osmosis. Lastly, the concentrate was filtered with a 0.45 μm membrane (Pall Acrodisc; www.pall.com) and stored in sealed dark polyethylene bottles (Nalgene; www.nalgene.com) at 4°C until the physical-chemical characterisation and fish experiments were performed.

The SGC DOC was first optically characterised shortly after collection in 2013 and the results were reported by Duarte *et al*. (2016). However, it was then stored at 4°C for *c.* 2 years until the performance of the present experiments in 2015, so we re-analysed it, using the same equipment (SpectraMax M2 fluorescence spectrophotometer, Molecular devices; www.moleculardevices.com; Agilent Cary Eclipse spectrophotometer; www.agilent.com) used in 2013, prior to use in the present study. The concentrated SGC solutions were at the same pH (7) and DOC concentration (5 mg C l^{-1}) for the optical characterisation in both analyses (2013 and 2015). The SAC_{340}, an indicator of aromaticity (Curtis & Schindler, 1997) and the fluorescent index (I_F; a simple ratio of emission intensity at 450 nm per emission intensity at 500 nm; both taken at an excitation wavelength of 370 nm), an indicator of origin (allochthonous *v.* autochthonous) were both determined according to McKnight *et al*. (2001); and the ratio of absorbance at 254 nm to that at 365 nm ($Abs_{254/365}$), an indicator of the molecular weight, was determined according to Dahlén *et al*. (1996). Parallel factor analysis (PARAFAC; Stedmon & Bro, 2008) was applied

to determine the relative abundance of fluorescent components of DOC, representing the humic-like, fulvic-like and proteinaceous materials (*e.g.*, tryptophan-like and tyrosine-like compounds), in the fluorescence excitation emission matrices (FEEM). For FEEM matrices, fluorescence was determined using excitation wavelength between 200 and 450 nm in 10 nm increments and for each excitation wavelength the emission wavelength was scanned between 250 and 600 nm at 1 nm increments. For excitation-emission fluorescence measurements, standard solutions were scanned together with the samples as a control procedure to check for instrument drift, where the standard solutions were, tryptophan (0.5 μM) and tyrosine (1.0 μM; Sigma Aldrich) and a mixed solution (10 mg of carbon l^{-1} of a well characterised DOM isolate plus 0.5 μM tryptophan and 1.0 μM tyrosine), in Milli-Q water (Merck-Millipore; www.merckmillipore.com). Scattering was removed by replacing it with not-a-number (NaN) in the data matrix. Inner filter corrections were not applied since the absorbance was found to be below the threshold, where inner-filter corrections are necessary (Ohno, 2002). The spectral FEEMs were modelled using the PLS Toolbox from Eigenvector Research Inc. (www.eigenvestoc.com) running on a Matlab platform (MathWorks; www.mathworks.com). PARAFAC assigned the fluorescence on a percentage basis based on the *a priori* assumption that there were four components (humic-like, fulvic-like, tyrosine-like and tryptophan-like) (Al-Reasi *et al*., 2012, 2013).

2.2 | Fish holding

Colossoma macropomum (mean ± SE total mass M_T = 100.5 g ± 4.0; n = 40) were obtained from a local fish farm (Fazenda Santo Antônio, Amazonas, Brazil) and held for approximately 1 month at INPA in outdoor 3000 l polyethylene tanks with a continuous flow of ion-poor water (IPW) (Na^+ 30, K^+ 17, Ca^{2+} 4, Mg^{2+} 1.4 μmol l^{-1}, DOC 1.1 mg l^{-1}; alkalinity 11.5 mg $CaCO_3$ l^{-1}; pH 6.0 and temperature 28°C) from a well on the INPA campus. During the acclimation period, fish were fed until satiation with dry food pellets (36% protein content, Nutripeixe, Purina; www.purina.com). Feeding was suspended two days prior to the experiments. All experiments were performed at the acclimation temperature of 28°C.

2.3 | Preparation of test solutions

The pH of the solutions was adjusted to neutral pH (7.0; 0.01 N KOH) or to acid pH (4.0; 0.01 N HNO_3) as appropriate. The test solutions were neutral IPW (pH 7.0); neutral IPW + DOC (IPW pH 7.0 + DOC); acid IPW (pH 4.0) and acid IPW + DOC (IPW pH 4.0 + DOC). Solutions of SGC DOC were prepared 24 h before the beginning of the experiments by diluting the DOC concentrate to a final concentration of 10 mg of carbon l^{-1} in IPW from INPA. The DOC concentration was checked in triplicate on a total carbon analyser (Apollo 9000 combustion TOC analyser, Teledyne Tekmar; www.teledynetekmar.com) using certified commercial standards (Phoenix TOC Validation Set, Teledyne Tekmar). The final concentrations of DOC in all treatments (IPW pH 7 + DOC and IPW pH 4 + DOC) were 9.89 ± 0.054 mg l^{-1}.

2.4 | Branchial transepithelial potential measurements

For the TEP measurements, fish (n = 8) were cannulated intraperitoneally with catheters as described by Wood and Grosell (2008). Before surgical procedures, fish were anesthetised with buffered MS-222 (Sigma-Aldrich) in IPW (pH 7.0 or pH 4.0). A saline-filled PE50 catheter (Clay-Adams; Becton–Dickinson; www.bd.com) was inserted 1–2 cm through the peritoneal wall into the coelom *via* a puncture site made with a 19 gauge needle, just lateral and anterior to the rectum. A 1 cm PE160 sleeve, heat-flared at both ends, was glued to the PE50 with cyanoacrylate resin and anchored to the body wall with several silk sutures, to prevent the catheter from changing depth. The fish were then transferred to individual 4 l chambers served with flowing, aerated INPA IPW, where they were held overnight, prior to TEP measurements. To minimise stress, the fish was gently transferred to a 2 l plastic chamber in which TEP measurements were made.

TEP measurements were first made in fresh IPW which was then replaced by four exchanges of the test solution (IPW pH 7.0; IPW pH 7.0 + DOC; IPW pH 4.0; IPW pH 4.0 + DOC). TEP measurements were taken 2 min after the solution change. TEP was measured using 3 M KCl–agar bridges connected by Ag–AgCl electrodes to a high impedance electrometer (pHM 82 m, Radiometer; www.radiometer.com). The measurement bridge was connected to the coelomic catheter (out of the water) and the reference bridge was placed in the surrounding water. The electrodes were checked for symmetry. TEP values (mV) were expressed relative to the water side as 0 mV after correction for junction potential.

2.5 | Experimental set-up for flux measurements and terminal plasma and gill sampling

Fish (n = 8 in each of the four treatment groups) were individually transferred to glass aquaria covered with black plastic and filled with 4 l of aerated experimental solution. These were either IPW pH 7.0, or IPW pH 7.0 + DOC, or IPW pH 4.0, or IPW pH 4.0 + DOC. Fish were allowed to settle for 1 h in the experimental glass aquaria before starting the experimental series. At the beginning of the experiment, 15 ml of water was removed with a pipette from each aquarium. This same procedure was repeated after 3, 9 and 24 h of exposure to the experimental solution, corresponding to 0–3; 3–9 and 9–24 flux periods, respectively. The flux chamber was aerated throughout and the pH was monitored hourly and adjusted as necessary by the addition of 0.01 N KOH or 0.01 N HNO_3. The average pH values (mean ± SE) in the chambers over the 24 h of experiment were 6.9 ± 0.02 for both IPW pH 7 and IPW pH 7 + DOC and 4.1 ± 0.01 for both IPW pH 4 and IPW pH 4 + DOC. Immediately after collection, water samples were stored at −20°C until analysis of Na^+, Cl^- and ammonia concentrations. At the end of 24 h of exposure, fish were anesthetised with buffered MS-222 as above in IPW (pH 7.0 or pH 4.0). Blood was sampled with a heparinised syringe from the caudal vein and then centrifuged (2000 *g*, 25°C, 5 min) for plasma separation, which was stored at −80°C until analysis of Na^+, Cl^-, ammonia and urea concentrations. Immediately after blood collection, the fish was euthanised

by cervical dislocation and then gill filaments were collected, frozen in liquid nitrogen and stored at −80°C until analysis of Na^+, K^+ ATPase, v-type H^+ ATPase and carbonic anhydrase specific activities.

2.6 | Plasma sodium, chloride, ammonia and urea concentrations

For determination of both sodium and chloride and urea concentrations, the plasma samples were diluted 1:1000 and 1:100, respectively. All dilutions were performed in Milli Q water (Milli-Q Integral 3, Millipore). The readings of sodium and chloride were performed by the same methods used for the water samples while plasma urea was measured by the colorimetric method of Rahmatullah and Boyde (1980). For ammonia, an enzymatic method was employed (Raichem commercial assay, Cliniqa Corporation; www.cliniqa.com) using the plasma without dilution. For all assays, measurements were made in triplicate, employing appropriate blanks, using certified commercial standards (Radiometer for Na^+ and Cl^-, Cliniqua for ammonia and Sigma-Aldrich for urea).

2.7 | Sodium, chloride and ammonia net fluxes

The net flux rates (J_{net}) of Na^+, Cl^- and total ammonia (T_{amm} = NH_3 + NH_4^+) were calculated as:

$$J_{net} = (X_1 - X_2)V(TM_T)^{-1}$$

where X_1 and X_2 werethe initial and final concentration of Na^+, Cl^- or total ammonia (μmol l^{-1}) in the water during the flux period, respectively, V was the volume (l) of the chamber, T was the duration of the flux period in hours and M_T was the fish mass in kg.

Sodium concentration was read on a Perkin-Elmer model 3100 atomic absorption spectrophotometer (AAS; www.perkinelmer.com). Chloride and total ammonia concentrations in water were measured by colorimetric methods described by Zall *et al*. (1956) and Verdouw *et al*. (1978), respectively. All measurements were made in triplicate using appropriate blanks and the same commercial certified standards as for plasma analyses.

2.8 | Enzyme activities in gill filaments

Na^+, K^+ ATPase (NKA) and v-type H^+ ATPase were measured according to Kültz and Somero (1995). Briefly, the assay is based on the inhibition of the NKA activity by the ouabain (2 mmol l^{-1}) and the v-type H^+ ATPase activity by N-ethylmaleimide (NEM, 2 mmol l^{-1}) in a reaction mixture (fresh made) containing 30 mmol l^{-1} imidazole, 45 mmol l^{-1} NaCl, 15 mmol l^{-1} KCl, 3 mmol l^{-1} $MgCl_2$, 0.4 mmol l^{-1} KCN, 1 mmol l^{-1} Na_2ATP, 0.2 mmol l^{-1} NADH, 0.1 mmol l^{-1} fructose 1,6-biphosphate, 2 mmol l^{-1} phosphoenolpyruvate (PEP), 3 IU ml^{-1} pyruvate kinase (PK) and 2 IU ml^{-1} lactate dehydrogenase (LDH). A reaction mixture without any inhibitor was used to measure the total ATPase activity. Frozen gill filaments were weighed and homogenised with the aid of an electric homogeniser (Dremel MultiPro 395JU; www.dremel.com) in 10 volumes of ice-cold buffer containing 150 mmol l^{-1} sucrose, 50 mmol l^{-1} imidazole, 10 mmol l^{-1} EDTA, 2.5 mmol l^{-1} deoxycholic acid, pH 7.5 and then centrifuged at 2000 *g* for 5 min at 4°C. The assay was performed at 25°C by combining 200 μl of the reaction mixture (with ouabain, with NEM, or without inhibitors) and 5 μl of the homogenate. The change in the absorbance at 340 nm was read over 10 min. NKA and v-type H^+ ATPase activities were calculated as the difference between total activity and activity with ouabain and NEM inhibitors, respectively. The activity is express as μmol ATP h^{-1} mg^{-1} protein.

Carbonic anhydrase (CA) activity was determined according to Vitale *et al*. (1999). Frozen gill filaments were weighed and homogenised with the aid of an MultiPro 395JU electric homogeniser in 10 volumes of ice-cold 10 mM phosphate buffer (pH 7.4). The homogenate was then centrifuged (10,000 g) for 5 min at 4°C and the supernatant was used for CA assay and protein content. The activity of the CA was quantified by the rate of pH drop upon addition of CO_2-saturated ice-cold water. In this procedure 50 μl of tissue homogenate were then added to a small beaker containing 7.5 ml of the assay buffer (225 mmol l^{-1} mannitol, 75 mmol l^{-1} sucrose, 10 mmol l^{-1} Tris base and 10 mmol l^{-1} sodium phosphate monobasic at pH 7.4). The rate of pH drop was measured following the addition to the beaker of 1 ml of ice-cold (4°C) CO_2-saturated distilled water. The pH was followed for 20 s, with readings every 4 s. The slope of the linear regression of pH against time corresponds to the rate of the catalysed reaction (catalysed rate, R_C). The non-catalysed rate (R_{NC}) was assessed as the rate of pH drop over time in the absence of tissue homogenate, with the addition of 50 μl of the sample dilution buffer. CA specific activity was calculated as: CA = $((R_C R_{NC}^{-1}) - 1)$ mg^{-1} total protein.

For all enzymatic activities, total protein concentration of the homogenates was determined according Bradford (1976), using bovine serum albumin (BSA) as standard and read at 595 nm.

2.9 | Statistics

All data are reported as means ± SE (n = 8). Statistical significance was accepted at $P < 0.05$. Significant differences among treatments in TEP were determined through one-way repeated measures ANOVA followed by the *a posteriori* Tukey multiple comparison test. Significant differences among treatments in Na^+, Cl^- and ammonia net fluxes, their plasma concentrations, as well as Na^+, K^+ ATPase; v-type H^+, ATPase and carbonic anhydrase specific activities in gill filaments were determined through a one-way ANOVA, followed by *a posteriori* Tukey multiple comparison test. In the case of a failed normality test, a non-parametric Kruskal-Wallis test was performed. All statistical analyses and graphics employed Sigma Stat 3.5 and Sigma Plot 11.0 software (Jandel Scientific; www.systatsoftware.com).

3 | RESULTS

3.1 | DOC characterisation

The physicochemical characteristics of the SGC, measured in 2015 at the time of the present study are summarised in Table 1 and compared with those measured in 2013 shortly after collection of the DOC, as reported by Duarte *et al*. (2016). Clearly, the physicochemical

TABLE 1 Summary of physicochemical properties of natural dissolved organic carbon (DOC) samples isolated by reverse osmosis from São Gabriel da Cachoeira (SGC) black water

				Relative abundance of DOC component (%)[c]			
Year	SAC_{340} (cm^2 mg^{-1})	$Abs_{254/365}$	I_F	FA	HA	Trp	Tyr
2013[a]	73.0	2.9	1.3	36.0	51.3	7.8	4.7
2015[b]	36.5	4.0	1.0	28.3	58.8	7.6	5.1

Note. SAC_{340}, The specific absorbance coefficient at 340 nm normalised to DOC; $Abs_{254/365}$, the ratio of absorbance at 254 nm to that at 365 nm. I_F, the index of fluorescence.
FA, fulvic acid; HA, humic acid; Trp, tryptophan; Tyr, tyrosine.
[a]Data from Duarte *et al.* (2016).
[b]Present study.
[c]Determined by PARAFAC analysis.

characteristics of the SGC DOC changed during storage; the sample became approximately half as dark as indicated by SAC_{340} although the relative contributions of fulvic acid, humic acid, tryptophan-like and tyrosine-like components were not as greatly altered. Humic fluorophores predominate in both the 2013 and 2015 characterisations, accounting for greater than half of the total observed fluorescence in both years, with approximately a third of the total fluorescence as fulvic acid-like fluorophores and the remainder made up of proteinaceous fluorophores.

3.2 | Branchial transepithelial potential

The TEP was *c.* −17 mV in neutral IPW (pH 7.0) but decreased significantly to −23 mV during exposure to SGC DOC under neutral conditions (pH 7.0 + DOC). When fish were transferred to acidic conditions in the absence of DOC (IPW pH 4.0), the TEP increased to −2 mV. The subsequent transfer of the fish to IPW pH 4.0 + DOC resulted in a significant decrease in TEP to −14 mV, which was not significantly different from the TEP in IPW at pH 7.0 (Figure 1). Thus, exposure to acidic pH significantly raised the TEP to less negative values in both the presence and absence of SGC DOC, while SGC DOC resulted in a significantly more negative TEP under both neutral and acidic conditions.

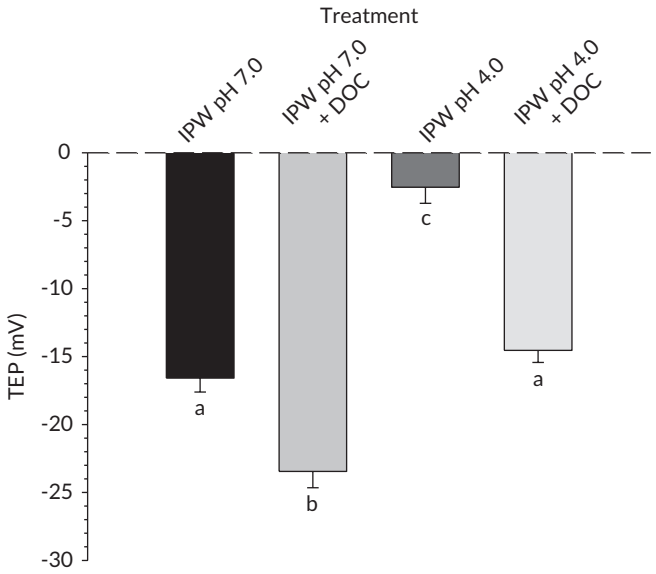

FIGURE 1 Mean (+ SE) response of gill transepithelial potential (TEP; inside relative to outside as 0 mV) of *Colossoma macropomum* to ion poor water (IPW) pH 7.0 and pH 4.0, with and without natural dissolved organic carbon (DOC) from São Gabriel da Cachoeira black water. Different lowercase letters indicate significant differences among the groups ($P < 0.05$)

3.3 | Sodium and chloride net flux rates

Colossoma macropomum exposed to IPW pH 7.0 were in slight negative balance for Na^+ and did not experience any significant alterations in $J_{Na.net}$ over time (0–3 h, 3–9 h and 9–24 h; Figure 2). Nevertheless, there was a general trend for Na^+ loss rates to become less negative over time in all treatments, an effect that was significant by 9–24 h in the other three groups. Thus, when *C. macropomum* were exposed to IPW pH 7.0 + DOC, the Na^+ net losses were reduced at 9–24 h in comparison to $J_{Na.net}$ seen in fish during the first 3 h of exposure. However, the presence of DOC had negligible other effect on Na^+ balance at neutral pH. Exposure to acidic conditions in the absence of DOC (IPW pH 4.0) caused overall increases in Na^+ net losses which were significant relative to the IPW pH 7.0 treatment only in the first 3 h of exposure; again, $J_{Na.net}$ was significantly reduced by 9–24 h (Figure 2). When *C. macropomum* were exposed to low pH in the presence of DOC, these Na^+ loss rates became even greater, although none of the time-specific differences were significant relative to the IPW pH 4.0 treatment, though they were significant at 0–3 h and 3–9 h relative to the IPW pH 7.0 + DOC treatment (Figure 2). Again, the pattern of significant attenuation of $J_{Na.net}$ over time was seen. These Na^+ net losses accounted for total mean losses over 24 h of −557, −529, −2256; and − 8455 μmol kg^{-1} for IPW pH 7.0, IPW pH 7.0 + DOC, IPW pH 4.0 and IPW pH 4.0 + DOC, respectively (Figure 2).

Net Cl^- balance (Figure 3) proved to be generally less negative than net Na^+ balance (Figure 2), though overall response patterns were similar. Thus, *C. macropomum* exposed to IPW pH 7.0 did not experience any significant alterations in $J_{Cl.net}$ over time. Exposure to IPW pH 7.0 + DOC did not cause any significant changes relative to exposure to IPW pH 7.0 alone. Acid exposure in the absence of DOC (IPW pH 4.0) caused significant increases in net Cl^- losses only in the 3–9 h period time. However acid exposure in the presence of DOC (IPW pH 4.0 + DOC) tended to exacerbate Cl^- loss rates. This effect was significant relative to the IPW pH 4.0 treatment only at 0–3 h, but it was significant relative to both the IPW pH 7.0 and IPW pH 7.0 treatments at 0–3 h and 3–9 h. These Cl^- net losses accounted for total mean losses over 24 h of −825, +155 (*i.e.*, net gain), −2143

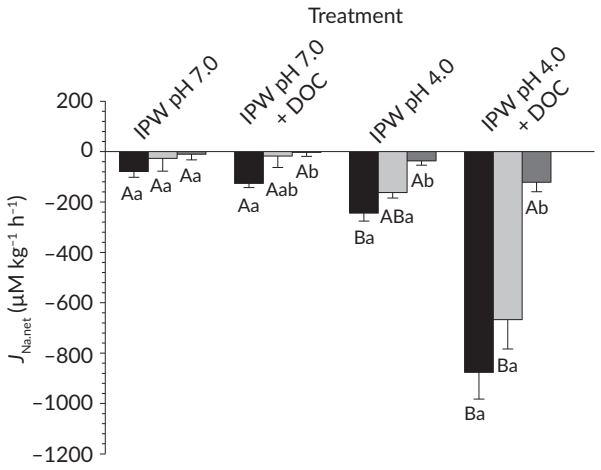

FIGURE 2 Mean (+ SE) net sodium fluxes rates ($J_{Na.net}$) of *Colossoma macropomum* in ion-poor water (IPW) pH 7.0 and 4.0, with and without natural dissolved organic carbon (DOC) from São Gabriel da Cachoeira black water over the flux periods of 0–3 h, 3–9 h and 9–24 h since the start of exposure. Different lowercase letters indicate significant differences of the mean values within the same treatment among the different flux periods ($P < 0.05$). Different capital letters indicate significant differences among the different treatments ($P < 0.05$) within the same flux period. (■) 0–3 h, (□) 3–9 h, and (■) 9–24 h

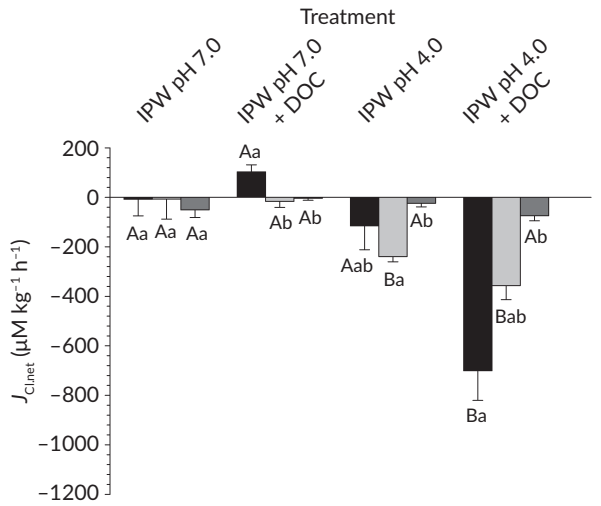

FIGURE 3 Mean (+ SE) net chloride fluxes rates ($J_{Cl.net}$) of *Colossoma macropomum* in ion-poor water (IPW) pH 7.0 and 4.0, with and without natural dissolved organic carbon (DOC) from São Gabriel da Cachoeira black water over the flux periods of 0–3 h, 3–9 h and 9–24 h since the start of exposure. Different lowercase letters indicate significant differences of the mean values within the same treatment among the different flux periods ($P < 0.05$). Different capital letters indicate significant differences among the different treatments ($P < 0.05$) within the same flux period. (■) 0–3 h, (□) 3–9 h, and (■) 9–24 h

and − 5360 μmol kg^{-1} for IPW pH 7.0, IPW pH 7.0 + DOC, IPW pH 4.0 and IPW pH 4.0 + DOC, respectively (Figure 3).

In summary, exposure to low pH caused the expected increases in Na^+ and Cl^- net loss rates, though they attenuated over time. The presence of SGC DOC had negligible effects at neutral pH but exacerbated both Na^+ and Cl^- loss rates at pH 4.0 Figures 2 and 3.

3.4 | Ammonia excretion rates

In contrast to Na^+ and Cl^- loss rates, which tended to become smaller over time (Figures 2 and 3), ammonia excretion rates tended to become greater over time, an effect that was significant by 9–24 h in both DOC treatments (Figure 4). *Colossoma macropomum* exposed to neutral (IPW pH 7.0) and acidic (IPW pH 4.0) water in the absence of DOC did not experience any alterations in $J_{amm.net}$ in any flux period and there were no significant differences between the two treatments. On the other hand, DOC exposure in neutral (IPW pH 7.0 + DOC) and acidic (IPW pH 4.0 + DOC) were associated with time-dependent increases in $J_{amm.net}$. In the latter treatment, the presence of DOC at low pH significantly increased the net ammonia excretion at both 0–3 h and 9–24 h, relative to both the (IPW pH 7.0) and IPW pH 7.0 + DOC treatments; indeed at 9–24 h, the elevation in ammonia net negative flux seen in the IPW pH 4.0 + DOC treatment was significant relative to all other treatments (Figure 4). In summary, exposure to low pH alone had negligible effect on ammonia excretion, whereas the presence of SGC DOC resulted in elevated $J_{amm.net}$ at pH 4.0, but not at pH 7.0.

3.5 | Plasma sodium, chloride, ammonia and urea concentrations

In accord with the increased net losses of Na^+ and Cl^-, the exposure to SGC DOC in IPW pH 4.0 caused marked significant decreases in plasma concentrations of these electrolytes in *C. macropomum* after 24 h of exposure (Table 2). There were no significant differences among the other three treatments. The absolute decrement in plasma (Cl^-) (*c.* 43 mmol l^{-1}) was larger but more variable than the absolute decrement in plasma (Na^+) (*c.* 18 mmol l^{-1}), in contrast with the pattern of net fluxes over 24 h (Figures 2 and 3). The increased ammonia

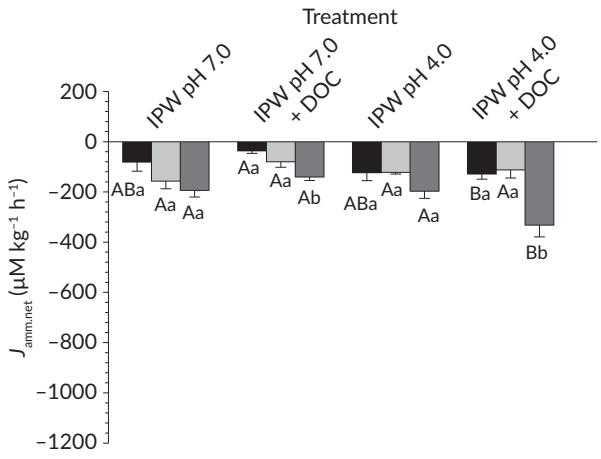

FIGURE 4 Mean (+ SE) net ammonia fluxes rates ($J_{amm.net}$) of *Colossoma macropomum* in ion-poor water (IPW) pH 7.0 and 4.0, with and without natural dissolved organic carbon (DOC) from São Gabriel da Cachoeira black water over the flux periods of 0–3 h, 3–9 h and 9–24 h since the start of exposure. Different lowercase letters indicate significant differences of the mean values within the same treatment among the different flux periods ($P < 0.05$). Different capital letters indicate significant differences among the different treatments ($P < 0.05$) within the same flux period. (■) 0–3 h, (□) 3–9 h, and (■) 9–24 h

TABLE 2 Major plasma ions, ammonia, and urea concentrations of *Colossoma macropomum* after 24 h of exposure to ion poor water (IPW) at pH 7.0 or pH 4.0, with and without natural dissolved organic carbon (DOC) from São Gabriel da Cachoeira black water

	Concentration (mean ± SE mmol l^{-1})			
Treatment	Na^+	Cl^-	Ammonia	Urea
IPW pH 7.0	159 ± 4[a]	127 ± 4[ab]	0.12 ± 0.01[a]	0.90 ± 0.12
IPW pH 7.0 + DOC	157 ± 5[a]	135 ± 6a	0.09 ± 0.01[a]	0.79 ± 0.12
IPW pH 4.0	161 ± 4[a]	107 ± 15[ab]	0.14 ± 0.02[ab]	1.03 ± 0.10
IPW pH 4.0 + DOC	140 ± 4[b]	86 ± 14[b]	0.22 ± 0.04[b]	0.85 ± 0.08

Note. Different superscript letters indicate significant differences among the groups ($P < 0.05$).

excretion rates seen in *C. macropomum* exposed to SGC DOC in IPW pH 4.0 were accompanied by a significant increase in plasma total ammonia after 24 h (Table 2). Again, there were no significant differences among the other three groups. The plasma urea concentration was not altered in *C. macropomum* in any treatment group (Table 3).

3.6 | Enzyme activities in gill filaments

No significant differences in the specific activities of Na^+, K^+ ATPase or v-type H^+, ATPase were found in gills of *C. macropomum* among the different experimental treatments (Table 3). The specific activity of v-type H^+, ATPase was consistently higher (by six to nine-fold) than that of Na^+, K^+ ATPase. On the other hand, *C. macropomum* exposed to acidic water in the presence of DOC (IPW pH 4.0 + DOC) had a higher value of the specific activity of carbonic anhydrase in the gills than fish exposed to neutral water either with DOC (IPW pH 7.0 + DOC) or without DOC (IPW pH 7.0) (Table 3).

4 | DISCUSSION

4.1 | DOC characterisation

In the present work, the SGC DOC molecules displayed optical properties (Table 1) indicating lower molecular weight (higher $Abs_{254/365}$) and lower aromaticity (lower SAC_{340}) in comparison with the values reported by Duarte *et al.* (2016) for the same DOC, but measured shortly after collection. The latter values were highly unusual for freshwater DOC in general (Duarte *et al.*, 2016, Table 1), indicative of very high aromaticity and very high molecular weight and were thought to reflect conditions in the upper Rio Negro close to major terrigenous sources of this allochthonous DOC. However, in both analyses, the I_F values were quite low confirming the allochthonous origin (Mcknight *et al.*, 2001). Furthermore, in both studies, the PARAFAC analysis of the relative contributions of major components to total fluorescence were similar, indicating a predominance of humic-like (51–59%) over fulvic like moities (28–36%) as the two major allochthonous components, but the tryptophan-like (8%) and tyrosine-like compounds (5%) were not insignificant, suggesting some autochthonous input.

As the techniques used for the two analyses were identical, the most likely explanation for the differences would be degradation over the period of time that the DOC was stored in the dark at 4°C, resulting in loss of aromatic compounds and reduction in mean molecular weight. Changes in DOC quality occurs spatially and temporally in nature between sites and even within the same sites in the Rio Negro (Holland *et al.*, 2017). Indeed, three recent investigations on Rio Negro DOC quality for samples collected 700–800 km downstream from the SGC collection site have reported $Abs_{254/365}$ and SAC_{340} values much closer to those of the stored SGC used in the present study (Holland *et al.*, 2017; Johannsson *et al.*, 2017; O.E. Johannsson, unpublished data).

TABLE 3 Specific activity of the Na^+, K^+ ATPase; v-type H^+ATPase and carbonic anhydrase of the gills of *Colossoma macropomum* after 24 h exposure to ion poor water (IPW) at pH 7.0 or pH 4.0, with and without natural dissolved organic carbon (DOC) from São Gabriel da Cachoeira black water

Treatment	Na^+, K^+ ATPase (mean ± SE µmol ATP h^{-1} mg^{-1} protein)	v-type H^+, ATPase (mean ± SE µmol ATP h^{-1} mg^{-1} protein)	Carbonic Anhydrase (mean ± SE specific activity mg^{-1} protein)
IPW pH 7.0	0.61 ± 0.08[a]	3.93 ± 0.38[a]	27.00 ± 1.17 [a]
IPW pH 7.0 + DOC	0.46 ± 0.07[a]	3.96 ± 0.38[a]	29.25 ± 1.03 [a]
IPW pH 4.0	0.55 ± 0.14[a]	3.76 ± 0.53[a]	29.72 ± 0.71 [ab]
IPW pH 4.0 + DOC	0.57 ± 0.12[a]	4.20 ± 0.43[a]	32.99 ± 0.85 [b]

Note. Values are. Different superscript letters indicate significant differences among the groups ($P < 0.05$).

4.2 | Branchial transepithelial potential (TEP), ion fluxes and effects of DOC

The TEP in freshwater fish can be interpreted as a diffusion potential predominantly regulated by the relative permeabilities of the gills to positively (mainly Na^+) and negatively (mainly Cl^-) charged ions (Galvez *et al.*, 2008; McWilliams & Potts, 1978). Under control conditions the negative potential in IPW pH 7.0 (Figure 1) would be expected to counter the preferential efflux of Na^+, which has a higher permeability than Cl^-. In the present work, acid exposure alone (IPW pH 4.0) caused a rise in TEP by about 14 mV in relation to neutral water, indicating that the relative permeabilities became more equal to one another at low pH. This response in TEP was very similar to, but in smaller magnitude, than those reported by Wood *et al.* (1998) for *C. macropomum* exposed to pH 4.0 in IPW without DOC and also to brown trout *Salmo trutta* L. 1758 exposed to low pH in water with low Ca^{2+} concentrations (McWilliams & Potts, 1978). This shift in potential is expected, since freshwater fish challenged by acidic waters usually display a large stimulation of diffusive losses of both Na^+ and Cl^-, in addition to an inhibition of active Na^+ and Cl^- uptake and thereby experience disturbances in both TEP and ionic regulation (Gonzalez *et al.*, 2005; Wood, 1989; Wood *et al.*, 2011). Thus, the rise in TEP seen in *C. macropomum* at IPW pH 4.0 might be explained by the stimulatory effects of increased H^+ on both J^{Na}_{net} and J^{Cl}_{net} of fish (Figures 2 and 3), as seen as by increased Na^+ (0–3 h) and Cl^- (3–9 h) net losses in *C. macropomum* at low pH in the absence of DOC. Interestingly, *C. macropomum* showed a pattern of decreasing loss over

time, resulting in cumulative net Na^+ (−2256 μmol kg^{-1}) and Cl^- losses (−2143 μmol kg^{-1}) about equal, which were not enough to promote disturbance in the plasma concentrations of both Na^+ and Cl^- in *C. macropomum* after 24 h under acidic conditions in the absence of DOC (IPW pH 4.0; Table 3). This was very comparable to the pattern reported by Wilson *et al.* (1999) in *C. macropomum* exposed to IPW pH 4.0 under similar conditions and indicates an ability to control ion losses over time, as seen in many acidophilic fish (Nelson, 2015; Wood, 1989).

The presence of SGC DOC clearly offered protection against the depolarising effect of low pH exposure in *C. macropomum* (Figure 1). At pH 7.0, the presence of DOC caused hyperpolarisation of the TEP by about −7 mV, a phenomenon previously reported in rainbow trout exposed to other allochthonous DOCs sources (Galvez *et al*., 2008). More importantly, the presence of DOC at pH 4.0 completely prevented the depolarisation of the TEP which occurred at low pH in the absence of DOC, allowing internal TEP to remain about 12 mV more negative in relation to fish at IPW pH 4.0, an effect that would be expected to preferentially help to retain Na^+. However, the Na^+ and Cl^- net losses were markedly stimulated when native *C. macropomum* were exposed to low pH in the presence of SGC DOC (IPW pH 4.0 + DOC) (Figures 2 and 3). Surprisingly, this occurred despite the protective effect of SGC DOC against the depolarisation of the TEP caused by low pH exposure (Figure 1). The exacerbation of ion losses by SGC DOC in the present study is completely different from the great protection against ion losses at low pH by freshly collected SGC DOC reported by Duarte *et al*. (2016) for non-native *D. rerio* and by Wood *et al*. (2003) for native potamotrygonids, where natural DOC-rich Rio Negro water provided great protection against influx inhibition and efflux stimulation by low pH, relative to similar IPW lacking DOC. Nevertheless, it is similar to the results of Glover *et al*. (2012) who reported that native DOC did not protect Na^+ balance and indeed exacerbated the inhibition of Na^+ uptake at low pH in the inanga *Galaxias maculatus* (Jenyns 1842), a fish native to acidic waters in New Zealand, as well as the report of Wood *et al*. (2003) that a commercial DOC (Aldrich humic acid) exacerbated the negative effects of low pH on potamotrygonids.

DOC molecules are known to directly interact with gill membranes and are also known to affect the permeability of cell membranes (Vigneault *et al*., 2000), where the interaction has been shown to become more intense at low pH (Campbell *et al*., 1997). How they might prevent or exacerbate ion losses under acid conditions, however, remains unknown. Using biogeochemical modelling, Wood *et al*. (2003) concluded that DOC could not protect the fish gill against H^+ binding but would reduce epithelial Ca^{2+} binding, so they hypothesised that DOC might actually substitute for Ca^{2+} in stabilising paracellular junctions, reducing tight junction permeability and thereby reducing diffusive ion losses (Gonzalez *et al*., 2005; Wood *et al*., 2003, 2011). Additionally, they suggested that DOC might concentrate Na^+ and Cl^- ions (by complexation) locally at the apical membrane of the gill in a similar manner to mucus (Handy, 1989), preferentially delivering the ions to the active uptake sites. In previous studies with *C. macropomum* (Wood *et al*., 1998) and *S. trutta* (McWilliams & Potts, 1978), the addition of Ca^{2+} to the IPW attenuated the potential shift caused by low pH exposure, while at the same time reducing the accompaning diffusive ion losses. However, it did the former by raising the TEP to a less negative value at pH 7.0, while having little effect on the value at pH 4.0. Therefore, the actions of DOC in the present study were different from those of elevated Ca^{2+}, because the TEP values at both pH 7.0 and pH 4.0 were both made more negative. Furthermore, ion losses at low pH were not reduced but rather exacerbated, as discussed above. From this, we conclude that the mechanisms of action of SGC DOC and Ca^{2+} in controlling the diffusive ionic losses at low pH differ from one another. Overall, no clear picture yet emerges, in accord with the conclusions of a recent review (Nelson, 2015).

Although the unexpected large stimulation in both Na^+ and Cl^- was seen mainly in the first 9 h of exposure to acidic conditions in the presence of SGC DOC, the ionic losses of *C. macropomum* were quite similar than those seen in fish following 9–24 h exposure to all other treatments, demonstrating that the fish were able to control ionic losses over time. However, at acidic conditions when SGC was present (IPW pH 4.0 + DOC) the cumulative net Na^+ (−8455 μmol kg^{-1}) and Cl^- (−5360 μmol kg^{-1}) losses of *C. macropomum* were markedly higher over 24 h, where the net loss of Na^+ exceeded net Cl^- losses by 57% (Figures 2 and 3). Nevertheless, there was a modest reduction in (Na^+) but a three-fold greater fall in (Cl^-) in plasma of *C. macropomum* at IPW pH 4.0 + DOC (Table 3). These data suggest that acid exposure was accompanied by a substantial redistribution in ions between intracellular and extracellular compartments and that this redistribution differed in the presence and absence of DOC. In future, it will be of interest to measure tissue ion levels during low pH exposure in *C. macropomum* .

Through the analysis of the DOC quality (discussed earlier), it is apparent that the SGC DOC after the storage became less aromatic (lower SAC_{340}) and the molecules became smaller (higher $Abs_{254/365}$; Table 1), which could change the way these molecules interact with biological membranes. Overall, our data suggest that the stored SGC DOC used here does not have an ameliorative effect to protect *C. macropomum* against ionoregulatory disturbances promoted by low pH exposure, as seen with fresh SGC in *D. rerio* by Duarte *et al*. (2016), but rather it exacerbates the ionic losses of both Na^+ and Cl^- in this species. The greater loss of Na^+ than of Cl^- is in accord with the more negative TEP. Although the exposure to stored SGC DOC at low pH helps fish to maintain a more negative TEP, it seems not to limit the overall gill permeability to Na^+ and Cl^-. These findings are contrary to McWilliams and Potts (1978) and Wood (1989), suggesting that the positive shift in TEP may not be the principal factor driving net Na^+ losses at low pH in this acid-tolerant fish species.

4.3 | Ammonia excretion, plasma ammonia and urea concentrations and branchial enzyme activities

The present data (Figure 4) confirm previous findings that *C. macropomum* are highly resistant to stimulation of $J_{Amm,net}$ under exposure to acidic waters (Wood *et al*., 1998, 2018), in contrast with many other freshwater fishes (Duarte *et al*., 2016; Gonzalez & Dunson, 1989; Gonzalez & Wilson, 2001; Kwong *et al*., 2014; Wood, 1989). In most freshwater fish, increased ammonia excretion at low pH is explained by the increased passive diffusion of NH_3, which is facilitated by the

acid-trapping of NH_3 in the boundary layer of the gills (Wilkie, 2002). The almost negligible NH_3 partial pressures in the ambient water serve as a perfect sink for excretion *via* NH_3 diffusion from blood to water (Gonzalez *et al*., 2005). Additionally, plasma ammonia levels often rise associated with increased metabolic production of ammonia when fish are stressed by acidity (Kwong *et al*., 2014). However, the plasma concentrations of ammonia in *C. macropomum* at IPW pH 4.0 did not increase and were kept at levels similar to those of animals under neutral pH (in either the presence or absence of DOC) and close to values previously reported to *C. macropomum* during exposure to IPW pH 4.0 (Wood *et al*., 1998). Therefore, the lack of elevation in $J_{Amm.net}$ seen in *C. macropomum* exposed to IPW pH 4.0 probably reflects a lack of elevation in ammonia gradient and a lack of elevated production rate in these acidophilic fish.

In contrast, increased ammonia excretion was seen in *C. macropomum* exposed to SGC DOC under acid conditions, particularly during 9–24 h of exposure. Increases in ammonia excretion were also reported in potamotrygonids of the Rio Negro and exposed to low pH in natural black water (Wood *et al*., 2003). Interestingly, in that study, increases in ammonia net fluxes were facilitated in reference water by the addition of Ca^{2+}, demonstrating that native DOC molecules may interact with gill membranes in the same manner as Ca^{2+} ions with respect to ammonia excretion mechanisms (Wood *et al*., 2003). In *D. rerio*, a stimulation in $J_{Amm.net}$ was seen in fish exposed to pH 4.0 in IPW in the presence of two different sources of DOC, which was thought to be essential for animals to keep Na^+ uptake coupled to ammonia excretion under acidic conditions (Duarte *et al*., 2016, 2018). This coupling was thought to be associated with an upregulation of the Rh-protein–Na^+–NH_4^+ exchange metabolon in the gills (Weihrauch *et al*., 2009; Wright & Wood, 2009). It is unknown whether this metabolon is operative in the *C. macropomum*. In *C. macropomum* exposed to pH 4.0 in IPW with SGC DOC, there was a marked increase in plasma total ammonia concentration (Table 3), which suggests an increment in oxidative metabolism towards enhanced proteolysis and amino-acid oxidation, resulting in elevated ammonia production (Sinha *et al*., 2013). Notably, the plasma concentration of urea, which comes largely from uricolysis rather than from protein–amino acid oxidation (Wood, 1993), was not elevated (Table 3). *Colossoma macropomum* are unusual in largely relying on protein–amino acid oxidation to maintain their aerobic metabolism (*e.g*., between 60% and 70% support; Pelster *et al*., 2014; Wood *et al*., 2017). Altogether, our data indicate that under low pH exposure in the presence of SGC DOC, *C. macropomum* enhanced ammonia production, resulting in both ammonia accumulation in plasma and increased ammonia excretion.

In three acidophilic characids of the Amazon Basin, tested in naturally acidic, DOC-rich water, Na^+ uptake appeared dependent on ammonia excretion and the mechanism appeared to be strongly dependent on intracellular carbonic anhydrase and basolateral Na^+, K^+ ATPase in the gill epithelium (Wood *et al*., 2014). In the present study, the activities of both Na^+, K^+ ATPase and v-type H^+, ATPase in the gills of *C. macropomum* were very similar in all four treatment groups (Table 3), suggesting that neither acidic conditions nor the presence of SGC DOC affected these enzymes. However, as seen for the increased ammonia excretion and concentration in plasma, the activity of carbonic anhydrase in the gills of *C. macropomum* was significantly stimulated in IPW pH 4.0 + DOC. Although the specific mechanisms for apical Na^+ uptake in *C. macropomum* are unknown, the present results suggest that SGC DOC may act to increase both carbonic anhydrase activity and ammonia excretion order to generate the required electrochemical gradient to drive Na^+ uptake under ion-poor acidic conditions. Further exploration of this topic is an important question for future examination in the *C. macropomum* and other acidophilic Amazon fishes.

Thus, in the present study, stored SGC DOC did not protect the native Amazon species *Colossoma macropomum* against the deleterious effects of low pH exposure. The positive shift in the TEP of *C. macropomum* exposed to SGC DOC at pH 4.0 demonstrated that this may not be an important factor driving net Na^+ losses at low pH. Indeed, the *C. macropomum* under these conditions experienced greater net Na^+ and Cl^- losses, decreases of Na^+ and Cl^- concentrations in plasma and elevated plasma ammonia levels and excretion rates, relative to those exposed in the absence of DOC. These findings could be related to species-specific differences and changes in DOC properties during storage. In future studies, it will be preferable to use DOC that has been freshly collected from the wild, rather than stored DOC. In addition, it will be of interest to investigate whether protective elements in the DOC are simply lost over time in storage, or whether they degraded into molecules that have the opposite action. Also, future studies should address the effects of DOC with different physico-chemical characteristics on the ionic and osmotic homeostasis of freshwater fishes under short-term acidic exposure and during acclimation to low pH, contributing to a better understand of the mechanistic action of DOC molecules on the gill physiology of freshwater fishes.

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