




RESEARCH ARTICLE

Physicochemical properties of the dissolved organic carbon can lead to different physiological responses of zebrafish (*Danio rerio*) under neutral and acidic conditions

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Abstract

Previous studies have suggested that the capacity of natural dissolved organic carbon (DOC) molecules to interact with biological membranes is associated with their aromaticity (SAC_{340}); origin (allochthonous versus autochthonous, FI); molecular weight ($Abs_{254/365}$); and relative fluorescence of DOC moieties (PARAFAC analysis). These interactions may be especially important when fish are challenged by acidic waters, which are known to inhibit the active uptake of Na^+ and Cl^- , while stimulating diffusive ion losses in freshwater fishes. Therefore, zebrafish were acclimated (7 days, pH 7.0) to five natural DOC sources (10 mg C/L), two from the Amazon Basin and three from Canada, together with a “no-added DOC” control. After the acclimation, fish were challenged by exposure to acidic water (pH 4.0) for 3 h. Osmoregulatory parameters were measured at pH 7.0 and 4.0. Acclimation to the five DOC sources did not disturb Na^+ , Cl^- and ammonia net fluxes, but resulted in differential elevations in Na^+ , K^+ ATPase and v-type H^+ ATPase activities in fish at pH 7.0. However, after transfer to pH 4.0, the control fish exhibited rapid increases in both enzymes. In contrast the DOC-acclimated animals exhibited unchanged (Na^+ , K^+ ATPase) or differentially increased (v-type H^+ ATPase) activities. Na^+ , Cl^- and ammonia net fluxes remained unchanged in the control fish, but were differentially elevated in most of the DOC treatments at pH 4.0, relative to the same DOC treatments at pH 7.0. Correlations between the osmoregulatory data the DOCs properties highlight that the DOC properties drive different effects on gill physiology.

KEYWORDS

Na^+ K^+ ATPase, osmoregulation, PARAFAC analysis, SAC_{340} , spectroscopic features, v-type H^+ ATPase.

1 | Introduction

Dissolved organic carbon (DOC) is a portion of the natural organic matter (NOM) present in all aquatic ecosystems and is functionally obtained by 0.45 μm filtration (Thurman, 1985). DOC is responsible

for the yellow to brown color of surface water in lakes and streams and is derived from the decomposition of lignin-rich plant material and dead organic biomass, and also from synthesis by aquatic microorganisms (Thurman, 1985). Its molecules are normally heterogeneous in structure, and encompass a wide range of molecular

weights where the humic substances are usually the major components, representing 40–90% of the total content of most aquatic DOCs (Rocha et al., 1999; Thurman, 1985; Wetzel, 2001). The humic substances are composed of a combination of high molecular weight humic acid and lower molecular weight fulvic acid, both of which contain a variety of carboxylic, phenolic and carbonyl groups (Thurman, 1985; Wetzel, 2001). As the DOC molecules are derived from the decomposition of organic materials, other less abundant components (e.g., tyrosine and tryptophan and similar amino acid-like compounds) may also be present. DOC can be classified as ranging from allochthonous (also sometimes termed terrigenous) to autochthonous, where autochthonous compounds are produced within aquatic systems (by algae or by microbial action) and allochthonous compounds generally originate from land-based sources and are mainly derived from the breakdown of lignins (Thurman, 1985). The source of the DOC (i.e. composition) can dictate its spectroscopic features. Autochthonous DOC tends to be optically lighter, and composed of smaller molecules with a lower content of aromatic ring structures, while allochthonous DOC tends to be optically darker, and composed of large molecules with more aromatic rings (i.e. more phenolic groups) (Thurman, 1985; Wetzel, 2001).

It is also known that the DOC, especially the humic substances, can interact with aquatic organisms in both direct and indirect manners (see Wood et al. (2011) and Morris et al. (2021) for reviews). Indirectly, DOC can play a key role in ameliorating the aquatic toxicity of many metals, reducing bioavailability to target surfaces such as the gills (Al-Reasi, Smith, et al., 2012; Al-Reasi, 2012; Duarte et al., 2021). Some investigations have demonstrated that DOC molecules can interact directly with biological membranes, altering their permeability (Galvez et al., 2008; Vigneault et al., 2000) as well as other physiological functions, such as ion regulation and nitrogenous waste excretion (Al-Reasi et al., 2016; Duarte et al., 2016; Duarte et al., 2018; Galvez et al., 2008; Sadauskas-Henrique et al., 2019; Wood et al., 2003), specially under acidic conditions. The high concentration of protons (H^+) is problematic because it has a powerful destabilizing effect on biological molecules (Nelson, 2015). Most fish exposed to freshwater acidic environments experience ionic disturbances due to elevated diffusive ion losses to the external environment and inhibition of the active uptake of Na^+ and Cl^- (Milligan & Wood, 1982). However, there is evidence that the DOC molecules can exert a protective effect regarding these ionic disturbances under acidic pH, and that this protection can be related to the DOC properties (Al-Reasi et al., 2013b, 2016; Duarte et al., 2016, 2018; Sadauskas-Henrique et al., 2019; Wood et al., 2011). At low pH the functional groups of the DOC molecules are protonated and uncharged, while at higher pH the functional groups dissociate and become negatively charged (Wit et al., 1993). Exactly how the DOC molecules interact with the biological membranes under acidic conditions, where the DOC is more effective, remains unclear. However, the DOC molecules are also generally amphiphilic, with hydrophilic and hydrophobic components, with the first one contributing to its lipophilicity (see Morris et al. (2021) for review).

The ability of the DOC molecules to interact with aquatic organisms appears to be associated with their specific functional groups and/or structural features which can be measured through their physicochemical properties (Al-Reasi et al., 2011; Al-Reasi et al., 2013a). For example, Al-Reasi et al. (2012) demonstrated that larger, optically darker, more lipophilic and aromatic DOC of terrigenous origin, with high humic-like content, was more protective against Cu toxicity to *Daphnia magna* than DOC of allochthonous origin. In other words, DOC of terrigenous origin reduced the gill binding and toxicity of metals. Similarly, but in a direct manner, the optically dark DOC from Rio Negro (Amazon, Brazil) “blackwater” provided almost perfect protection of the zebrafish (*Danio rerio*) against ionoregulatory disturbances associated with acute exposure to acidic ion-poor water (pH 4.0) (Duarte et al., 2016). On the other hand, Sadauskas-Henrique et al. (2019) demonstrated that an acidophilic Amazon fish, the tambaqui (*Colossoma macropomum*), actually experienced greater net Na^+ and Cl^- losses, decreases of Na^+ and Cl^- concentrations in plasma, and elevated plasma ammonia levels and excretion rates, when exposed to the same Amazon “blackwater” DOC as used by Duarte et al. (2016). The authors attributed these significant differences in the ability of this DOC to ameliorate the effects of the acid exposure to species-specific differences and/or to changes in the properties of the DOC during storage, such as lower molecular weight (higher $Abs_{254/365}$), and lower aromaticity (lower SAC_{340}) in comparison with the values reported by Duarte et al. (2016).

With this background in mind, the present study aimed to characterize the spectroscopic properties of five natural DOC sources: two from the Amazon Basin in Brazil (“blackwater” river type - Rio Negro at São Gabriel da Cachoeira and “whitewater” river type - Solimões River) and three from Canada (Luther Marsh and Bannister Lake in Ontario, and “home-made” DOC derived from the leaves of maple trees).

We employed zebrafish as our experimental model. In nature, zebrafish are found across a range of habitat types that vary considerably in their physico-chemical properties as a result of local geology and pronounced seasonal fluctuations in rainfall patterns (Aleström et al., 2019; Lawrence, 2007). Therefore they are a useful experimental model due to their tolerance of a wide range of environmental conditions in captivity, and their importance as vertebrate model organisms in genetics, developmental biology, neurophysiology and biomedicine (Spence et al., 2008).

We acclimated adult zebrafish (*Danio rerio*) to these five DOC sources, under circumneutral pH (7.0) in ion-poor soft water. Subsequently, we tested if they influenced various osmoregulatory parameters (Na^+ , Cl^- and ammonia net fluxes; gill Na^+ , K^+ ATPase, v-type H^+ ATPase activities) at circumneutral pH and when challenged by acidic water (pH 4.0). Then, we evaluated whether the observed responses were associated with various spectroscopic properties of the DOC, considering that the ability of the DOC molecules to interact with aquatic organisms appears to be asso-

ciated with their physicochemical properties, and with the acidic environmental conditions. We hypothesized that the more allochthonous DOC sources would present more interactions with the biological membranes, as measured through the Na^+ , Cl^- and ammonia net fluxes, and gill Na^+ , K^+ ATPase, and v-type H^+ ATPase activities at acidic water (pH 4.0).

2 | Material and Methods

2.1 | Water collection and DOC concentration and characterization

Water samples for DOC collection and concentration were obtained in Brazil and Canada. In Brazil, the water was collected in black- and white-water bodies from the Amazon. The black water was collected from the upper Rio Negro at São Gabriel da Cachoeira (SGC) (0°07'S 67°05'W) in 2014, and the white water was collected from the Solimões River, in Iranduba city (3°15'S 60°14'W) in 2015. In Canada the water was collected in 2010 in Luther Marsh (43°37'N 80°26'W) and Bannister Lake (43°30'N 80°38'W) both located in Ontario. Additionally, maple tree leaf-derived DOC was extracted in 2008, as described by Crémazy et al. (2017), and studied as an additional DOC source. The natural waters were collected and concentrated by reverse osmosis as described by Duarte et al. (2016). After that, the concentrates were treated with a cation exchange resin (Amberlite IR – 118 (H), Sigma-Aldrich, St. Louis, USA) to remove the influence of the cations, which had been concentrated together with the DOC by reverse osmosis. Lastly, the concentrate was filtered with a 0.45 μm membrane (Acrodisc™, Pall, Ann Arbor, USA) and stored in sealed dark polyethylene bottles (Nalgene®) at 4°C until the spectroscopic characterization and fish experiments were performed.

Details on the optical techniques used for DOC characterization as well as the control procedures for the excitation-emission fluorescence measurements have been described by Sadauskas-Henrique et al. (2019). In brief, the specific absorbance coefficient at 340 nm (SAC_{340}) as an indicator of aromaticity (Curtis & Schindler, 1997) and the fluorescent index (FI, a simple ratio of emission intensity at 450 nm/emission intensity at 500 nm; both taken at an excitation wavelength of 370 nm) as an indicator of origin (allochthonous versus autochthonous) were both determined according to Mcknight et al. (2001). The ratio of absorbance at 254 nm to that at 365 nm ($\text{Abs}_{254/365}$) as an indicator of 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 (humic-like, fulvic-like, and tryptophan-like and tyrosine-like) in the fluorescence excitation emission matrices (FEEM). We assume the quantum yields of our fluorophores are approximately the same (Hawes et al., 1992) to facilitate comparisons between samples and other already published articles (Al-Reasi

et al., 2011, 2013a, 2016, Duarte et al., 2016, 2018; Sadauskas-Henrique et al., 2019).

2.2 | Fish holding

All experimental procedures with zebrafish were performed in 2015. The experimental and holding procedures were approved by the University of British Columbia Animal Research Ethics Board (A14-0251, A18-0271) and followed the guidelines of "The care and use of the fish in research, teaching and testing" of the Canadian Council for Animal Care (2005). Zebrafish (*Danio rerio*, weight: 0.276 ± 0.007 , mean \pm SEM; $N = 112$) were obtained from a local producer and acclimated to dechlorinated Vancouver tap water for approximately one week in a 50-L aquaria. Laboratory conditions were: temperature: 25.03 ± 0.11 °C; DOC: 1.06 ± 0.08 mg of carbon L^{-1} ; pH: 7.07 ± 0.03 ; Na^+ : 93.2 ± 12.4 $\mu\text{mol L}^{-1}$; Cl^- : 100.4 ± 4.4 $\mu\text{mol L}^{-1}$; Ca^{2+} : 101 ± 6.8 $\mu\text{mol L}^{-1}$. During the acclimation period, the fish were fed daily until satiation with a commercial food (New Life Spectrum, Homestead, USA). All experiments were performed at the acclimation temperature of 25 °C.

2.3 | Preparation of experimental solutions

The pH values of the DOC stocks from the five different sources were adjusted to neutral pH (7.0; 0.01 N KOH) or to acid pH (4.0; 0.01 N HNO_3) as appropriate. Experimental solutions were prepared 24 h before the beginning of the experiments by diluting the DOC stocks to an environmentally-relevant concentration of 10 mg L^{-1} (e.g. Morris et al., 2021; Thurman, 1985), using Vancouver dechlorinated tap water.

The DOC concentrations were checked with a Total Organic Carbon analyzer (V-series TOC analyzer, Shimadzu, Kyoto, Japan). Instrument calibrations were verified with certified reference waters TMDA-54.5 and TM-25.4 from Natural Resources Canada (percent recovery was > 95%). The control group were exposed only to the dechlorinated water used to dilute the DOC solutions. Dissolved O_2 (DO) concentrations were checked throughout all experiments using a hand-held Accumet® meter and polarographic electrode (Fisher Scientific, Toronto, ON, Canada, and remained close to air saturation values.

2.4 | Acclimation effects of the five DOC sources on Na^+ , K^+ ATPase and v-type H^+ ATPase of zebrafish at neutral pH (7.0)

Fish ($N = 16$ in each of the five DOC sources and the control group) were kept in glass aquaria with temperature control (25°C) and constant aeration. The glass aquaria were covered with black plastic and filled with 3 liters of aerated experimental solutions. During the seven days of acclimation to the five DOC sources and the control

treatment at neutral pH (7.0), 50% of the experimental solution was changed daily and fish were fed until satiation daily. The physico-chemical water characteristics of the experimental water during the seven days of acclimation are shown in Table 1. After the acclimation period, 8 fish in each treatment were euthanized with an overdose of neutralized MS-222 solution (Sigma Aldrich, St. Louis, MO, USA) for 5 s, then gill filaments were collected, frozen in liquid nitrogen and stored at -80°C until analysis of Na⁺, K⁺ATPase and v-type H⁺ATPase activities.

2.5 | Effects of acclimation to five DOC sources on ion regulation and ammonia excretion at pH 7.0 and 4.0, and Na⁺, K⁺ATPase and v-type H⁺ATPase at pH 4.0

After the acclimation for seven days to the five DOC sources and the control treatment at neutral pH (7.0), fish (N = 8) from each experimental group were transferred to individual aerated plastic chambers (20 ml) containing the same aerated experimental solutions (pH 7.0, 25°C) as used in the experiment described in Section 2.4. Fish were allowed to settle for 1 h in the experimental containers before starting the experimental procedures. At the beginning of the experiment, and again after 3 h, 8 ml of water were removed with a pipette from each chamber, corresponding to a 3-h flux period. After that, the experimental solutions (pH 7.0) were exchanged for the same experimental solutions but now adjusted to pH 4.0. A 3-h flux experiment at pH 4.0 was then performed, with identical methodology. During the flux periods, the pH was checked and adjusted at pH 7.0 and pH 4.0, as necessary. Immediately after collection, water samples were stored at -20°C until analysis of ammonia, Na⁺ and Cl⁻ concentrations. At the end of the Series 2, fish were euthanized with an overdose of neutralized MS-222 solution for 5 s, then gill filaments were collected, frozen in liquid nitrogen and stored at -80°C until analysis of Na⁺, K⁺ATPase, v-type H⁺ATPase activities.

2.5.1 | Ammonia, sodium and chloride net fluxes

The net flux rates (J_{net}) of total ammonia ($T_{\text{amm}} = \text{NH}_3 + \text{NH}_4^+$), Na⁺ and Cl⁻ were calculated as:

$$J_{\text{net}} = (X_1 - X_2) \times V \times (T \times W)^{-1}$$

where X_1 and X_2 were, respectively, the initial and final concentrations of total ammonia, Na⁺ or Cl⁻ ($\mu\text{mol L}^{-1}$) in the water during the flux period, V was the volume in L of the chamber, T was the duration of the flux period in hours, and W was the fish weight in kg.

Sodium concentration in water was measured, without dilution, by atomic absorption spectrometry (AA240 FS, Varian, Mulgrave, Australia) in flame mode. Chloride and total ammonia concentrations in water, without dilution, 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.

2.5.2 | Enzyme activities in gill filaments

All reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA). 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 MgCl₂, 0.4 mmol L⁻¹ KCN, 1 mmol L⁻¹ Na₂ATP, 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, Racine, USA) 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

TABLE 1 Experimental water physicochemical characteristics (N = 7, means ± SEM) during the acclimation period (7 days) to the five DOC sources

	pH	Temp (°C) ^a	$\frac{\text{mg L}^{-1}}{\text{DOC}^b}$	DO ^c	$\frac{\mu\text{Molar}}{\text{Na}^+}$	Cl ⁻
Control	7.07 ± 0.03	25.03 ± 0.11	1.06 ± 0.08	7.05 ± 0.14	116 ± 2.30	173 ± 6.90
Rio Negro at São Gabriel da Cachoeira (SGC)	6.99 ± 0.01	24.88 ± 0.17	10.03 ± 0.24	7.12 ± 0.11	140 ± 22.9	148 ± 31.6
Solimões River (SOL)	6.99 ± 0.01	24.76 ± 0.23	11.09 ± 0.22	7.27 ± 0.16	122 ± 11.3	155 ± 9.20
Maple tree-derived DOC (MP)	6.98 ± 0.017	24.9 ± 0.16	9.71 ± 0.39	7.23 ± 0.11	108 ± 3.70	184 ± 8.10
Luther Marsh (LM)	6.99 ± 0.02	24.9 ± 0.19	11.3 ± 0.12	7.3 ± 0.12	104 ± 0.77	184 ± 16.5
Bannister Lake (BL)	7.03 ± 0.01	24.4 ± 0.10	9.77 ± 0.08	7.39 ± 0.20	249 ± 2.96	567 ± 17.5

^aTemperature (Temp).

^bDissolved organic carbon (DOC).

^cDissolved oxygen (DO).

deoxycholic acid, pH 7.5, and then centrifuged for 5 minutes (2000 g) at 4 °C. The assay was performed at 25 °C by combining 200 µl of the reaction mixture (with ouabain, or 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. Total protein concentration of the homogenates was determined according to Bradford (1976), using bovine serum albumin (BSA) as standard and read at 595 nm. The enzyme activities were expressed as µmol ATP h⁻¹ mg protein⁻¹.

2.6 | Statistical analysis

All data are reported as means ± SEM (N = 8). Statistical significance was accepted at P < 0.05. All the data were tested for normality and variance, when the data did not meet these assumptions, a non-parametric test were applied. Significant differences among treatments in ammonia, Na⁺ and Cl⁻ net fluxes were determined through a two-way ANOVA (factors: DOC and pH), followed by the *a posteriori* Holm-Sidak multiple comparison test. Significant differences among treatments in Na⁺, K⁺ATPase and v-type H⁺ATPase activities in gill filaments were determined through a one-way ANOVA, followed by the *a posteriori* Tukey multiple comparison test. Spearman correlation was applied to identify the interactions between DOC physicochemical

characteristics and the physiological responses. All statistical analyses and graphics employed Sigma Stat (version 3.5) and Sigma Plot (version 11.0) software (Jandel Scientific, San Jose, USA).

3 | Results

3.1 | DOC characterization

The physicochemical characteristics of the São Gabriel da Cachoeira (SGC), Solimões (SOL), the “home-made” Maple tree-derivate DOC (MP), Luther Marsh (LM) and Bannister lake (BL) DOCs are summarized in Table 2.

The MP had the highest SAC₃₄₀ followed by LM, SGC, SOL, and BL DOC sources. These results indicated highly variable aromatic content of organic matter from the different DOC sources. The BL had higher Abs_{254/365} than SOL followed by SGC, LM and MP. Low values of Abs_{254/365} indicate a high mean molecular weight of the DOC molecules, and *vice versa*. The FI values of the DOC sources used in this study were fairly uniform (0.9 – 1.5), where the MP had higher FI than SOL followed by BL, SGC and LM. The FI is an indicator of DOC source, with lower values representative of more allochthonous (terrigenous) origin, and higher values indicative of more autochthonous origin.

Based on PARAFAC analysis, the BL had a higher relative fulvic-like content (FA) than the MP followed by SOL, SGC and LM. On the

TABLE 2 Summary of physicochemical properties of natural dissolved organic carbon (DOC) samples isolated by reverse osmosis from Rio Negro at São Gabriel da Cachoeira (SGC), Solimões River (SOL), Maple tree-derived DOC (MP), Luther Marsh (LM) and Bannister lake (BL). Earlier published measurements on the same sources are summarized in the lower part of the Table

DOC source	Type	SAC ₃₄₀ ^a	Abs _{254/365} ^b	FI ^c	Relative fluorescence of DOC components (%) ^d				Reference
					FA	HA	Trp	Tyr	
SGC	Terrigenous	36.6	4.1	1.0	28.3	58.8	7.6	5.1	This work
SOL	Autochthonous	21.9	5.5	1.3	33.8	60.8	4.0	1.3	This work
MP	Terrigenous	45.5	3.2	1.4	37.9	22.6	17.2	22.1	This work
LM	Terrigenous	39.7	3.6	0.9	14.7	78.5	3.3	3.4	This work
BL	Autochthonous	20.1	5.9	1.2	41.3	52.2	4.6	1.8	This work
SGC	Terrigenous	73.0	2.9	1.3	51.3	36	7.8	4.7	Duarte et al. (2016)
SGC	Terrigenous	36.5	4.0	1.0	28.3	58.8	7.6	5.1	Sadauskas-Henrique et al. (2019)
SOL	Autochthonous	64.95-33.41	3.45-4.55	1.54-1.52	48.45-45.6	45.89-51.54	3.18-1.2	2.48-1.66	Holland et al. (2017)
LM	Terrigenous	39.3	3.72	1.19	9.7-5.0	95-84.7	>1.0	-	Al-Reasi et al. (2012, 2011)
BL	Autochthonous	14.16	6.31	1.5	-	-	-	-	Al-Reasi, Smith, et al. (2012)

^aSAC₃₄₀ (cm² mg⁻¹) is the specific absorbance coefficient at 340 nm normalized to DOC.

^bAbs_{254/365} is the ratio of absorbance at 254 nm to that at 365 nm.

^cFI is the fluorescence index.

^d% is the relative abundance of each DOC component determined via PARAFAC analysis (FA = fulvic acid-like; HA = humic acid-like; Trp = tryptophan-like; Tyr = tyrosine-like).

TABLE 3 Observed Spearman correlations between the spectroscopic characteristics of five dissolved organic carbon (DOC) sources isolated by reverse osmosis from Rio Negro at São Gabriel da Cachoeira (SGC), Solimões River (SOL), Maple tree-derived DOC (MP), Luther Marsh (LM) and Bannister lake (BL). Bold values represent significant correlations ($P < 0.05$)

	SAC ₃₄₀	Abs _{254/365}	FI	FA	HA	Trp	Tyr
Abs _{254/365}	-1.00						
FI	-0.10	-0.100					
FA	-0.40	0.40	0.70	-	-	-	-
HA	-0.10	0.10	-0.70	-0.80	-	-	-
Trp	0.30	-0.30	0.60	0.50	-0.90	-	-
Tyr	0.80	-0.80	0.10	-0.10	-0.50	0.70	-

other hand, LM had higher humic-like content (HA) than SOL followed by SGC, BL and the MP. With regard to amino acid-like composition (i.e., Tyr and Trp), the MP had higher amounts of both amino acid-like components, followed by the SGC, indicating some autochthonous input for these DOC sources.

Analyzing the values of the SAC₃₄₀, Abs_{254/365}, and the PAR-AFAC we can infer that the SGC, LM and the MP are from more allochthonous (terrestrial) sources while SOL and BL are from more autochthonous sources (Table 2).

Significant correlations ($P < 0.05$) were found among the physicochemical characteristics of the five DOC sources (Table 3). Aromaticity (SAC₃₄₀) was negatively correlated with the Abs_{254/365} and with the fulvic-like content (FA) and positively correlated with the amino acid contents (Tyr and Trp). The molecular weight ratio (Abs_{254/365}) was positively correlated with the fulvic-like content (FA) and negatively correlated with the amino acid contents (Tyr and Trp). The FI was positively correlated with the fulvic-like content and the amino acid contents (Tyr and Trp), and negatively correlated with the humic-like content (HA). The fulvic-like content (FA) was negatively correlated with humic-like content (HA) and positively correlated just with the Trp, while the humic-like content (HA) was negatively correlated with both amino-acids, Tyr and Trp. The amino acid-like contents (Tyr and Trp) were positively correlated with each other (Table 3).

3.2 | 3.2. Acclimation effects of the five DOC sources on Na⁺, K⁺ATPase and v-type H⁺ATPase at neutral pH (7.0) and acidic pH (4.0)

After 7 days acclimation at pH 7.0, significant increases in the specific activity of Na⁺, K⁺ATPase relative to the control were found in gills of the zebrafish exposed to SOL (2.7-fold) and MP (2.5-fold), while the 2.1-fold increase with SGC was below statistical significance ($P = 0.197$) (Figure 1a). The specific activities of v-type H⁺ATPase were significantly higher in gills of the zebrafish exposed to SOL (3.7-fold) and SGC (2.5-fold) against the control (Figure 2a).

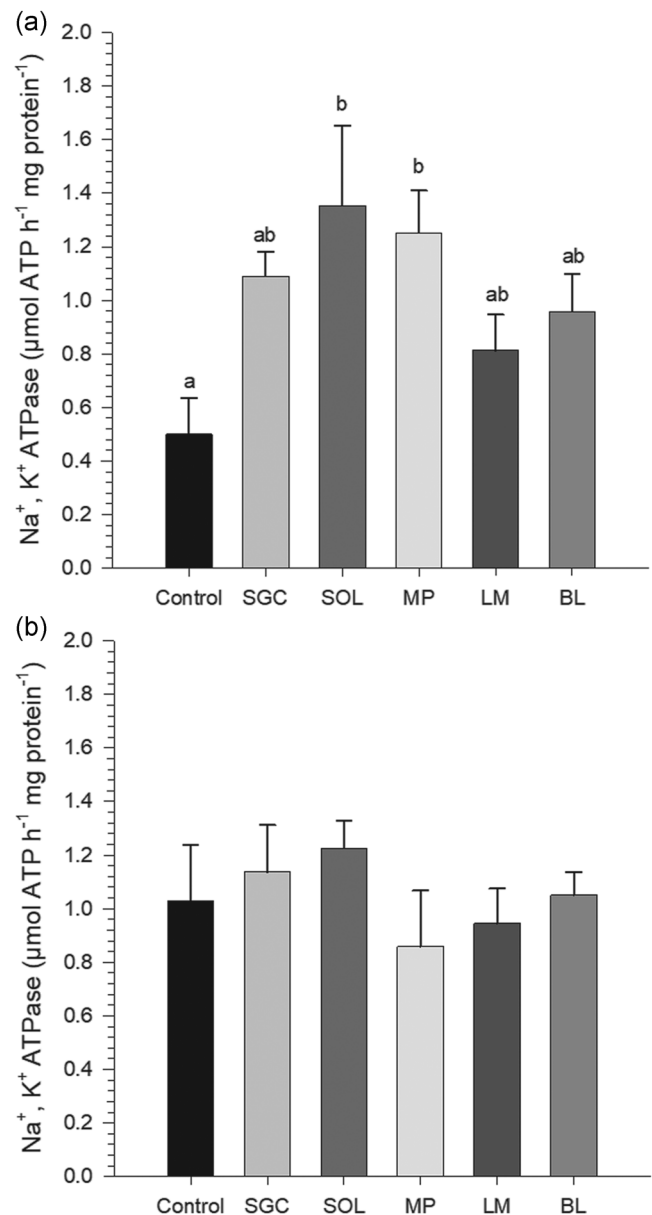


FIGURE 1 Specific activity of the Na⁺, K⁺ATPase of the gills of *Danio rerio* (A) after seven days of acclimation at pH 7.0 and (B) exposure for 3 h at pH 4.0, after seven days of acclimation to pH 7.0. Data for the no-added DOC control group and five dissolved organic carbon (DOC) treatments are shown: São Gabriel da Cachoeira (SGC), Solimões (SOL), Maple tree-derivate DOC (MP), Luther Marsh (LM) and Bannister lake (BL). Means not sharing the same letters are significantly different among treatments ($P < 0.05$)

No significant correlations ($P > 0.05$) were found between the Na⁺, K⁺ATPase and v-type H⁺ATPase activities (under neutral conditions) and the physicochemical characteristics of the five DOCs (SGC; SOL; MP; LM and BL).

When the zebrafish that had been acclimated to the five different DOCs at pH 7.0 for 1 week were subsequently exposed to pH 4.0 for 3.0 h in the continuing presence of the same DOCs, there were no significant changes in the specific activities of Na⁺,

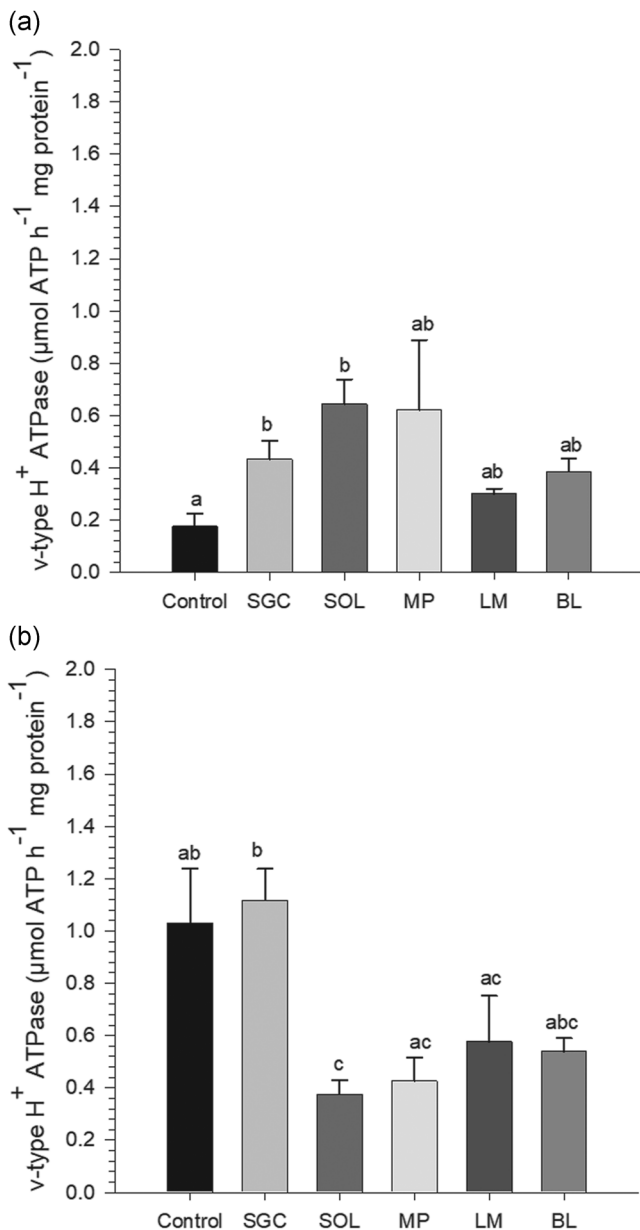


FIGURE 2 Specific activity of the v-type H⁺-ATPase of the gills of *Danio rerio* (A) after seven days of acclimation at pH 7.0 and (B) exposure for 3 h at pH 4.0, after seven days of acclimation to pH 7.0. Data for the no-added DOC control group and five dissolved organic carbon (DOC) treatments are shown: São Gabriel da Cachoeira (SGC), Solimões (SOL), Maple tree-derivate DOC (MP), Luther Marsh (LM) and Bannister lake (BL). Means not sharing the same letters are significantly different among treatments ($P < 0.05$)

K⁺ATPase (Figure 1b). The one marked exception was for the control group, in which the pH 4.0 challenge resulted in an approximate doubling of the Na⁺, K⁺ATPase activity, bringing it to the same approximate level as in the DOC treatments. There were no significant differences among the six treatments at pH 4.0.

The response patterns in the specific activity of v-type H⁺ATPase in these same fish were very different (Figure 2). Exposure to pH 4.0

for 3 h caused a 6-fold increase in the v-type H⁺ATPase activity of the control group. There were also increases caused by low pH challenge in the SGC (2.6-fold in relation to pH 7.0), whereas v-type H⁺ATPase activity decreased significantly by 40% at low pH in the SOL and MP groups. As a result, the highest activities at pH 4.0 were in the control and SGC treatments with significantly lower activities in SOL and LM (Figure 2b).

There was a negative relationship between FI and v-type H⁺ATPase activity (Table 4). Otherwise, no other correlation was found between these enzymes (Na⁺, K⁺ATPase and v-type H⁺ATPase) and the DOC physicochemical properties.

3.3 | Acclimation effects of the five DOC sources on ion regulation and ammonia excretion of zebrafish at neutral (7.0) and low pH (4.0)

There were no significant differences among the treatments in the $J_{\text{net}}^{\text{Amm}}$ values of the zebrafish that had been acclimated to the five different DOCs at pH 7.0 for 1 week (Figure 3a). In the control treatment (no added DOC), there was also no change in $J_{\text{net}}^{\text{Amm}}$ when these zebrafish were subsequently exposed to pH 4.0. On the other hand, subsequent exposures of animals acclimated to the various DOCs at pH 7.0, and then transferred to pH 4.0 in the continued presence of the same DOCs resulted in increases in net negative $J_{\text{net}}^{\text{Amm}}$ in every case. These elevations (2 to 3-fold) were greatest in SGC, SOL, and BL but significant (pH 7.0 versus pH 4.0) in all DOC treatments (Figure 3a).

Zebrafish exposed to neutral water were in slight negative Na⁺ balance in all groups, including the control. At pH 7.0, the DOC treatments did not cause any significant alterations in $J_{\text{net}}^{\text{Na}}$ relative to the control and there were no significant differences among the five DOC sources (Figure 3b). Subsequent exposure to pH 4.0 did not significantly alter $J_{\text{net}}^{\text{Na}}$ in the control group. However, in all of the DOC treatments except BL, there were significant elevations in $J_{\text{net}}^{\text{Na}}$, some of which were quite large. For example, the SGC, SOL, MP, and LM treatments experienced increases in $J_{\text{net}}^{\text{Na}}$ which were about 9-, 6-, 3- and 5-fold respectively, relative to the pH 4.0 control treatment. The 2.5-fold increase in the BL group was not significant (Figure 3b).

As for net Na⁺ balance, zebrafish exposed to neutral water were in slight negative Cl⁻ balance in all groups, and there were no significant variations in $J_{\text{net}}^{\text{Cl}}$ among the various treatments (Figure 3c). Similarly, when transferred to pH 4.0, there was no significant change in the control group, but again there was a clear trend for large elevations of Cl⁻ loss rates in 4 of the 5 DOC treatments (Figure 3c). These loss rates were generally even higher than for Na⁺ (cf. Figure 3b), representing increases of about 16-, 2-, 7-, and 9-fold, respectively). The 1.5-fold increase in the BL treatment was not significant (Figure 3c).

Correlations between the various spectroscopic characteristics of the different DOCs and the observed osmoregulatory parameters

	SAC ₃₄₀	Abs _{254/365}	FI	FA	HA	Trp	Tyr
Na ⁺ , K ⁺ ATPase pH 4.0	-0.28	0.28	-0.01	0.01	0.10	-0.07	-0.25
v-type H ⁺ ATPase pH 4.0	-0.07	-0.07	-0.31	-0.14	0.06	-0.23	-0.24
J _{Na.net} pH 7.0	-0.01	0.01	-0.05	0.007	-0.09	0.138	0.118
J _{Na.net} pH 4.0	-0.11	0.11	0.31	0.56	-0.47	0.23	0.04
J _{Cl.net} pH 7.0	0.28	-0.20	-0.09	-0.01	-0.16	0.19	0.34
J _{Cl.net} pH 4.0	-0.59	0.59	0.28	0.66	-0.29	0.004	-0.43
J _{amm.net} pH 7.0	0.66	-0.66	-0.06	-0.37	-0.09	0.01	0.45
J _{amm.net} pH 4.0	0.48	-0.48	0.03	-0.04	-0.07	-0.01	0.32

TABLE 4 Observed Spearman correlations between the osmoregulatory parameters of zebrafish (*Danio rerio*) exposed to acidic pH (4.0), after 7 days of acclimation under neutral pH (7.0), and the spectroscopic characteristics of five dissolved organic carbon (DOC) sources. Bold values represent significant correlations ($P < 0.05$)

are summarized in Table 4, with significant relationships illustrated in Supplementary Information (Figures A–P). Positive correlations ($P < 0.05$) were found between SAC₃₄₀ and J_{amm.net} at both pH 7.0 and pH 4.0 (Table 4, Supplementary Fig. S1 graphs J and N). This means that as SAC₃₄₀ (a general indicator of aromaticity) decreases, net ammonia excretion rates become higher (more negative net flux values). In an opposite manner, negative correlations occurred between Abs_{254/365} and J_{amm.net} at both pH 7.0 and pH 4.0, indicating that as molecular weight decreases, net ammonia excretion rate increases (more negative net flux values) (Table 4, Supplementary Fig. S1 graphs K and O). In other words, with the decreases of the aromaticity and consequent decreases of the MW (which will increase the Abs_{254/365}), the net ammonia excretion rates become higher (more negative net flux values). In agreement, with the increases of the low MW component FA (fulvic acid), net ammonia excretion rate (J_{amm.net}) increases (more negative net flux values), though this is significant only at pH 7.0 (Table 4, Supplementary Fig. S1 graph L). At pH 4, an opposite effect of FA on net Cl[−] and Na⁺ fluxes (i.e. greater loss rates at low FA content) was seen (Table 4, Supplementary Fig. S1 graphs H and C). For HA content, the greater the HA content, the higher were the Na⁺ fluxes at pH 4.0 (Table 4, Supplementary Fig. S1 graph D and C). The only significant correlation ($P < 0.05$) with gill enzyme activity was the negative relationship between FI (an indicator of the allochthonous versus autochthonous origin of DOC) and v-type H⁺ATPase activity at pH 4.0 (Table 4, Supplementary Fig. S1 graph A). This suggests that a greater allochthonous (lower FI) character is associated with greater v-type H⁺ATPase activity at low pH. Notably, FI also exhibited significant positive correlation ($P < 0.05$) with J_{Na.net} at low pH (Table 4, Supplementary Fig. S1 graph B). This suggests that a greater allochthonous character (lower FI) is also associated with higher loss rate of Na⁺ under acidic conditions. Finally, within the DOC amino acid-like components (Trp and Tyr), the only significant correlations were positive relationships between the Tyr component and J_{amm.net} and J_{Cl.net} at both pH 7.0 and pH 4.0 (Table 4, Supplementary Fig. S1 graphs M, P, and E). However, a negative relationship between the Tyr component and J_{Cl.net} at pH 4.0 was found (Table 4, Supplementary Fig. S1 graphs I). Therefore, as the relative tyrosine-like content increases, ammonia (pH 7.0 and 4.0) and Cl[−] excretion (pH

7.0) rates decrease (less negative net flux values) while the Cl[−] excretion at pH 4.0 increases (more negative flux values).

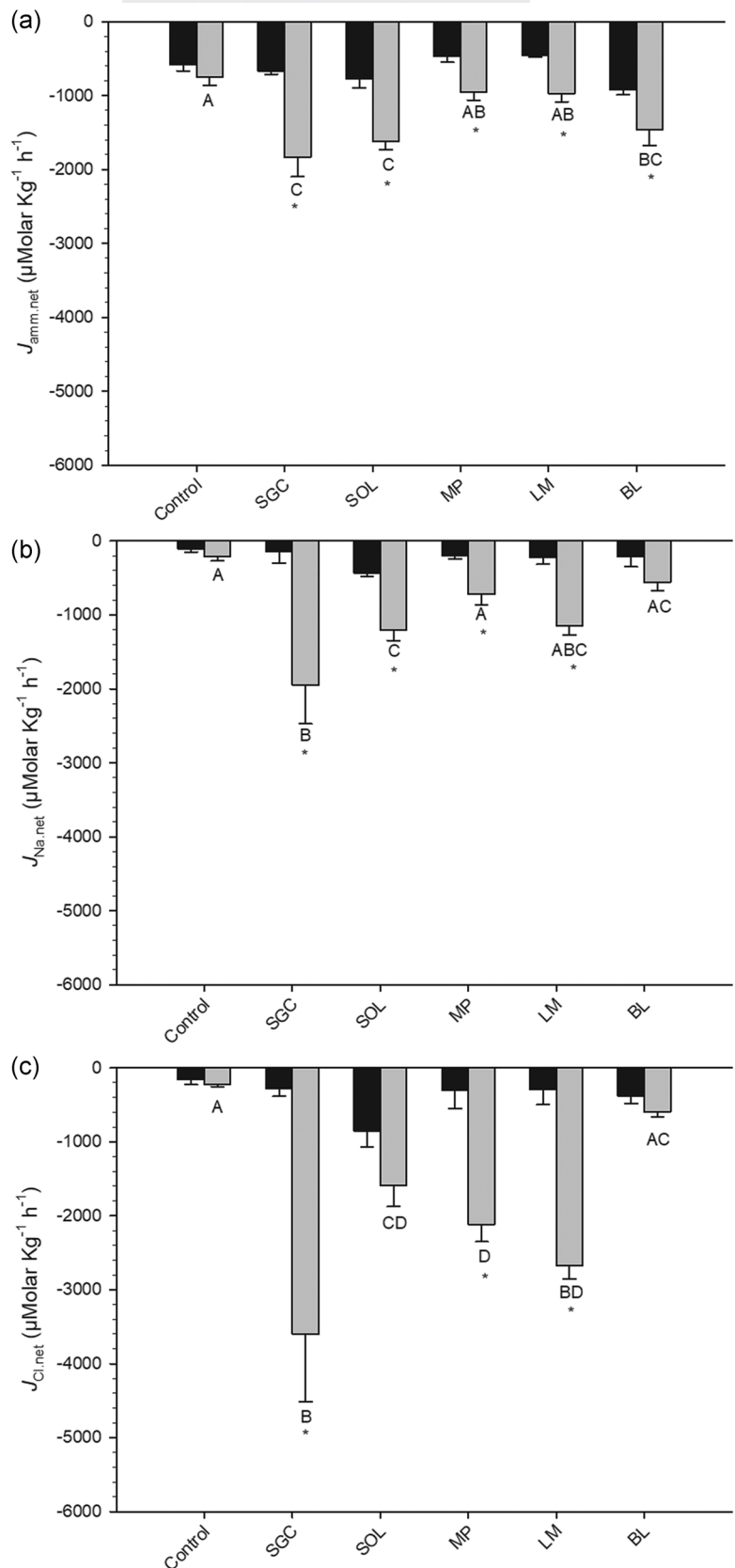
4 | Discussion

In the present study, changes in physicochemical properties of the five DOC sources over time during storage seem to be related with the changes in physiological responses of zebrafish, specially under acidic conditions. This was especially true for the aromaticity, indicated by SAC₃₄₀, where lower aromaticity potentiated Na⁺ and Cl[−] losses at low pH. Correlations between the measured physiological parameters and DOC spectroscopical features elucidated that all components (with the exception of Trp) were associated with physiological responses of zebrafish under neutral and acid conditions. These and other findings are discussed on the next following sections.

4.1 | DOC characterization

In natural waters, SAC₃₄₀ values ranging between 1 and 73 cm² mg^{−1} have been reported, with most waters exhibiting values less than 40 cm² mg^{−1} (Al-Reasi et al., 2011, 2012; Johannsson et al., 2017), with exceptions for naturally acidic waters (Duarte et al., 2016; Holland et al., 2018). The aromaticity, as indicated by SAC₃₄₀, is generally greatest for DOC from naturally acidic waters of tropical and sub-tropical regions, such as those found in the Amazon (Duarte et al., 2016) and Australia (Holland et al., 2018). In the present study the maple tree-derived DOC (MP) had the highest SAC₃₄₀ (Table 2), indicating a higher presence of ringed aromatic chromophores (ter-rigenous type), followed by LM (terrigenous type), SGC (terrigenous type), while the SAC₃₄₀ values were lower in SOL (autochthonous type) and BL (autochthonous type). Also, the fluorescence indices (FI) were quite low for MP, LM and SGC, indicative of terrestrial origin, and the Abs_{254/365} values were very low for MP, LM and SGC, signalling a high mean MW for these DOCs molecules (Table 2). In fact, in the present study, the SAC₃₄₀ was negatively correlated with the Abs_{254/365} and with the relative FA fluorescence (humic substances

FIGURE 3 Mean (\pm SEM) net ammonia fluxes rates ($J_{\text{amm.net}}$) (A), net sodium fluxes rates ($J_{\text{Na.net}}$) (B) and net chloride fluxes rates ($J_{\text{Cl.net}}$) (C) of *Danio rerio* at pH 7.0 (black bars) and pH 4.0 (grey bars), after seven days of acclimation at pH 7.0 (black bars). Data for the no-added DOC control group and five dissolved organic carbon (DOC) treatments are shown: São Gabriel da Cachoeira (SGC), Solimões (SOL), Maple tree-derivate DOC (MP), Luther Marsh (LM) and Bannister lake (BL) during the flux periods of 0–3 h since the start of exposure. Different letters indicate significant differences of the mean values among the different DOC sources at the same pH ($P < 0.05$). Means not sharing the same letters are significantly different among treatments ($P < 0.05$). Asterisk (*) indicate significant differences among the pH 7.0 and 4.0 within the same treatment ($P < 0.05$)



with lower molecular weight) but positively correlated with the amino acid fluorescence (Table 3). In other words, the DOCs from a more terrigenous origin (allochthonous) (i.e. SGC, MP and LM) are composed of larger molecules with more aromatic rings (higher SAC_{340} , lower $Abs_{254/365}$ and FI, higher relative HA and amino acid content) (Thurman, 1985; Wetzel, 2001).

It is known that a natural degradation of the DOC can occur over time during its storage, which might alter the original physicochemical properties (Peacock et al., 2015; Sadauskas-Henrique et al., 2019). For example, Sadauskas-Henrique et al. (2019) reported lower aromaticity ($SAC_{340} = 36.5 \text{ cm}^2 \text{ mg}^{-1}$) in comparison with the values reported by Duarte et al. (2016) ($SAC_{340} = 73.0 \text{ cm}^2 \text{ mg}^{-1}$) for the same SGC DOC but measured shortly after collection. As the techniques used for the two analyses were identical, the authors attributed the differences to the degradation over the period of time that the DOC was stored under refrigeration, resulting in loss of aromatic compounds and reduction in mean molecular weight. In fact, the $Abs_{254/365}$ was higher (lower molecular weight) in the present work and in Sadauskas-Henrique et al. (2019) in relation to Duarte et al. (2016) (Table 2). In the present study, the SAC_{340} for SGC was almost identical with that found by Sadauskas-Henrique et al. (2019) ($36.6 \text{ cm}^2 \text{ mg}^{-1}$) (Table 2), since the interval of storage of the SGC DOC between these two studies (present study and Sadauskas-Henrique et al., 2019) was relatively short (~ 6 months) in comparison with Duarte et al. (2016) versus Sadauskas-Henrique et al. (2019) (~ 2 years).

These physicochemical changes in the spectroscopic characteristics of the SGC DOC during the storage were reflected in the fish ionoregulatory responses when challenged by acidic water (pH 4.0). Duarte et al. (2016) reported almost perfect protection by the SGC DOC against ionoregulatory disturbances for the zebrafish (*Danio rerio*) acutely exposed to acidic ion-poor water (pH 4.0). However, this was not observed for the acidophilic tambaqui (*Colossoma macropomum*) where SGC DOC potentiated Na^+ and Cl^- losses at low pH (Sadauskas-Henrique et al., 2019). Similarly, in the present study, zebrafish exhibited greater net Na^+ and Cl^- losses, and elevated ammonia excretion rates, when exposed to all DOC sources under acidic environmental pH (4.0). This was especially true for autochthonous DOCs sources, as discussed in the next sections.

4.2 | Acclimation effects of the five DOC sources on Na^+ , K^+ ATPase and v-type H^+ ATPase at neutral pH (7.0) and acidic pH (4.0)

In the present study, the response of the two enzymes was unexpected. After 7 days of acclimation to the five DOC sources or the control condition under neutral pH (7.0), zebrafish acclimated to SOL and MP had significantly higher activity of the Na^+ , K^+ ATPase in relation to control (Figure 1a), and indeed there were similar high activities in the other three DOC treatments. There is at least one previous study where acclimation to elevated DOC at circumneutral pH resulted in elevated gill Na^+ , K^+ ATPase activity in rainbow trout,

though a commercial DOC rather than a DOC collected from nature, was used (McGeer et al., 2002). In the present study, these differences were no longer present when zebrafish were exposed to acidic pH (4.0) for 3 h (Figure 1b). However, this lack of difference was due to the Na^+ , K^+ ATPase activity in the controls now being 2-fold higher than at pH 7.0, whereas activities in the five DOC treatments did not change (Figure 1b versus Figure 1a). The control activity had been quickly upregulated by pH 4.0 exposure to levels already seen at pH 7.0 when DOCs were present. The Na^+ , K^+ ATPase located in the basolateral membrane is thought to provide a major energy input to power overall uptake of major ions as well as to provide an exit step for the Na^+ from the branchial epithelial cell into the extracellular fluids (Evans et al., 2005). Because Na^+ uptake in freshwater fish is coupled to H^+ secretion through the actions of the Na^+/H^+ exchanger (NHE) and the v-type H^+ ATPase (Evans et al., 2005), a reduction in environmental pH level would reduce the gradient to drive Na^+ influx, which can be compensated by the increases in the Na^+ , K^+ ATPase activity (Kwong et al., 2014). In this sense, the zebrafish exposed to pH 4.0 without DOC elevated their Na^+ , K^+ ATPase activity in attempt to control their net ion losses, and this was achieved for both Na^+ and Cl^- loss rates (Figure 3b). To our knowledge, this is the first demonstration of upregulated Na^+ , K^+ ATPase at low pH in the zebrafish, though it has been seen on a slower basis at the mRNA level (Esbaugh et al., 2019) and activity level (Craig et al., 2007) when zebrafish were transferred to ion-poor softwater.

In contrast, no increases in branchial Na^+ , K^+ ATPase activity upon transfer to pH 4.0 were observed for zebrafish acclimated to the five DOC sources, despite the fact that Na^+ and Cl^- losses were elevated in all cases (Figure 1a, b). While this result (elevation in Na^+ and Cl^- losses at low pH in the presence of DOCs) agrees with our previous study (Sadauskas-Henrique et al., 2019) on the tambaqui using stored, naturally collected DOCs, it conflicts with others showing the protective effect of natural, freshly collected DOC against ion losses at low pH (Al-Reasi et al., 2016; Duarte et al., 2016; Gonzalez et al., 2002; Wood et al., 2003). Indeed some of these authors have demonstrated that the DOC can ameliorate the effects of the low pH exposure by modulating various aspects of the branchial Na^+ transport system and the electrical properties of fish gill epithelium, and this ability seems to be directly related with spectroscopic characteristics of the DOC (Al-Reasi et al., 2013b; Al-Reasi et al., 2016; Duarte et al., 2016; Galvez et al., 2008; Wood et al., 2011). In the present study, no correlations were found between the Na^+ , K^+ ATPase activity and the DOC spectroscopic characteristics (Table 4). Moreover, significantly higher Na^+ , K^+ ATPase activities in relation to control were found only for one source of allochthonous (MP) and one source of autochthonous (SOL) DOC, which have very different spectroscopical features (Table 2). Similarly, Al-Reasi et al. (2016) found changes in sodium regulation of zebrafish exposed to DOC sources under neutral (7.0–8.0) and acidic (5.0) pH, only some of which were related to their physicochemical characteristics.

The v-type H^+ ATPase of zebrafish is located apically in the H^+ ATPase rich cells (Kwong et al., 2014) and is considered to play an

important role in acid excretion and Na^+ uptake coupled to acid excretion (Esaki et al., 2009). Previous studies on zebrafish have emphasized the importance of v-type H^+ ATPase upregulation as a compensatory strategy to maintain Na^+ uptake and acid-base balance at low environmental pH (reviewed by Kwong et al., 2014; Shir-Mohammadi & Perry, 2020). However, as with Na^+ , K^+ ATPase activity, the speed of the upregulation (3 h) in the present study was remarkable.

Similar to Na^+ , K^+ ATPase activity, acclimation to the various DOCs at pH 7.0 resulted in a general trend for increases in v-type H^+ ATPase activity, but this was significant only for SGC and SOL, which have respectively allochthonous and autochthonous characteristics (Figure 2a). To our knowledge, this is the first demonstration that chronic DOC exposure can alter v-type H^+ ATPase activity in any fish. Furthermore, when zebrafish were exposed for 3 h to acidic pH (4.0), the v-type H^+ ATPase activity increased by 6-fold in the control treatment (Figure 2b), an even greater change than seen for Na^+ , K^+ ATPase activity in the control treatment (Figure 1b). As with Na^+ , K^+ ATPase, this could help explain the lack of elevated Na^+ and Cl^- losses at low pH in the control fish (Figure 3b, c). This rapid upregulation of v-type H^+ ATPase activity at pH 4.0 was also seen in the presence of SGC, but not for the other DOCs. Indeed v-type H^+ ATPase activity was even lowered at pH 4.0 in the presence of SOL and MP (Figure 2b), two DOCs of dissimilar characteristics (Table 2). A similar rapid inhibition of v-type H^+ ATPase activity upon exposure to pH 4.0 was seen in an Amazonian cichlid, the angelfish, though added DOC was not present in that study (Duarte et al., 2013).

Notably, of all the optical indices, only the FI was correlated (negatively) with the v-type H^+ ATPase activity, and only at low pH (Table 4). In fact, the SGC DOC was associated with the highest v-type H^+ ATPase activity under acidic pH (together with the control). In the case of the SGC DOC, perhaps the massive net Na^+ losses caused by SGC DOC (Figure 3b) resulted in feedback upregulation of the branchial v-type H^+ ATPase activity (Figure 2b).

4.3 | Acclimation effects of the five DOC sources on ion regulation and ammonia excretion of zebrafish at neutral (7.0) and low pH (4.0)

Zebrafish acclimated to the five DOC sources under neutral pH (7.0) exhibited no significant alterations in ion regulation and ammonia excretion (Figure 3). Similarly, zebrafish acclimated to moderately acidic pH (5.0) for one week experienced a steady-state with respect to Na^+ and Cl^- efflux rates and ammonia excretion (Al-Reasi et al., 2016). On the other hand, in the present study, exposure to acidic pH (4.0) caused net ion losses and elevated ammonia excretion in the presence of almost all of the DOC sources, in contrast to the regulation of these parameters seen in the control treatment at acidic pH (Figure 3). It is important to note that net flux rates of Na^+ and Cl^- represent the arithmetic difference between active influx and passive efflux rates. As reviewed by Morris et al. (2021), previous studies

have shown that natural DOCs may be supportive at low pH both by protecting active influx rates from inhibition, and by limiting the stimulation of passive efflux rates by low pH. The fact that at pH 7.0, branchial Na^+ , K^+ ATPase and v-type H^+ ATPase activities, the two enzymes critical in driving ion influxes, were generally raised by DOCs, above the levels seen in the control fish (Figures 1a and 2a), suggests that some of that protective action on influx persisted despite the storage issue discussed subsequently (see Section 4.4 below). However, at low pH, the changeover to increased Na^+ and Cl^- losses in the presence of added DOC (Figure 3b, c) suggests that the protective action on influx was lost at low pH, and/or that the DOCs became toxic, stimulating rather than reducing diffusive efflux rates. Radioisotopic experiments measuring unidirectional Na^+ and Cl^- fluxes would be needed to answer this question. Furthermore, net Cl^- losses generally exceeded net Na^+ losses in zebrafish in the presence of DOC at pH 4.0, suggesting net acidic equivalent excretion by Strong Ion Difference (SID) theory (Stewart, 1983), though direct water titration measurements would be needed to confirm this.

The zebrafish is known as an acid-tolerant model, surviving well in acidic waters as low as pH 4.0 (Al-Reasi et al., 2016; Duarte et al., 2016; Duarte et al., 2018; Horng et al., 2007, 2009; Kumai & Perry, 2011; Kwong & Perry, 2013; Kwong et al., 2014). However, all the studied DOC sources (with exception of BL) were associated with increases of Na^+ and Cl^- net fluxes, where these increases were accompanied with increases in ammonia excretion rate (Figure 3). Recent studies (Duarte et al., 2016; Duarte et al., 2018; Kumai & Perry, 2011; Kumai & Bahubeshi, Steele, et al., 2011; Kwong & Perry, 2013) with zebrafish exposed to acidic pH (4.0) suggest a functional coupling of the stimulation of Na^+ uptake with the ammonia excretion during low pH exposure. Al-Reasi et al. (2016) found that acclimation of zebrafish to less extreme acidity (pH 5.0) resulted in a modest but significant overall stimulation of ammonia excretion. In the present study, lacking unidirectional flux measurements, it is not possible to analyze if a functional coupling of the stimulation of Na^+ uptake with the elevated ammonia excretion occurs during low pH exposure. However, zebrafish exposed to BL DOC under acidic pH (4.0) show no elevation on Na^+ flux rate while increases in ammonia excretion were observed (Figure 3a, b). Similarly, Al-Reasi et al. (2016) demonstrated that reductions, relative to control, in the Na^+ efflux rates (0–3 h) for zebrafish exposed to BL DOC were statistically significant only at acidic pH (5.0), but were not coupled with increases in ammonia excretion. Also, these authors observed that all studied DOCs including LM, tended to reduce Na^+ efflux rates in all treatments relative to control at both pH (7.0 and 5.0). Other authors also found that the natural, freshly collected DOCs (SGC and LM) in water reduced both Na^+ and Cl^- efflux rates across the gills of zebrafish yet supported ammonia excretion at extremely low pH (4.0) (Duarte et al., 2018, Duarte et al., 2016, respectively). Importantly, Al-Reasi et al. (2016) found a positive correlation between the buffer capacity of DOCs and Na^+ influx. This suggests that the DOC can stabilize the pH of the branchial micro-environment, allowing the Rh protein- Na^+ transporter metabolon, already described for fish (Wright & Wood, 2009), to function.

Overall, the present results differ from those of Al-Reasi et al. (2016) and Duarte et al. (2016) and Duarte et al. (2018), where these same DOCs (with exception of BL) protected against Na^+ and Cl^- net losses yet stimulated ammonia excretion. Indeed, they are more similar to our previous work on tambaqui where stored SGC DOC had no effect at pH 7.0, did not protect against Na^+ and Cl^- losses at pH 4.0, but stimulated ammonia excretion at pH 4.0 (Sadauskas-Henrique et al., 2019). In that study, it was not clear if the lack of protectiveness against ion losses was due to species difference, or to DOC changes during storage. The present results, now on the same species (zebrafish) as used by Duarte et al. (2016) and Duarte et al. (2018) point strongly to the changes during storage explanation.

4.4 | DOC properties and potential changes during storage

It is known that a natural degradation of the DOC can occur over time during its storage, which would alter the original physicochemical properties (Peacock et al., 2015; Sadauskas-Henrique et al., 2019). Clearly not all beneficial effects are lost: for example, the hyperpolarizing effects of DOC on transepithelial potential (Galvez et al., 2008; Sadauskas-Henrique et al., 2019) and the stimulatory effects on gill Na^+ , K^+ ATPase and v-type H^+ ATPase activities (Figures 1a and 2a) are still present to some degree. There may be an analogy here, inasmuch as commercial DOC sources can be effective in raising Na^+ , K^+ ATPase activity (McGeer et al., 2002), yet ineffective or deleterious to ionoregulation at low pH (Wood et al., 2003; da Costa et al., 2015; da Costa et al., 2017). The physicochemical changes in the spectroscopic characteristics of the DOCs during the storage (or during commercial purification) may reflect in the way that it interacts with the biological membranes. For example, Wood et al. (2011) argued that unknown aspects of the phenolic ring structure of dissolved organic matter (DOC) were important in promoting Na^+ uptake capacity. In accord with this idea, Galvez et al. (2008) found that SAC_{340} correlates directly with a more negative transepithelial potential across the gills, which will favor Na^+ uptake (Galvez et al., 2008). The lack of a relationship between Na^+ efflux with any of these parameters (Al-Reasi et al., 2016) suggests that all natural DOCs reduce Na^+ efflux in a non-specific phenomenon, perhaps associated with binding of these amphiphilic molecules to the gill surface (Campbell et al., 1997), exerting a Ca^{2+} -like effect. Wood et al. (2003) reported that added Ca^{2+} duplicated some of the protective effects of natural DOC in Amazonian stingrays at low pH. However, the fact that DOC generally hyperpolarizes TEP, whereas Ca^{2+} generally depolarizes TEP (Morris et al., 2021) argues against this explanation.

How the DOC molecules might prevent or exacerbate ion losses under acid conditions remains unknown. Analysing the changes in the physicochemical properties of the DOCs (Table 2), we can infer that changes in the aromaticity (SAC_{340}), the molecular weight ($\text{Abs}_{254/365}$), the DOC origin character (FI), the content of HA and FA, and the proteinaceous material (Tyr) may play important roles in physiological effects mediated by DOC on gill functions of freshwater fishes. In the present study, lower aromaticity, and MW (higher $\text{Abs}_{254/365}$) tended to increase

the net ammonia excretion rates at both pH 7.0 and pH 4.0. Moreover, a more allochthonous (lower FI) nature of the DOC was associated with greater v-type H^+ ATPase activity at low pH. Also, lower FI was associated with higher (more negative values) loss rates of Na^+ under acidic conditions. In other words, DOC with a more allochthonous origin (i.e. SGC; MP and LM) induced increases in ammonia excretion rates of zebrafish under neutral (pH 7.0) and acidic pH (4.0), yet aggravated net Na^+ losses at low pH. Thus, low FA increases the net Na^+ losses while low HA decreases the net Na^+ losses at low pH. Lower FA increases the net Cl^- losses at low pH, but decreases the net ammonia losses at neutral pH. High content of protein-like material (Tyr) caused a decrease of the Cl^- net losses (more positive values) at neutral pH while at acid pH, caused a stimulation (more negative values). On the other hand, higher the tyr-like material, lower the ammonia net fluxes (more positive values) at both pH 7.0 and 4.0. These results contrast with some previous studies using the same DOCs when freshly collected, indicating that changes critical to physiological impact may occur during storage (Al-Reasi et al., 2013b, 2016, Duarte et al., 2016, 2018). Comparative studies of the osmoregulatory responses mediated in zebrafish by DOC from different sources highlights that the quality and properties of the DOC drive different effects on gill physiology.

5 | Conclusions

Although there is an extensive literature on the zebrafish as a model for understanding low pH tolerance (reviewed by Kwong et al., 2014), the effects of DOC have been largely overlooked. The present study provides the first evidence that acclimation to natural DOCs at circumneutral pH can stimulate both gill Na^+ , K^+ ATPase and v-type H^+ ATPase activities, and also that acute exposure to pH 4.0 in the absence of DOC can upregulate the activities of both enzymes within 3 h. None of the DOCs were protective at low pH, and indeed all five tended to increase net loss rates of Na^+ and Cl^- and promoted increases in ammonia excretion, at pH 4.0, to variable extents. Comparison with past studies using some of these DOC sources when freshly collected indicates that spectroscopic and physiological properties of the DOCs may occur during storage. The physicochemical properties of the DOCs (i.e. aromaticity (SAC_{340}), molecular weight ($\text{Abs}_{254/365}$), origin (FI), and the content of HA, FA and tyr-like components seem to play important roles in interacting with the zebrafish gill membranes since they were correlated with Na^+ and Cl^- net fluxes and ammonia excretion.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request. The authors declare that the data will be available to be shared if requested.

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