Received: 30 September 2024 | Revised: 17 December 2024 | Accepted: 20 December 2024

DOI: 10.1111/jfb.16054

REGULAR ARTICLE

JOURNAL OF FISH BIOLOGY fsbi

Dissolved organic carbon from "white" waters in Amazon basin (Brazil) and the osmoregulatory responses of tambaqui (*Colossoma macropomum*) in neutral and acidic environments

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Funding information
Fundação de Amparo à Pesquisa do Estado do Amazonas; Conselho Nacional de Desenvolvimento Científico e Tecnológico; Instituto Nacional de Ciência e Tecnologia Centro de Estudos das Adaptações da Biota Aquática da Amazônia; Natural Sciences and Engineering Research Council of Canada, Grant/Award Numbers: RGPIN/473-2012, RGPIN-2017-03843, RGPIN-2023-03714; Ciência sem Fronteiras

Abstract

Optical characterization of dissolved organic carbon (DOC) freshly collected from the circumneutral "white water" of the Rio Solimoes revealed that it had lower aromaticity, lower molecular weight, and a greater autochthonous content than DOC from the acidic "black water" of the Rio Negro. The tambaqui (*Colossoma macropomum*), a characid member of the Serrasalmidae, is a model neotropical fish that migrates annually between the two rivers. We analysed ionoregulatory responses of the tambaqui over 24 h in ion-poor water at pH 7.0 and pH 4.0 in the absence and presence of 10 mg L^{-1} Rio Solimoes DOC ("SOL DOC"). Measured parameters included trans-epithelial potential (TEP) across the gills, net flux rates, and plasma concentrations of Na$^+$, Cl$^-$, ammonia, and urea, and branchial Na$^+$, K$^+$ –ATPase, H$^+$, ATPase, and carbonic anhydrase activities. Results were compared to our earlier study using similar protocols to examine the ionoregulatory effects of 10 mg L^{-1} DOC ("SGC DOC") collected from black water at São Gabriel da Cachoeira (SGC) in the upper Rio Negro. At pH 7.0, SOL DOC had no effect on the negative TEP across the gills. Exposure to pH 4.0 caused a marked depolarization of the TEP to positive values that was not ameliorated by the presence of SOL DOC. This contrasts with SGC DOC that drove TEP more negative at pH 7.0 and fully protected against the depolarization at pH 4.0. However, SOL DOC promoted positive balance of Na$^+$ and Cl$^-$ at pH 7.0 and helped ameliorate the negative balance of these ions seen at pH 4.0. This again contrasts with SGC DOC that exacerbated ion losses at pH 4.0. The protective effects of SOL DOC on ion balance maybe related to increased v-type H$^+$ ATPase activity in the gills, and unrelated to TEP. The very different responses to the two DOCs are discussed with respect to their optical properties, the time that they were in storage prior to testing (SOL <1 month, SGC 2 years), and the life history of the tambaqui in the two rivers.

KEYWORDS
acidic water, Amazon "white water", net fluxes, osmoregulation, SAC$_{340}$

1 | INTRODUCTION

The Amazon region presents heterogeneous characteristics in its different aquatic environments, which differ mainly in their chemical and physical properties (Sioli, 1984). These properties of Amazon waters allowed their classification into "black," "white," and "clear" waters (Junk, 1983; Val & Almeida-Val, 1995; Wallace, 1889), which mainly reflects differences in their content of dissolved ions (particularly, Na^+, Cl^-, and Ca^{2+}), the concentration of dissolved organic carbon (DOC), and pH (Hedges et al., 1994; Leenheer, 1980). The DOC present in these aquatic environments can be classified as allochthonous, autochthonous (Thurman, 1985), or a mixture of both types (McKnight et al., 2001). Allochthonous DOCs are derived from the degradation of land-based plant materials, have a dark (*black tea*) color, and their molecules have high molecular weight. The autochthonous DOCs, produced in waterbodies by bacterial action or photoconversion, tend to be clearer (*cafe-au-lait*), and their molecules have lower molecular weight (Leenheer, 1980). The major components of aquatic DOC are the humic substances (50%–90% of total content), which are a heterogeneous combination of higher-molecular-weight humic acids (HA) and lower-molecular-weight fulvic acids (FA) (Al-Reasi et al., 2013). The black waters are the most dilute and most acidic, with pHs often in the range of 3.5–5.0 due to their high concentrations of mainly allochthonous DOC. The white waters also contain allochthonous DOC, but they may also have an autochthonous component, and total DOC concentrations are lower than in black waters. White waters also have much higher concentrations of ions, particles, and natural alkalinity, resulting in pHs that are usually close to neutrality.

Spectroscopic measurements have been used to differentiate physicochemical properties of DOCs from various freshwater sources (Al-Reasi et al., 2011; Morris et al., 2021) and employed to provide qualitative insights about the chemical reactivity of freshwater DOC molecules. For example, the absorbance ratio as $Abs_{254/365}$, the specific absorbance coefficient (SAC_{340}), and the fluorescence index (FI) have been used as estimators of the molecular weight (Dahlén et al., 1996), aromaticity (Curtis & Schindler, 1997), and origin of DOC molecules (McKnight et al., 2001), respectively. Moreover, the relative abundance of fluorescence components of DOC that represent the humic-like, fulvic-like, and proteinaceous materials (the latter as tryptophan-like and tyrosine-like compounds) can be estimated by parallel factor analysis (PARAFAC). Through spectral deconvolution of a stack of fluorescence excitation-emission matrixes (FEEMs), PARAFAC quantifies a minimum number of user-defined fluorescent components to describe each FEEM (Stedmon & Bro, 2008). Each FEEM is considered as a unique fingerprint of the DOC source, providing qualitative information on the presence of the fluorescent groups (e.g., humic-like fractions, fulvic-like fractions, protein-like fractions) (Stedmon & Bro, 2008).

The functional properties of the DOC, which are associated with the spectroscopic characteristics, can predict the capacity of the DOC molecules to interact, directly or indirectly, with biological surfaces affecting many physiological processes of the aquatic biota. Indirect interactions are related with the ability of the DOC to complex and sequester metals, or other pollutants, reducing the bioavailability of contaminants and, consequently, their toxicity to aquatic animals (Cremazy et al., 2016; Haitzer et al., 1998; Holland et al., 2017; Matsuo et al., 2006; Sadauskas-Henrique et al., 2016; Wood et al., 2011). On the contrary, direct interactions are related with the ability of the DOC to interact with the aquatic organisms themselves, altering gill membrane permeability (Galvez et al., 2008; Vigneault et al., 2000) and affecting basic physiological functions (Duarte et al., 2016; Galvez et al., 2008; Wood et al., 2003). In most cases, the DOCs appear to provide protection at the gills, particularly against the damaging effects of low pH on ionoregulation (Duarte et al., 2016; Galvez et al., 2008; Gonzalez et al., 1998, 2002; Matsuo et al., 2006; Val & Wood, 2022; Wood et al., 2003, 2011). In both cases, these abilities of the DOC are related to its concentration and spectroscopic characteristics, like aromaticity (SAC_{340}) and the proton binding capacities (PBI) (Al-Reasi et al., 2013; Duarte et al., 2016; Galvez et al., 2008; Morris, Zulian, Brauner, et al., 2024).

Most fish encountering acidic conditions in fresh water experience disturbances in their ionic homeostasis, as demonstrated by inhibition of active Na^+ and Cl^- uptake, stimulation of massive diffusive ionic losses, and reversal of the transepithelial potential (TEP) in gills (Gonzalez et al., 2005; Sadauskas-Henrique et al., 2016, 2019, 2021). These responses can lead to ionic imbalances and consequent decreases in plasma concentrations of major ions (Milligan & Wood, 1982; Sadauskas-Henrique et al., 2019). However, several Amazon fish species endemic to naturally acidic Amazon black waters seem to have developed mechanisms to avoid these ionic disturbances at low pH (Gonzalez et al., 2006; Val & Wood, 2022). Among these species, the tambaqui (*Colossoma macropomum*, Cuvier 1816), a characid fish from the Serrasalmidae family, is considered one of the most acid-tolerant species (Gonzalez & Wilson, 2001; Wilson et al., 1999). The tambaqui is particularly interesting, because it lives in both black water and white water, migrating annually between the two in its natural life history (Araújo-Lima & Goulding, 1998).

In an earlier study, the effects of DOC from the São Gabriel da Cachoeira (SGC) region of the upper Rio Negro (black water) on a range of ionoregulatory parameters in tambaqui in ion-poor water (IPW) were examined (Sadauskas-Henrique et al., 2019). SGC DOC ($10\ mg\ L^{-1}$) had negligible effects at pH 7.0 despite driving the TEP across the gills to a more negative value. Under acidic conditions, SGC prevented the depolarization of TEP caused by exposure to pH 4.0, consistent with the prediction that it would be protective. However, at pH 4.0, the presence of SGC DOC unexpectedly exacerbated net Na^+ and Cl^- loss rates and disrupted ammonia excretion, contrary to the initial prediction. This was surprising, because an earlier study on zebrafish in IPW had demonstrated protective actions of SGC DOC against ionoregulatory disturbances at pH 4.0 (Duarte et al., 2016).

With this background in mind, in the present study, we employed very similar methods to those of Sadauskas-Henrique et al. (2019) to examine the ionoregulatory effects of Rio Solimoes (SOL) DOC collected from white water on tambaqui. Experiments were conducted in IPW at pH 7.0 and pH 4.0, as in Sadauskas-Henrique et al. (2019).

Our specific goals were (i) to investigate the spectroscopic characteristics of this SOL DOC from white water of the Amazon basin and (ii) to investigate the influences of this DOC on the osmoregulatory parameters (TEP, Na^+, Cl^-, ammonia, and urea net fluxes; Na^+, Cl^-, ammonia, and urea plasma concentrations, and the activity of the Na^+, K^+ ATPase [NKA]; v-type H^+ ATPase [VHA]; and carbonic anhydrase [CA] in gill filaments) of tambaqui in both neutral (pH 7.0) and acidic (pH 4.0) environments.

2 | MATERIALS AND METHODS

2.1 | Fish holding

Tambaqui (*C. macropomum*, Cuvier 1816) were obtained from a local fish farm (Fazenda Santo Antônio, Amazonas, Brazil) and held for approximately 1 month in outdoor 3000-L polyethylene tanks with continuous-flow IPW sourced from Instituto Nacional de Pesquisas da Amazônia (INPA) (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).

During the acclimation period, fish were fed until satiation with dry food pellets (26% protein content, Nutripeixe-Purina). Feeding was suspended 2 days prior to the experiments. All experimental and holding procedures followed Brazilian animal care guidelines and were previously approved by INPA's animal care committee (registration number 047/2012).

2.2 | Water collection and DOC concentration and characterization

Water for DOC collection and concentration was obtained from the Rio Solimões (SOL, "white water" 3°15′20″ S 60°14′52″ W) in Iranduba city in April 2015. The "white water" (Na^+ 123, K^+ 20, Ca^{2+} 22, Mg^{2+} 40 μmol L^{-1}, DOC 4.2 mg L^{-1}; pH 6.8 and temperature 28°C) was pumped from the main channel, with the aid of a submerged pump. Due to the high quantity of suspended solids in the Rio Solimões, prior serial filtrations were performed using polypropylene filters (Permution) with pore sizes of 25, 10, and 5 μm. After the filtrations, the water was pumped through a 1-μm polypropylene filter to a reverse osmosis unit (Vontron ULP21-4021 polyamide membrane, Permution, model PEOS-0001, Curitiba, Brazil). To remove the cations, which were concentrated together with the DOC during the reverse osmosis, the concentrate was treated with a cation exchange resin (Amberlite IR–118 [H], Sigma-Aldrich, St. Louis, MO, USA). Lastly, the concentrate was filtered using a 0.45-μm membrane (Acrodisc, Pall, Ann Arbor, MI, USA) and stored at 4°C until the physical–chemical characterization and fish experiments. Both were performed within 1 month of the collection.

SOL water was submitted for the optical measurements (SpectraMax M2 fluorescence spectrophotometer, Molecular devices; www.moleculardevices.com; Agilent Cary Eclipse spectrophotometer; www.agilent.com). The specific absorbance coefficient at 340 nm (SAC_{340}) and the fluorescent index (FI) were determined as described by McKnight et al. (2001), and the ratio absorbance at 254 nm to that at 365 nm ($Abs_{254/365}$) was determined according to Dahlén et al. (1996). PARAFAC was applied to the previously determined FEEMs. The spectral FEEMs were modeled using the PLS Toolbox from Eigenvector Research Inc. (Wenatchee, WA, USA) running on a Matlab platform. 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).

2.3 | Experimental setup for flux, plasma, and tissue measurements

Solutions of DOC isolated from SOL were prepared 24 h before the beginning of the experiments by diluting the concentrated DOCs to a final concentration of 10 mg of carbon L^{-1} in IPW from INPA. The DOC concentration was read on a total carbon analyser (Apollo 9000 combustion TOC analyser, Teledine Tekmar). 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. Tambaqui ($n = 8$, weight 129 ± 4 g, mean ± SE of the mean [SEM]) was individually transferred into glass aquaria shielded with black plastic and filled with 4 L of aerated experimental solution: IPW pH 7.0, IPW pH 7.0 + DOC, IPW pH 4.0, and IPW pH 4 + DOC. Fish were kept for 1 h in the experimental glass aquaria before the experimental series was started. 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. Immediately after collection, water samples were stored at −20°C until analysis for the concentrations of Na^+, Cl^-, ammonia, and urea. At the end of 24 h, fish were terminally anaesthetized with neutral buffered MS-222 (0.5 g of MS-222 L^{-1} and 2.0 g of $NaHCO_3$ L^{-1}, Sigma Aldrich) for blood collection. Blood was collected from a puncture of the caudal vein and then centrifuged (2000 g, 25°C, 5 min) for plasma separation, which was stored at −80°C until the measurement of Na^+, Cl^-, ammonia, and urea concentrations. Immediately after blood collection, the fish was euthanized by cervical dislocation, and then gill filaments were collected, frozen in liquid nitrogen, and stored at −80°C for analysis of Na^+, K^+, and ATPase; VHA and CA activities and protein concentrations.

2.4 | Experimental setup for transepithelial potential measurements

For the TEP measurements, a separate set of tambaqui was used ($n = 8$; weight: 157 ± 9 g, mean ± SEM). Fish were fitted with intraperitoneal catheters as described by Wood and Grosell (2008). Fish were anaesthetized using neutral buffered MS-222 (0.5 g of MS-222 L^{-1} and 2.0 g of $NaHCO_3$ L^{-1}, Sigma Aldrich) in IPW (pH 7.0 or pH 4.0 as appropriate). A saline-filled PE50 catheter (Clay-Adams; Becton–Dickinson, Sparks, MD, USA) 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 revived by returning them to IPW and then transferred into individual 4-L chambers where they were held overnight prior to TEP measurements. To minimize stress, the fish was gently transferred into a 2-L plastic chamber in which TEP measurements were made. In each series, TEP measurements were first made in freshly IPW at 28°C, which was then replaced by four exchanges of the test solution (IPW pH 7.0, IPW pH 7.0 + DOC, IPW pH 4.0, and IPW pH + 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 (Radiometer pHM meter, Copenhagen, Denmark). 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) are expressed relative to the water side as 0 mV after correction for junction potential.

2.5 | Sodium, chloride, ammonia, and urea-N net fluxes

The net flux rates (J_{net}) of Na^+, Cl^-, total ammonia, and urea-N were calculated as

$$J_{net} = (X1 - X2) \times V\,(T \times W)^{-1},$$

where X1 and X2 were, respectively, the initial and final concentration of Na^+, Cl^-, total ammonia, and urea (μmol L^{-1}) in the water during the flux period. *V* is the volume in liters of the chamber; *T* is the interval of flux period in hours, and *W* is the fish weight in kilograms. Thus, net losses from the fish are negative, and uptakes by the fish are positive.

Water sodium concentrations were measured using a Perkin-Elmer model 3100 Atomic Absorption Spectrophotometer (AAS). Total chloride, ammonia, and urea were measured by colorimetric methods described by Rahmatullah and Boyde (1980), Verdouw et al. (1978), and Zall et al. (1956), respectively.

2.6 | Sodium, chloride, ammonia, and urea-N plasma concentrations

For determination of sodium and chloride concentrations, the plasma was diluted in a ratio of 1:1000 (plasma: water). For urea concentration, the plasma was diluted in a ratio of 1:100 (plasma: water). The concentrations were determined by the same methods used for the water samples. For plasma ammonia, an enzymatic method was employed (Raichem commercial assay, Cliniqa Corporation, San Marcos, CA, USA) using the plasma without dilution.

2.7 | Na^+, K^+ ATPase, v-type H^+ ATPase, and carbonic anhydrase in gill filaments

NKA and VHA were measured according to Kültz and Somero (1995). The assay is based on the inhibition of the NKA activity by ouabain (2 mMol L^{-1}), and the VHA 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, and 2 IU ml^{-1} LDH. A reaction mixture without any inhibitor was used to measure the total ATPase activity. Tissue samples were homogenized (1:10 for gills) in a 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 assays were performed at 25°C by combining 200 μL of the reaction mixture (with ouabain, NEM, and without inhibitors) and 5 μL of the homogenate. The change in the absorbance was read over 10 min at 340 nm. NKA and VHA activities were calculated as the difference between total activity and activity with ouabain and NEM inhibitors.

CA was determined according to Vitale et al. (1999). Activity of the CA was quantified by the rate of pH drop upon addition of CO_2-saturated ice-cold water. The slope of the linear regression of pH against time corresponds to the rate of the catalyzed reaction (catalyzed rate [CR]). The non-catalyzed rate (NCR) was assessed as the rate of pH drop over time in the absence of tissue homogenate, with the addition of 0.05 mL of the sample dilution buffer (225 mMol mannitol, 75 mMol sucrose, and 10 mMol trisphosphate at pH 7.4). CA-specific activity was calculated as: CAA = [(CR/NCR) − 1] mg total protein^{-1} in the sample (Vitale et al., 1999).

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

2.8 | Statistics

All data are reported as mean ± SEM. Statistical significance was accepted at $p < 0.05$. Significant differences in TEP were determined using one-way repeated-measures analysis of variance 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 used Sigma Stat and Sigma Plot software (Jandel Scientific, San Jose, USA).

3 | RESULTS

3.1 | DOC characterization

The optical properties of the Rio Solimoes DOC used in the present study are summarized in Table 1. For comparison, optical properties

TABLE 1 Summary of physicochemical properties of natural dissolved organic carbon (DOC) samples isolated by reverse osmosis from the Rio Solimoes (SOL) "white water."

						%[d]			
DOC source	**Coordinates**	**DOC collection date**	**SAC_{340} (cm^2 mg^{-1})[a]**	**$Abs_{254/365}$[b]**	**FI[c]**	**FA**	**HA**	**Trp**	**Tyr**
SOL	3°15′ S 60°14′ W	April 2015	22.9	5.6	1.3	33.7	60.9	4.0	1.3
SOL[e]	3°15′ S 60°14′ W	May–July 2015	33.4	4.5	1.5	45.6	51.5	1.2	1.6
SGC[f]	0° 07′ S 67° 05′ W	Collected 2013 and analysed 2015	36.5	4.0	1.0	28.3	58.8	7.6	5.1

Abbreviations: DOC, dissolved organic carbon; FA, fulvic acid-like; HA, humic acid-like; PARAFAC, parallel factor analysis; SAC_{340}, specific absorbance coefficient; SGC, São Gabriel da Cachoeira; SOL, Rio Solimões; Trp, tryptophan-like; Tyr, tyrosine-like.

[a] SAC_{340} is the specific absorbance coefficient at 340 nm normalized to DOC.

[b] $Abs_{254/365}$ is the ratio of absorbance at 254 nm to that at 365 nm.

[c] FI is the fluorescence index.

[d] % relative abundance of each DOC component determined using PARAFAC analysis.

[e] Data from Holland et al. (2017).

[f] Data from Sadauskas-Henrique et al. (2019).

(determined with similar methods) of another sample of DOC collected from the Rio Solimoes (Holland et al., 2017) somewhat later in the year (though both were collected during the wet season) are also shown in Table 1. The properties of the SGC DOC collected from the upper Rio Negro and used in the experiments of Sadauskas-Henrique et al. (2019) are also tabulated. For SOL DOC used in the present study, the ratio of the absorbance at 254/365 nm ($Abs_{254/365}$), which is considered an inverse index of molecular weight, was 5.6 (Table 1). The specific absorbance coefficient (SAC_{340}, cm^2 mg^{-1}), which is considered an aromaticity index, was 22.9. The FI, which provides information about the source and origins of the DOC, was 1.3. The contents of fulvic-like and humic-like compounds (%) were 60.9 and 33.7, respectively. The tryptophan-like and tyrosine-like contents (%) of the samples were 4.0 and 1.3, respectively (Table 1). The SOL DOC used in Holland et al. (2017) exhibited a more allochthonous characteristics (e.g., higher SAC_{340}, molecular weight, and FI) with higher HA and tyrosine-like compounds and lower FA and tryptophan-like compounds (Table 1). Both SOL DOC sources (present and used in Holland et al., 2017) were collected in the same season and site. The present SOL DOC was freshly used in the DOC characterization and experiments. However, the present SOL DOC presented some characteristics similar to the aged SGC DOC collected from the upper Rio Negro in 2013 and used in the experiments of Sadauskas-Henrique et al. (2019) in 2015. The aged SGC DOC and the fresh SOL DOC presented low FI and FA and high HA and tyrosine-like compounds (Table 1).

3.2 | Branchial transepithelial potential

The TEP was around −18 mV in IPW at pH 7.0 and did not change significantly in the presence of SOL DOC (10 mg C L^{-1}) (Figure 1). When tambaqui were transferred to IPW at pH 4.0, a strong depolarization (increase) occurred, raising the TEP to about +2.5 mV. Again, the presence of SOL DOC did not affect the response (Figure 1).

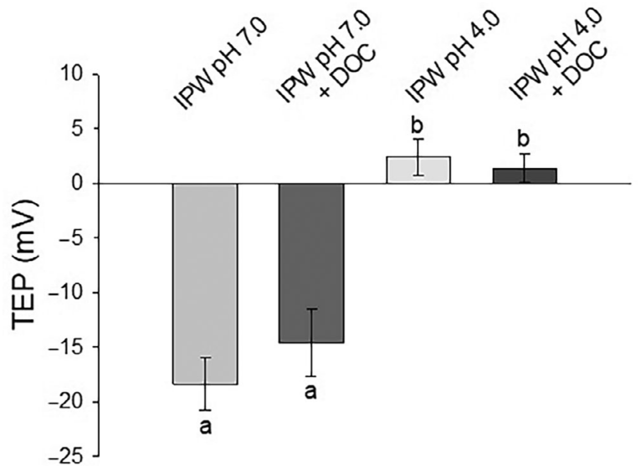

FIGURE 1 Mean (± SE of the mean [SEM], $N = 8$) response of gill transepithelial potential (TEP; inside relative to outside as 0 mV) of *Colossoma macropomum* to ion-poor water (IPW) pH 7.0 and pH 4.0, with and without natural dissolved organic carbon (DOC) from Rio Solimoes water. Different lowercase letters indicate significant differences ($p < 0.05$) among the treatment groups.

3.3 | Sodium, chloride, ammonia, and urea-N net fluxes

Tambaqui exposed to pH 7.0 IPW presented a negative balance for Na^+ over the first hours (0–3 h and 3–9 h), but there was a general trend for net Na^+ balance to became significantly positive by 9–24 h (Figure 2a). The same temporal trend from negative to positive Na^+

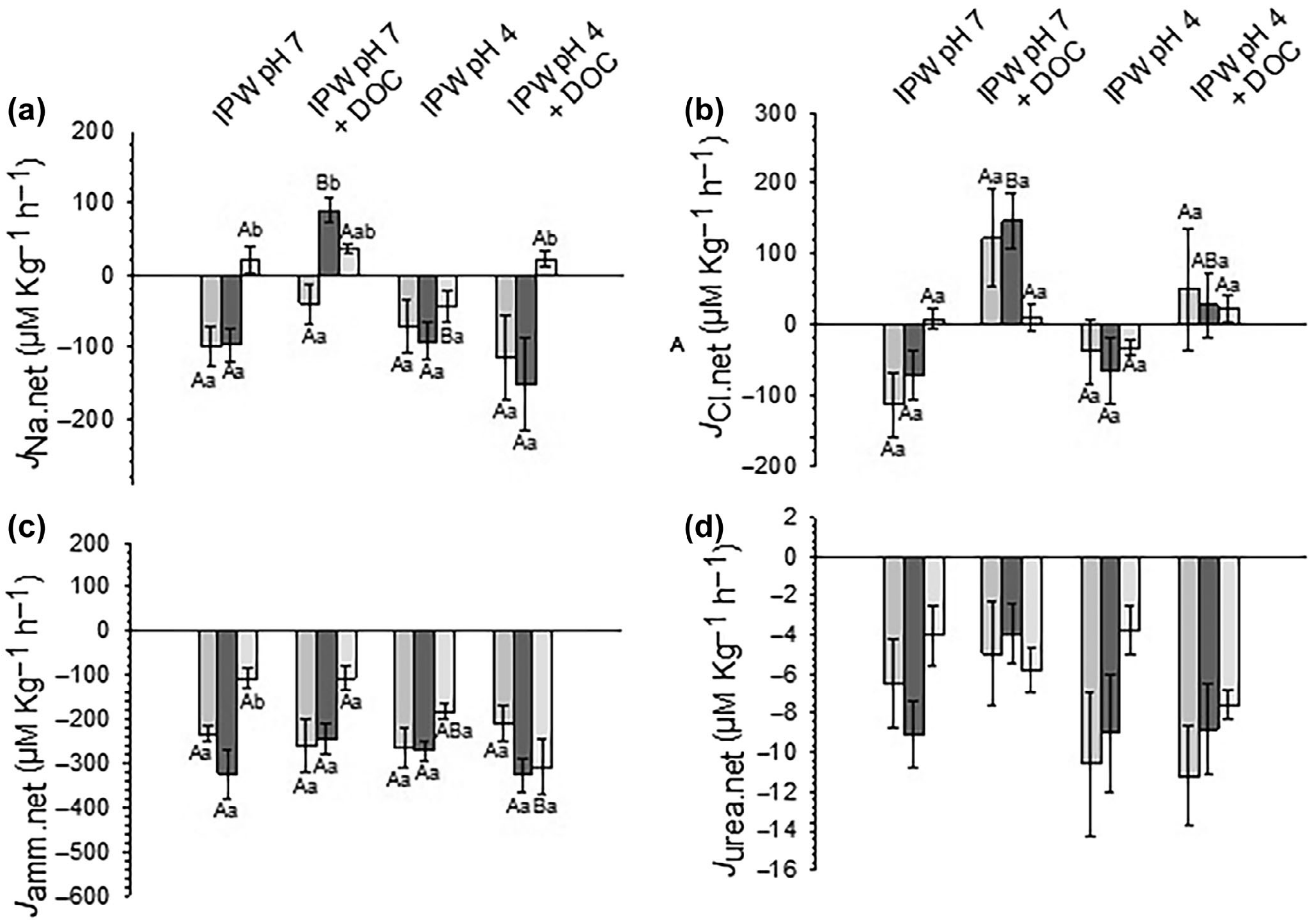

FIGURE 2 Mean (± SE of the mean [SEM], $N = 8$) of (a) net sodium flux rates ($J_{\mathrm{Na.net}}$), (b) net chloride flux rates ($J_{\mathrm{Cl.net}}$), (c) net ammonia flux rates ($J_{\mathrm{Amm.net}}$), and (d) net urea flux rates ($J_{\mathrm{Urea.net}}$) of *Colossoma macropomum* in ion-poor water (IPW) pH 7.0 and pH 4.0, with and without natural dissolved organic carbon (DOC) from the Rio Solimoes 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 ($p < 0.05$) of the mean values within the same treatment among the different flux periods (0–3 h, 3–9 h, and 9–24 h). Different capital letters indicate significant differences ($p < 0.05$) among the different treatment groups (IPW pH 7, IPW pH 7 + DOC, IPW pH 4, and IPW pH 4 + DOC) within the same flux period.

balance was observed when tambaqui were exposed to pH 7.0 IPW + DOC. However, the presence of DOC in neutral IPW water reversed the Na^+ loss rate more quickly, as the balance became positive at 3–9 h (Figure 2a). Exposure to acidic IPW water without DOC (IPW pH 4.0) caused increases in Na^+ net losses, which were significant in relation to neutral IPW, both with and without DOC. Na^+ balance remained negative in IPW at pH 4.0 throughout the 24-h observation period. When the exposure to pH 4.0 was performed in the presence of SOL DOC (IPW pH 4.0 + DOC), Na^+ balance was again negative in the first 9 h but became significantly positive at 9–24 h (Figure 2a).

Net Cl^- balance of tambaqui under neutral IPW without DOC (IPW pH 7.0) exhibited the same temporal trend as for Na^+ balance (Figure 2b). However, in the presence of SOL DOC under neutral condition (IPW pH 7.0 + DOC), net Cl^- balance was significantly positive throughout all three periods (0–3 h, 3–9 h, and 9–24 h). Exposure to acidic pH in IPW without DOC (IPW pH 4.0) caused negative net Cl^- flux rates without recovery over time (0–3 h, 3–9 h, and 9–24 h; Figure 2b), similar to the pattern seen with net Na^+ flux rates under this treatment (Figure 2a). However, the presence of DOC under acidic conditions (IPW pH 4.0 + DOC) turned net Cl^- balance positive at all time periods (0–3 h, 3–9 h, and 9–24 h), though the data were rather variable, so the differences were not significant (Figure 2b).

Ammonia excretion rates tended to become lower over time by 9–24 h under neutral conditions in IPW both with (IPW pH 7.0 + DOC) and without DOC (IPW pH 7.0) (Figure 2c). Exposure to pH 4.0 had no marked effect on ammonia flux rates, but the presence of SOL DOC under acidic conditions (IPW pH 4.0 + DOC) increased the ammonia excretion rates of tambaqui by 9–24 h in relation to neutral IPW without (pH 7.0 IPW) and with DOC (pH 7.0 IPW + DOC) (Figure 2c).

Tambaqui under neutral and acidic conditions in the absence and presence of DOC exhibited urea excretion rates that were only about 3% of ammonia excretion rates in terms of N content, and there were no significant differences among treatments or time periods (Figure 2d).

3.4 | Sodium, chloride, ammonia, and urea plasma concentrations

Besides the increased net losses of Na^+ and Cl^-, the exposure to acidic IPW did not decrease the plasma concentrations of these electrolytes at the end of the 24-h flux experiments. The greater ammonia excretion rates seen at 9–24 h in tambaqui exposed to the IPW pH 4.0 + DOC treatment did not alter the plasma ammonia concentration (Table 2). Similarly, no differences in plasma urea concentration were found in tambaqui in neutral or acidic IPW, with or without SOL DOC (Table 2). Plasma urea concentrations were about 30-fold higher than ammonia concentrations in terms of N-content.

3.5 | Na^+, K^+ ATPase; v-type H^+ ATPase; and carbonic anhydrase in gill filaments

No differences in the activities of the NKA were found in the gills of tambaqui in any of the experimental treatments (Figure 3a). On the contrary, the VHA activity increased in both IPW pH 7.0 and pH 4.0 with DOC in relation to the same treatments without DOC (Figure 3b).

TABLE 2 Plasma main ions, ammonia, and urea concentrations of *Colossoma macropomum* after 24 h of exposure to ion-poor water (IPW) pH 7.0 and pH 4.0, with and without dissolved organic carbon (DOC) from the Rio Solimoes.

	Mmol L^{-1}			
	Na^+	Cl^-	Ammonia	Urea
IPW pH 7.0	154.9 ± 10.2	112.6 ± 6.7	0.037 ± 0.006	1.32 ± 0.09
IPW pH 7.0 + DOC	157.3 ± 4.4	117.4 ± 3.1	0.038 ± 0.007	1.23 ± 0.04
IPW pH 4.0	166.8 ± 9.59	112 ± 2.6	0.045 ± 0.009	1.26 ± 0.1
IPW pH 4.0 + DOC	153.6 ± 4	118 ± 3.6	0.033 ± 0.009	1.22 ± 0.07

Note: Values are means ± SE of the mean (SEM) ($N = 8$).

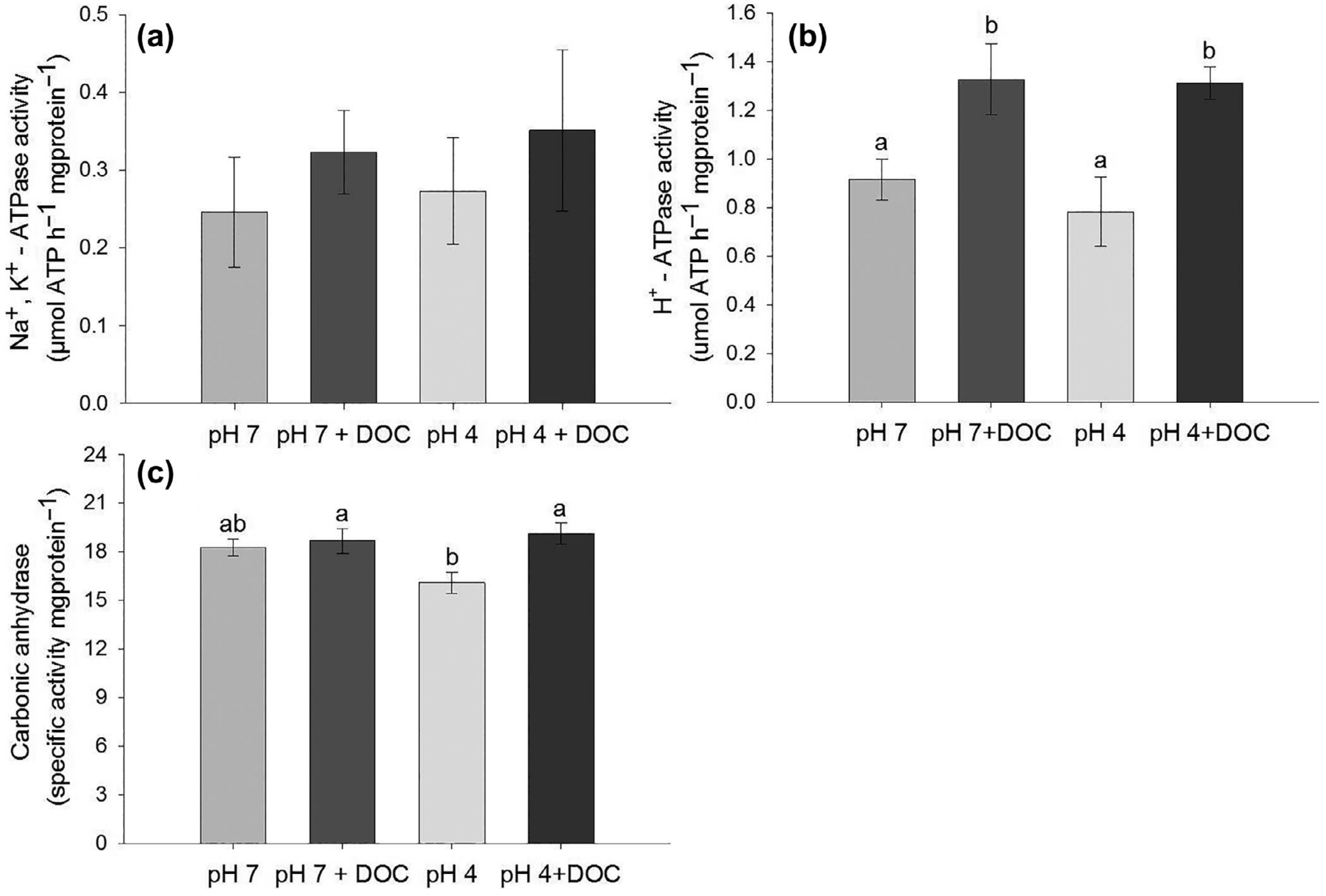

FIGURE 3 Specific activity of the (a) Na^+, K^+ ATPase; (b) v-type H^+ ATPase; and (c) carbonic anhydrase of the gills of *Colossoma macropomum* after 24-h exposure to ion-poor water (IPW) at pH 7.0 or pH 4.0, with and without natural dissolved organic carbon (DOC) from the Rio Solimoes. Means ± SE of the mean (SEM) ($N = 8$). Different letters indicate significant differences ($p < 0.05$) among the different treatments (IPW pH 7, IPW pH 7 + DOC, IPW pH 4, and IPW pH 4 + DOC).

The exposure to pH 4.0 IPW decreased the activity of the CA in relation to pH 7.0 and pH 4.0 IPW water with DOC (Figure 3c).

4 | DISCUSSION

4.1 | DOC characterization

The physicochemical characteristics of Solimoes River water were in accordance with previous published studies of the same rivers (Holland et al., 2017) and other classical analysis of Amazon water characteristics (Junk, 1983; Val & Almeida-Val, 1995; Wallace, 1889). The Solimões River contained lower levels of DOC, higher ionic composition, and neutral pH.

The spectroscopical features of SOL DOC in the present study demonstrated that the DOC molecules have lower SAC (22.8 cm^2 mg^{-1}) and molecular weight (high $Abs_{254/365} = 5.6$), and these values were similar to those reported by Sadauskas-Henrique et al. (2021) (SAC 22.8 cm^2 mg^{-1} and $Abs_{254/365}$ 5.6) and different from Holland et al. (2017) (SAC 33.4 cm^2 mg^{-1} and $Abs_{254/365}$ 4.5). The SOL DOC used in the present study and in Sadauskas-Henrique et al. (2021) was collected in the same site and time and used within 1 month after collection. Although these SOL DOC sources (present; Sadauskas-Henrique et al., 2021; Holland et al., 2017) were collected in the same site and season (wet season), it is already known that the rainfall can increase the input of allochthonous organic matter. This result demonstrates that even in the same season, there are some variabilities in the DOC quality due to the increased flushing of the surrounding soils and forest inputs with consequent increased input of allochthonous organic matter (Holland et al., 2017; Minor et al., 2006). Allochthonous DOC have larger molecules, such as observed in Holland et al. (2017). That study also reported decreases in tryptophan-like and tyrosine-like compounds (representative of protein compounds, free or bound amino acids) between wet and dry season, indicating higher inputs of allochthonous organic material during the wet season. In the present study, the tryptophan-like and tyrosine-like compounds were not insignificant, suggesting some autochthonous input.

Differences in DOC quality can occur during different flooding seasons (Holland et al., 2017). However, it is already known that the DOC quality can change over time due to degradation, which results in smaller and less aromatic molecules (Peacock et al., 2015; Sadauskas-Henrique et al., 2019, 2021). The SOL DOC in the present study presented intermediated spectroscopical DOC characteristics between SOL DOC in Holland et al. (2017) and SGC DOC in Sadauskas-Henrique et al. (2019). The present SOL DOC is less allochthonous (lower SAC_{340}, lower molecular weight, and lower FI) compared to Holland et al. (2017), yet it has a higher percentage of HA and lower percentage of FA, more similar to the aged SGC DOC in Sadauskas-Henrique et al. (2019). These differences in the spectroscopic features that dictate the DOC quality may explain, and be involved in, the physiological and biochemical responses of the aquatic organisms, especially under acidic conditions, as observed in the tambaqui in the present work, as described below.

4.2 | Transepithelial potential, Na^+, and Cl^- net loss

The TEP in freshwater fish can be interpreted as a diffusion potential predominantly regulated by the relative permeability of the gills to positively (mainly Na^+) and negatively (mainly Cl^-) charged ions (McWilliams & Potts, 1978; Potts, 1984). In the present work, exposure to pH 4.0 caused an increase in TEP by about 20 mV (from −18 to +2 mV) in relation to baseline TEP at pH 7.0 (Figure 1). Both values were similar to those seen at pH 7.0 and pH 4.0 in a different batch of tambaqui in IPW in our earlier study (Sadauskas-Henrique et al., 2019). A much earlier study (Wood et al., 1998) on larger tambaqui reported a similar baseline TEP at pH 7.0 in IPW, but a much greater depolarization to about +18 mV at pH 4.0. McWilliams and Potts (1978) also reported a shift to a positive TEP in brown trout exposed to acidic conditions in IPW. Thus, fish exposed to acidic fresh waters experience depolarization due to the high presence of protons (H^+) with consequent disturbances of ionic regulation. The active Na^+ and Cl^- uptake mechanisms are inhibited, whereas massive diffusive losses of Na^+ and Cl^- are stimulated (Gonzalez et al., 2005; McWilliams & Potts, 1978; Val & Wood, 2022). The rise in TEP of tambaqui exposed to acidic pH in IPW in the absence of DOC was associated with increases in net Na^+ losses (9–24 h; Figure 2a), whereas net Cl^- losses increased by 3–9 h in relation to neutral water in the absence of DOC (IPW pH 7.0; Figure 2b). Although there were some quantitative differences, the overall patterns were similar to those reported in our earlier study on tambaqui in IPW with no added DOC (Sadauskas-Henrique et al., 2019), indicating an ability to gradually control ion losses over time, typical of acidophilic species (Gonzalez et al., 1998; Kwong et al., 2014; Nelson, 2015; Wood et al., 1998). Notably the ion losses were not great enough to promote disturbances in the plasma concentrations of Na^+ and Cl^- over 24 h in the present study (Table 1).

On the contrary, the responses to SOL DOC in the present experiments contrasted greatly with the responses to SGC DOC at the same concentration (10 mg C L^{-1}) reported by Sadauskas-Henrique et al. (2019). First, there was no significant effect of SOL DOC on TEP in neutral water (IPW pH 7.0 + DOC; Figure 1), very different from the marked hyperpolarization to more negative values seen with SGC DOC in this treatment. Second, under acidic conditions, the presence of SOL DOC did not prevent the depolarization of TEP (Figure 1), whereas SGC had been fully protective against this disturbance (Sadauskas-Henrique et al., 2019). Third, the presence of SOL DOC, in contrast to SGC DOC, did not greatly exacerbate Na^+ loss rates at low pH (Figure 2a) and tended to improve Cl^- balance at pH 4.0 (Figure 2b). Certainly, the decreases in plasma Na^+ and Cl^- concentrations reported by Sadauskas-Henrique et al. (2019) when SGC was present at pH 4.0 did not occur when SOL DOC was present at pH 4.0.

Overall, these results demonstrate that different DOCs can have very different physiological effects in confirmation of previous studies (Al-Reasi et al., 2013; Galvez et al., 2008; Morris et al., 2021; Morris, Zulian, Smith, et al., 2024; Sadauskas-Henrique et al., 2021; Wood et al., 2003). Very interestingly, for the first time, they suggest that the effects of DOCs on TEP may not be directly coupled to their effects on net ion flux rates, because SOL DOC did not protect TEP against the depolarizing effects of low pH yet had no effect or offered modest protection to ion flux rates at low pH. This was very different from SGC that protected TEP yet exacerbated ion loss rates at low pH. This topic clearly deserves future investigation. One possible explanation is that net ion flux rates represent the difference between active ion uptake rates that are thought to be electroneutral and passive diffusive loss rates that are not (Potts, 1984). At low pH, the former are usually inhibited, and the latter may be differentially exacerbated. The limited evidence available suggests that DOC differentially influences both influx and efflux rates, especially at low pH (Al-Reasi et al., 2016; Duarte et al., 2016; Duarte et al., 2018; Wood et al., 2003). Further complications arise from the fact that these actions are related to the optical properties of DOC (Al-Reasi et al., 2016; Galvez et al., 2008; Wood et al., 2014), and recent studies demonstrated that these properties change differentially with pH (Morris, Zulian, Brauner, et al., 2024), as do the effects of DOC on TEP (Morris, Zulian, Smith, et al., 2024).

In terms of real-world relevance, the present experiments were performed with freshly collected SOL DOC. It must also be remembered that a tambaqui migrating between the Rio Solimoes and the Rio Negro would likely encounter SOL DOC only at circumneutral pH, where it appears to be helpful in promoting positive ion balance (Figure 2a,b). Furthermore, the fish would likely encounter SGC DOC only at low pH, where SGC DOC appears to be unfavorable to net ion balance (Sadauskas-Henrique et al., 2019). However, it must be appreciated that the latter study used SGC DOC that had been aged for 2 years in storage, unlike the freshly collected SOL DOC employed in the present study. During this time, the optical properties of the SGC DOC had changed markedly, with a decrease in mean molecular weight, and a 50% loss of SAC_{340} (Sadauskas-Henrique et al., 2019), SAC_{340} is spectroscopic metric that appears to be a prime indicator of the physiological effectiveness of DOC (Al-Reasi et al., 2013; Galvez et al., 2008; Morris, Zulian, Smith, et al., 2024). When freshly collected SGC DOC was first tested in the zebrafish, it was extremely protective against both the inhibition of active uptake and the stimulation of diffusive ion losses caused by exposure to pH 4.0 (Duarte et al., 2016). However, when tested after about 2.5 years of storage, it greatly exacerbated net Na^+ and Cl^- losses at pH 4.0 in zebrafish (Sadauskas-Henrique et al., 2021). Thus, the responses reported with SGC DOC by Sadauskas-Henrique et al. (2019) may not have reflected real-world conditions.

4.3 | Ammonia and urea-N net fluxes

Tambaqui showed a tendency to decrease ammonia excretion by 9–24 h when exposed to neutral water both in the presence and absence of DOC (pH 7.0 IPW and pH 7.0 IPW + DOC) and when exposed to acidic water in the absence of DOC (pH 4.0 IPW). The only significant response to experimental treatment was the greater ammonia excretion rate seen at 9–24 h in tambaqui exposed to the IPW pH 4.0 + DOC (Figure 2c). A similar response at this time period was reported by Sadauskas-Henrique et al. (2019) when aged SGC was present at pH 4.0 The absence of increased ammonia excretion at low pH in tambaqui in the absence of added DOC has been reported previously (Sadauskas-Henrique et al., 2019; Wilson et al., 1999; Wood et al., 2018) and probably reflects the acidophilic nature of this species, as discussed in detail by Sadauskas-Henrique et al. (2019). In fact, as in the present study (Table 2), no alterations in the ammonia concentration in plasma were observed for tambaqui under those conditions (Wood et al., 1998). The stimulation of ammonia excretion at pH 4.0 by both fresh SOL DOC and aged SGC DOC has also been seen in zebrafish with DOCs of various origins and ages (Duarte et al., 2016, 2018), as well as in Amazon stingrays with fresh Rio Negro DOC (Wood et al., 2003). Its significance remains unclear; potentially it could be involved in protective alkalization of the gill boundary layer by NH_3 efflux (Wood et al., 2003) and/or coupling of Na^+ uptake to increased NH_4^+ efflux (Duarte et al., 2016).

Moreover, no increases in urea excretion (Figure 2d) or urea concentrations in plasma (Table 2) were observed, and the same response was found for tambaqui in neutral and acidic water in the absence and presence of aged SGC DOC (Sadauskas-Henrique et al., 2019). This lack of response is expected, as tambaqui are unusual in largely relying on protein-amino acid oxidation resulting in ammonia excretion to maintain their aerobic metabolism (e.g., between 60% and 70% support) (Pelster et al., 2015; Wood et al., 2017), and most of the urea comes largely from uricolysis rather than from protein-amino acid oxidation (Wood, 1993).

4.4 | Na^+, K^+ ATPase; v-type H^+ ATPase; and carbonic anhydrase in gill filaments

In freshwater fish, active Na^+ uptake, through NKA activity, is coupled with H^+ and ammonia excretion through the actions of the Na^+/H^+ exchanger (NHE), the VHA, and the Rh proteins (Evans et al., 2005; Wright & Wood, 2009). Acidic conditions can inhibit these mechanisms by decreasing the driving H^+ gradient (Kwong et al., 2014). Active Cl^- uptake is likely coupled indirectly with these processes. However, in the present study, tambaqui in acidic water in the absence of SOL DOC exhibited no changes in NKA (Figure 3a) and VHA (Figure 3b) activities. Similarly, Sadauskas-Henrique et al. (2019) found no alteration in the activities of these enzymes in the gills of tambaqui after exposure to IPW pH 4.0. As an acidophilic fish species, tambaqui can control ion losses, as observed in the Na^+ and Cl^- net flux rates in both the present study and Sadauskas-Henrique et al. (2019) (Figure 2a,b), and no significant alterations in plasma Na^+ and concentrations were observed (Table 2). Notably, the presence of fresh SOL DOC under both neutral and acidic conditions led to increases in the VHA activity, which could be a significant contributor to the more positive Na^+ and Cl^- balances observed at both pH 7.0 and pH 4.0 (Figure 2a,b). For previous studies with zebrafish, the VHA upregulation can be considered as a compensatory strategy to maintain Na^+ uptake and acid–base balance at low environmental pH, as reviewed by Kwong et al. (2014). Notably, this did not occur with aged SGC DOC (Sadauskas-Henrique et al., 2019).

CA activity in the gills is essential for active Na^+ and Cl^- uptake, as well as ammonia excretion. This enzyme generates H^+ ions for exchange with Na^+, and the HCO_3^- ions for exchange with Cl^-, while also producing H^+ ions for intracellular NH_4^+ formation and/or extracellular diffusion trapping of NH_3 excretion (Evans et al., 2005; Wright & Wood, 2009). In the present study, CA activity was slightly inhibited by exposure to low pH alone (IPW pH 4.0), but its activity was restored when fresh SOL DOC was added (IPW pH 4.0 + DOC; Figure 3d). In a previous study by Sadauskas-Henrique et al. (2019), CA was slightly stimulated by the presence of aged SGC DOC at pH 4.0. This suggests that the protection and/or stimulation of CA activity may facilitate ammonia, H^+, and HCO_3^- efflux, thereby enhancing Na^+ and Cl^- uptake and ammonia excretion under ion-poor acidic conditions. Further investigation into this mechanism is a crucial area of study for future research in *C. macropomum* and other acidophilic Amazonian fish species.

5 | CONCLUSIONS

DOC freshly collected from the white waters of the Solimoes River was shown to have lower aromaticity, lower molecular weight, and a higher autochthonous content compared to DOC from the black waters of the Rio Negro. Acidic exposure resulted in a depolarization of the TEP across the gills, which was not mitigated by the presence of SOL DOC. However, under both neutral and acidic conditions, the presence of SOL DOC reduced net Na^+ and Cl^- losses. This effect appears to be associated with increased VHA activity in the gills but unrelated to changes in TEP.

AUTHOR CONTRIBUTIONS

Helen Sadauskas-Henrique, Adalberto L. Val, and Chris M. Wood designed the study. Chris M. Wood and Adalberto L. Val obtained funding. Helen Sadauskas-Henrique and Luciana R. de Souza-Bastos performed the experiments. D. Scott Smith analysed the physical–chemical and spectroscopical characteristics of the dissolved organic carbon. Helen Sadauskas-Henrique wrote the first draft, and all authors edited it and approved the final manuscript.

FUNDING INFORMATION

Supported by FAPEAM and CNPq-Brasil through the INCT-ADAPTA grant to Adalberto L. Val, a Ciência sem Fronteiras grant to Adalberto L. Val and Chris M. Wood, and NSERC (Canada) Discovery grants to Chris M. Wood (RGPIN/473-2012, RGPIN-2017-03843, and RGPIN-2023-03714). Helen Sadauskas-Henrique and Luciana R. de Souza-Bastos were the recipient of a CNPq postdoctoral fellowship (CNPq-Brasil). Chris M. Wood was supported by the Canada Research Chairs programme and was the recipient of a visiting fellowship from the Ciência sem Fronteiras Program (CNPq-Brasil). Adalberto L. Val was the recipient of a research fellowship from CNPq-Brasil.

CONFLICT OF INTEREST STATEMENT

No competing interests declared.

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How to cite this article: Sadauskas-Henrique, H., de Souza-Bastos, L. R., Smith, D. S., Val, A. L., & Wood, C. M. (2025). Dissolved organic carbon from “white” waters in Amazon basin (Brazil) and the osmoregulatory responses of tambaqui (*Colossoma macropomum*) in neutral and acidic environments. *Journal of Fish Biology*, 1–12. https://doi.org/10.1111/jfb.16054