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REVIEW

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# The physiology of fish in acidic waters rich in dissolved organic carbon, with specific reference to the Amazon basin: Ionoregulation, acid–base regulation, ammonia excretion, and metal toxicity

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## Abstract

Although blackwaters, named for their rich content of dissolved organic carbon (DOC), are often very poor in ions and very acidic, they support great fish biodiversity. Indeed, about 8% of all freshwater fish species live in the blackwaters of the Rio Negro watershed in the Amazon basin. We review how native fish survive these harsh conditions that would kill most freshwater fish, with a particular focus on the role of DOC, a water quality parameter that has been relatively understudied. DOC, which is functionally defined by its ability to pass through a 0.45-μm filter, comprises a diverse range of compounds formed by the breakdown of organic matter and is quantified by its carbon component that is approximately 50% by mass. Adaptations of fish to acidic blackwaters include minimal acid–base disturbances associated with a unique, largely unknown, high-affinity $Na^+$ uptake system that is resistant to inhibition by low pH in members of the Characiformes, and very tight regulation of $Na^+$ efflux at low pH in the Cichliformes. Allochthonous (terrigenous) DOC, which predominates in blackwaters, consists of larger, more highly colored, reactive molecules than autochthonous DOC. The dissociation of protons from allochthonous components such as humic and fulvic acids is largely responsible for the acidity of these blackwaters, yet at the same time, these components may help protect organisms against the damaging effects of low water pH. DOC lowers the transepithelial potential (TEP), mitigates the inhibition of $Na^+$ uptake and ammonia excretion, and protects against the elevation of diffusive $Na^+$ loss in fish exposed to acidic waters. It also reduces the gill binding and toxicity of metals. At least in part, these actions reflect direct biological effects of DOC on the gills that are beneficial to ionoregulation. After chronic exposure to DOC, some of these protective effects persist even in the absence of DOC. Two characteristics of allochthonous DOC, the specific absorbance coefficient at 340 nm (determined optically) and the PBI (determined by titration), are indicative of both the biological effectiveness of DOC and the ability to protect against metal toxicity. Future research needs are highlighted, including a greater mechanistic understanding of the actions of DOCs on gill ionoregulatory function, morphology, TEP, and metal toxicity. These should be investigated in a wider range of native fish Orders that inhabit one of the world's greatest biodiversity hotspots for freshwater fishes.

KEYWORDS

acid–base regulation, Amazon basin, ammonia excretion, dissolved organic carbon, ionoregulation, low pH, metal toxicity, transepithelial potential

## 1 | INTRODUCTION

Dissolved organic matter, often loosely referred to as dissolved organic carbon (DOC), is central to the function, structure, and diversity of aquatic ecosystems (Findlay & Parr, 2017; Lindeman & Lindeman, 2007). It is defined by passage through a 0.45-μm filter (therefore "dissolved"), and quantified by its carbon content (therefore "DOC"), which generally makes up about 50% of the molecules by mass (Wood et al., 2011). When molar units are used, these are based only on the moles of carbon content (molecular weight [MW] = 12.01 Da), because the exact structures of most DOC molecules are unknown. It should be recognized that there are many carbon atoms in each DOC molecule. These heterogeneous compounds are naturally occurring transformed products of the breakdown of organic matter by microbes, light, heat, and enzymes in soil and water (Zara et al., 2006). Allochthonous DOCs (also known as terrigenous) are formed largely on land from the breakdown of plant products such as lignins, whereas autochthonous DOCs are formed largely in lakes and rivers by the metabolism of very small organisms (invertebrates, plankton, and bacteria). DOC is often the most abundant dissolved component in freshwater. It provides essential nutrients to organisms and influences the productivity of trophic interactions within an ecosystem (Findlay & Parr, 2017; Lindeman & Lindeman, 2007). The effects of fluctuations in other water quality parameters (e.g., pH, alkalinity, Ca, Na, salinity) on gill function have been extensively studied by physiologists but the effects of DOC on the ion transporting properties of the gill remain relatively unexplored. DOC is a critically important water quality parameter to consider for ecological understanding and risk assessment as it may have comparable or greater effects on freshwater fish physiology (e.g., ionoregulation, acid–base balance, and acute metal toxicity) than the previously mentioned and well-studied water quality parameters (Duarte et al., 2016; Gonzalez et al., 2002; Sadauskas-Henrique et al., 2019; Wood et al., 2011; Wood et al., 2003, 1998).

The dissociation of protons from allochthonous DOC is the major cause of acidity in peat bogs and many other natural blackwaters. However, there is now extensive evidence in freshwater fish that natural DOCs may actually mitigate the ionoregulatory disturbances of the ambient low pH which they help to create (Duarte et al., 2016, 2018; Gonzalez et al., 2002; Wood et al., 2003, 1998). It has even been suggested that DOCs may affect ventilation (Holland et al., 2014; Perry et al., 2021) and respiratory gas exchange ($O_2$, $CO_2$, and ammonia) at the gills (Matsuo et al., 2004), and at least for ammonia, there are data that support this (Duarte et al., 2016, 2018; Wood et al., 2003). Here, our major focus is on ionoregulatory physiology and associated functions (acid–base balance, ammonia excretion), where there remain significant gaps in our understanding of the effect(s) of DOC on ion transport in freshwater fish. DOC source, physicochemical properties, and the species of interest all influence the effect of DOC on freshwater organisms under acidic conditions (Wood et al., 2011). We will first review the nature, origins, ecological roles, and chemistry of DOC, and then provide an overview of previous investigations on the effects of DOC in acidic water on ion transport including active ion uptake and passive ion loss, acid–base balance, electrical properties of the gill (transepithelial potential [TEP]), ammonia excretion, and the amelioration of metal toxicity. Blackwater systems are found globally from tropical peatlands in Southeast Asia, European peat bogs, swamps along the Atlantic coast of North America to South American tropical rainforests (Gandois et al., 2020; Robroek et al., 2017; Sabater et al., 1993). Blackwater systems have a characteristically high concentration of DOC, low pH, and often low-ion concentration. Our focus will be on the best studied of these habitats, the Amazon basin, particularly the fish native to Rio Negro blackwaters.

## 2 | PHYSICOCHEMICAL PROPERTIES OF DOC

Humification is a poorly understood biological process where DOC is formed by the microbial processing of lignin-rich plant materials and the decay of organic remains of animals (Ertel et al., 1984; Hatcher & Spiker, 1988). Humification yields complex chemical structures that are more stable than the original compounds from which they are derived (Boggs et al., 1985). DOC is often the largest component by mass of the dissolved components of natural waters. However, there is a wide variation in concentrations (5–35 mg/L in Rio Negro; Table 1) found within freshwater systems that may fluctuate seasonally and differ among regions (Boggs et al., 1985; Holland et al., 2018, 2017; Thurman, 1985; Tipping, 2002). DOC is a combination of humic substances which are generally larger molecules than nonhumic substances such as amino acids, fatty acids, phenols, sterols, natural sugars, hydrocarbons, urea, and porphyrins (Boggs et al., 1985; Gaffney et al., 1996). Humic substances are primarily composed of carbon, nitrogen, oxygen, and sulfur that make up organic acids that have highly oxygenated functional groups

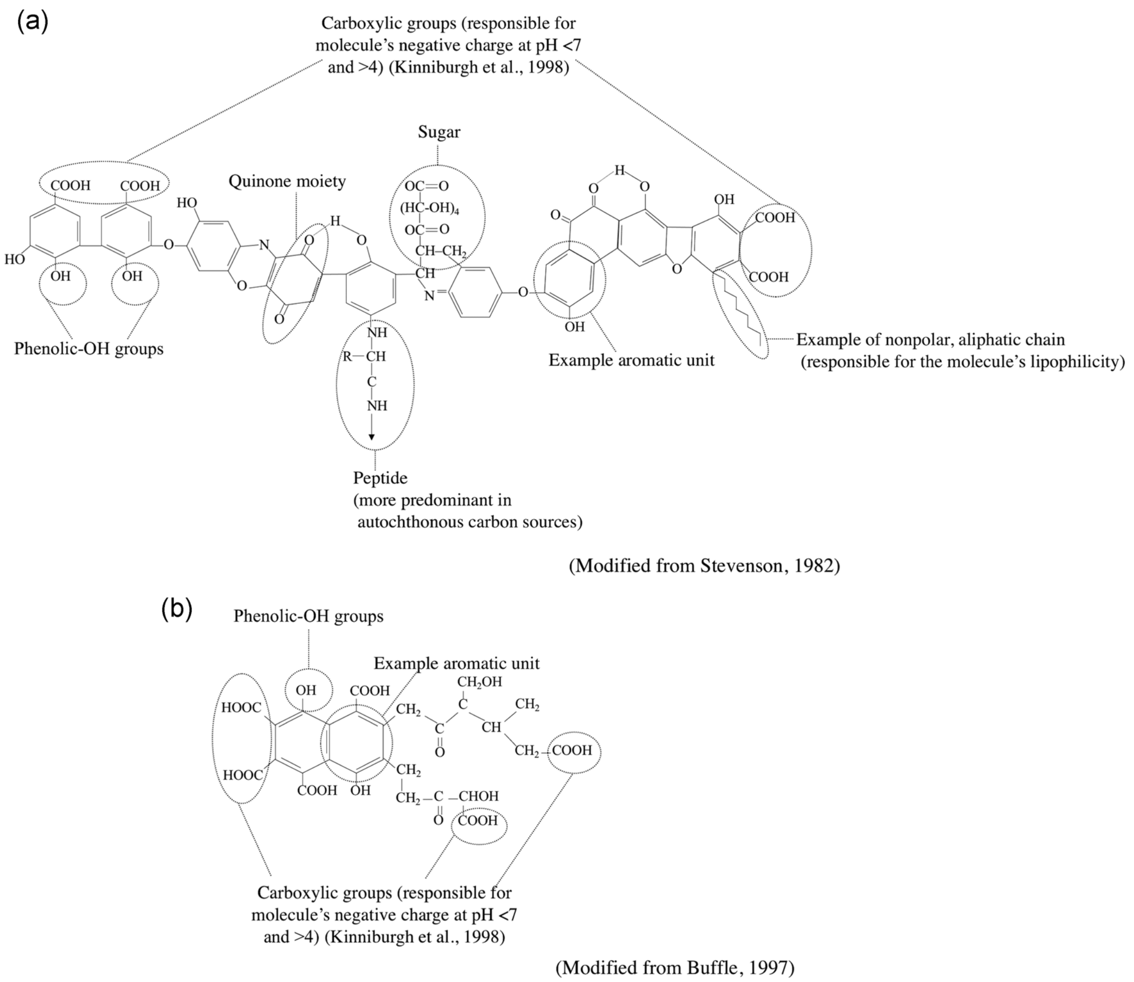
**TABLE 1** General water chemistry ranges for acidic, DOC-rich black waters of the Rio Negro watershed, based on the experience of the authors and data of Duarte (2012) (in μmol/L, except DOC which is in mg C/L)

| Parameter | Range |
|---|---|
| DOC | 5–35 |
| Na | 5–50 |
| K | 0.2–20 |
| Cl | 8–70 |
| Ca | 0.1–20 |
| Mg | 0.3–10 |
| pH | 3.0–6.0 |

Abbreviation: DOC, dissolved organic carbon.

(Boggs et al., 1985; Zara et al., 2006). Operationally, humic substances are fractionated into "humic acids" which precipitate at low pH (1.0–2.0) and "fulvic" acids which do not (Figure 1). Humic acid DOC is more aromatic and has a higher MW compared to fulvic acid DOC. The general structure of the humic substance macromolecule found in aquatic systems, a flexible polyelectrolyte, depends on the conditions of the system including electrolyte concentration and pH (Figure 1; Holland et al., 2018; Zara et al., 2006). Naturally acidic waters have higher concentrations of humic substances and appear to be optically darker indicating a different quality DOC than found in circumneutral water (Holland et al., 2018). DOC can be distinguished by origin, optical characteristics, or molecular properties.

Allochthonous DOC, composed of ~50%–90% humic substances, is produced on land and washed into nearby natural waters (Thurman, 1985; Tipping, 2002). The transport of DOC from terrestrial to aquatic environments varies spatially and temporally,



(Modified from Stevenson, 1982)

(Modified from Buffle, 1997)

**FIGURE 1** The typical structure of (a) humic and (b) fulvic acids which typically comprise a substantial proportion of allochthonous DOC molecules. Functional groups are circled (carboxylic groups, phenolic-OH groups, aromatic units, quinone, sugar, aliphatic chains, and peptides). Functional groups responsible for characteristics of DOC (negative charge and lipophilicity) are noted (Buffle, 1997; Kinniburgh et al., 1998; Stevenson, 1982). DOC, dissolved organic carbon creating variation in DOC characteristics (Kullberg et al., 1993). Autochthonous DOC is generated within the water column by algae and bacteria and is much lighter in color compared to allochthonous DOC (Tipping, 2002). Optical properties such as aromatic composition (quantified by specific absorbance coefficients), fluorescence signatures, and parallel factor analysis (PARAFAC) have been used to describe molecular variability of DOC (Al-Reasi et al., 2011). DOCs identified by these methods are often referred to as chromophoric DOCs because they absorb light (Holland et al., 2018; Nimptsch et al., 2014).

Humic fractions can be distinguished from fulvic fractions as they produce longer wavelength emissions (Wu et al., 2007). The specific absorption coefficient (SAC) is the absorbance of DOC at 300–350 nm normalized to DOC concentration, and is used as an index of aromatic composition (Curtis & Schindler, 1997), with the higher the value, the greater the aromaticity (i.e., phenol ring content). The specific absorption coefficient at 340 nm ($SAC_{340}$) appears to be a very useful indicator of the ability of DOC to both bind metals (Al-Reasi et al., 2012, 2011) and exert physiological effects on aquatic organisms (Al-Reasi, Wood et al., 2013). Fluorescence index (FI) may indicate if organic matter originates from different sources. It is the ratio of fluorescence $intensity_{450\,nm}$/fluorescence $intensity_{500\,nm}$ at an excitation wavelength of 370 nm, with high values indicating an autochthonous origin, and low numbers an allochthonous origin (McKnight et al., 2001). Overall, Al-Reasi et al. (2011) concluded that FI was less successful than SAC in predicting the ability of different DOCs to protect against metal toxicity. This may reflect spatial and temporal variations during humification (Al-Reasi et al., 2011). Parallel factor analysis is a computational model based on the fluorescence excitation-emission fingerprint of a DOC sample that uses a multivariate statistical approach (Kroonenberg & Heiser, 1998; Stedmon & Bro, 2008). It allows for greater molecular discrimination than other methods (e.g., FI and SAC) because of its advanced spectral resolution based on excitation-emission fluorescence spectroscopy. This method yields relative percent fractionation into user-defined components, and generally assumes 2–4 such components as representative fluorophores in each sample—for example, a humic-like, a fulvic-like, and two different protein/amino acid-like components, and then produces summary numbers representing organic matter quality (Al-Reasi et al., 2011). The protective effect of DOC against metal toxicity and the molecular variability between samples from different environments can be tracked based on the wavelengths of light emitted and the resulting fluorescent composition. Samples that are high in humic- and fulvic-like content are generally allochthonous and highly protective, whereas autochthonous samples are high in amino-acid-like fluorescence and are generally much less protective (Al-Reasi et al., 2011). Overall, SAC appears to be positively correlated with the humic-like component and negatively correlated with the fulvic-like component calculated by PARAFAC, whereas the opposite is true for FI (Al-Reasi et al., 2011).

Lipophilicity and binding capacity of DOC have also been investigated to determine their role in affecting ionoregulatory physiology and metal toxicity in fish (Al-Reasi et al., 2011; Wood et al., 2011). Lipophilicity has been estimated by two methods, reverse-phase high-pressure liquid chromatography and the octanol–water partition coefficient ($K_{ow}$) (Egeberg & Alberts, 2002; Gjessing et al., 1991). When these two isolation procedures were performed on the same samples, different lipophilicity ranges were reported suggesting that the lipophilic nature of DOC is dependent on the isolation procedure (Namjesnik-Dejanovic & Cabaniss, 2004). The lipophilic nature of DOC is likewise dependent on pH (Egeberg & Alberts, 2002; Gjessing et al., 1991; Namjesnik-Dejanovic & Cabaniss, 2004). The overall conclusion appears to be that there is no relationship between lipophilicity and the ability to bind metals (Al-Reasi et al., 2011). There has been a large matrix of methods employed to determine the binding capacity properties of DOC with debate over the importance of the specific binding ligands involved (Alberts et al., 1984). Ultrafiltration through a 1000 Da MW filter followed by a series of titrations as well as measurements with ion-selective microelectrodes have been used (Alberts et al., 1984; Smith & Kramer, 1999; Takács et al., 1999). With respect to protection against metal toxicity, no clear association between $H^+$- or metal-binding site density was apparent in earlier analyses (Al-Reasi et al., 2011). However, Al-Reasi, Wood et al. (2013) developed a new metric, the proton binding index (PBI), as a general indicator of the chemical reactivity of DOC, based on acid–base titration over a wide range of pK values. There was a strong positive correlation between PBI and $SAC_{340}$, such that DOCs with high PBI values were both protective against metal toxicity and supportive of ionoregulation. Photo-oxidation breaks down DOC into smaller, less aromatic molecules with generally lower binding capacities for metals (Patel-Sorrentino et al., 2004; Shiller et al., 2006) and therefore lower protective abilities against metal toxicity to fish, although some exceptions to this trend have also been observed (Brooks, McKnight et al., 2007, Brooks, Meyer, & Boese 2007, Brooks, Meyer, McKnight 2007).

Finally, it is important to note that while commercially available humic acids, which are usually derived from coal or peat, can be characterized and classified by the above approaches, it is now clear that they are fundamentally different from humic substances occurring in natural waters (Chiou et al., 1987; Malcolm & MacCarthy, 1986; Rodríguez et al., 2014). Typically, they have been treated with strong chelating agents to strip away metals, and they may have unusually high ash content, both of which may give them unusually high cation binding capacity and reactivity with biological membranes. There may also be structural differences. Results obtained with commercially available humic acids can be misleading and should be interpreted cautiously. For example, Aldrich humic acid, at the same total DOC concentration as natural compounds in Rio Negro blackwater, exacerbated rather than protected against the effects of low pH on ion balance in Amazonian stingrays (Wood et al., 2003). Similarly, it disturbed the chemosensory control of ventilation, whereas natural DOC did not (Perry et al., 2021). Aldrich humic acid was also much less protective against Ag toxicity to daphnia (Glover, Pane, 2005) and less effective on reducing urea excretion at low pH in daphnia (Al-Reasi, Yusuf et al., 2013) than would have been predicted by its optical properties. Likewise, exposure to environmentally relevant concentrations of Aldrich humic acid at low pH decreased the survival of the silver catfish (*Rhamdia quelen*) and altered gill morphology, suggesting ionic and respiratory disturbances (da Costa et al., 2017, 2015).

## 3 | DOC AS A GENERAL ECOLOGICAL DRIVING FACTOR IN AQUATIC ECOSYSTEMS

DOC concentrations are presently increasing in freshwater systems on a worldwide basis, though with great temporal and spatial variability. Driving factors include changes in air temperature, increased precipitation, changes in land usage, increased atmospheric carbon dioxide, decreased atmospheric sulfur deposition, and accumulation of atmospherically deposited nitrogen (reviewed by Pagano et al., 2014; Sucker & Krause, 2010).

DOC is an environmental regulator of biotic and abiotic processes in freshwater ecosystems (Al-Reasi et al., 2011; Kullberg et al., 1993; Petersen, 1991; Steinberg et al., 2006). The full extent of the ecological significance of DOC in freshwater systems is not yet understood. Aquatic ecosystems are very sensitive to changes in DOC concentrations as these molecules interact with complex biological processes including ultraviolet screening, the carbon cycle, $CO_2$ generation and $O_2$ consumption by photo-oxidation, production of reactive oxygen species (ROS), acidification, nutrient transport, and metabolic processes (Holland et al., 2018; Johannsson et al., 2020, 2017; Kullberg et al., 1993; Sucker & Krause, 2010; Wood et al., 2011). While autochthonous DOC is usually colorless, high concentrations of allochthonous DOC give water a brown hue which decreases UV penetration and influences water temperature, productivity, stratification, food web structure, and organism distribution within the water column (Hessen & Tranvik, 1998; Keller et al., 2003; Sucker & Krause, 2010). The photo-mineralization of allochthonous DOC produces $CO_2$ which has been estimated to be responsible for up to 10% of global greenhouse gas emissions (Koehler et al., 2016; Panneer Selvam et al., 2019). Acidification of water has been reported to increase DOC photodegradation (Anesio & Granéli, 2003; Koehler et al., 2016). The aromatic nature of DOC derived from the land makes it particularly photoreactive compared to DOC derived within the water column, therefore, it is likely a greater contributor to the carbon cycle (Anesio & Granéli, 2003). While DOC contributes to the acidity of the surface water, it may also buffer against acidification (Kullberg et al., 1993). While there is a general accord that most DOCs buffer effectively around circumneutral pH, and then again at very low pHs (<4.5) (Leenheer, 1980; Wood et al., 2003), detailed titration studies have shown that a multiplicity of pK values exist within each type of DOC, reflecting different moieties within the molecules (variable amongst types). The overall buffer characteristics that are seen represent averages of these. Some DOCs can also have toxic effects to fish populations that are exacerbated at low pH (Kullberg et al., 1993), while others are protective (Wood et al., 2011). DOC delivers nutrients and energy to freshwater bacteria which are transferred to zooplankton that have a high reliance on allochthonous carbon, and in turn, these are subsequently transferred to fish (Jones, 2005; Steinberg et al., 2006). Overall, the influence of DOC on these processes depends on the quality of DOC itself (Holland et al., 2018).

While it has been argued that DOC molecules can be taken up across the gills of aquatic organisms (Steinberg et al., 2006, 2003), there appears to be no definitive evidence in fish on this point, though there is evidence that DOC molecules can bind to the external surfaces of gill cells (Campbell et al., 1997). Much of the focus has been placed on the binding capabilities of DOC with regard to chelating xenobiotics and metals, thereby reducing their bioavailability and toxicity (Al-Reasi et al., 2011; Duarte et al., 2009; Glover, Pane, 2005; Hollis et al., 2001; Niyogi & Wood, 2004; Steinberg et al., 2003). The functional groups that interact with the toxicants appear to be the same groups that interact with biological systems (Al-Reasi, Wood et al., 2013), thus DOCs can act as xenobiotics themselves (Steinberg et al., 2003). Specifically, DOC exposures have been shown to provoke chemical and oxidative stress responses such as the induction of defense proteins (e.g., HSP70), and the creation of free radicals and ROS with subsequent lipid peroxidation (Steinberg et al., 2006). As discussed subsequently, there also appear to be significant beneficial effects of DOC on the physiology of aquatic organisms.

## 4 | CHEMISTRY OF RIO NEGRO BLACKWATER

The blackwaters of the Rio Negro, a major tributary of the Amazon River, are characteristically dilute yet deeply colored (Val & Almeida-Val, 1995). The soil surrounding the river is largely composed of silicate sand and due to its binding properties, has been long since been stripped of its major cations, leaving the water in this region extremely ion-poor (Benedetti et al., 2003; Seyler & Boaventura, 2003). The unique geochemistry of the headwaters and breakdown of jungle vegetation are reflected in the water composition, making Rio Negro blackwaters some of the most naturally acidic, DOC-rich, and ion-poor waters anywhere in the world (Furch, 1984; Val & Almeida-Val, 1995; Walker & Henderson, 1996). The pH is typically 4.0–6.0 but can be as low as ~3.0 (Table 1) in nearby forest streams and riverside lakes, likely due to poor buffering capacity as well as the input of decaying tropical plant material (Furch, 1984; Walker & Henderson, 1996). It is this decaying vegetation that results in a high DOC concentration, typically 5–15 mg/L and occasionally as high as 35 mg/L (Table 1) in blackwaters. In contrast, the Rio Solimoes (often called whitewater because of its silt burden from the Andes mountains) typically has only ~2.5 mg/L DOC. $Na^+$, $Cl^-$, $Mg^{2+}$, and $Ca^{2+}$ levels in the Rio Negro are usually less than 30 μmol/L (Table 1) and water conductivity less than 10 μS/L (Furch, 1984; Küchler et al., 2000; Wood et al., 2003), whereas typical levels in the Rio Solimoes are 10-fold higher or more. On average, $Na^+$, $Cl^-$, $Mg^{2+}$, and $Ca^{2+}$ concentrations within Rio Negro waters are only 6%, 22%, 3%, 1% of the mean values in global rivers (Gonzalez et al., 2006). As outlined below, the exceptionally low $Ca^{2+}$ concentration may be an especially important influence on the physiology of Rio Negro fish.

There is considerable seasonal variability between the wet (May–July) and dry (October–November) seasons in DOC and major ion concentrations. Concentrations of DOC, $Na^+$, $Mg^{2+}$, and $Cl^-$ were higher in the wet season relative to the dry whereas trends in $Ca^{2+}$, $K^+$ concentrations were variable (Duarte, 2012; Holland et al., 2017). Fluctuations in $O_2$ concentration occur between the day and night driving many species of fish to migrate laterally to the river from the flooded forest before nightfall. At night, the flooded forest $O_2$ levels may reach as low as zero whereas the running river is generally normoxic (Val, 2019).

DOC-rich waters that drain from the forest soils, rocks, and the plants growing there may have high concentrations of metals, reflecting the preferential binding of metals to humic substances (Benedetti et al., 2003; Seyler & Boaventura, 2003). Rainfall is an additional source of metals to Amazonian river systems which may partially explain the relatively high concentrations of Al, Sn, Se, Th, Rb, and Cd in the Rio Negro (Konhauser et al., 1994). Konhauser et al. (1994) reported that Al, Cd, Sn, P, and V were precipitated at more than 10-fold the concentration of the surface waters based on analysis of collected rainfall compared with the concentration of dissolved metals in the Rio Negro. In summary, the chemistry of these blackwaters is so severe that they would likely be toxic to most freshwater fish, yet the ichthyofauna of the Rio Negro watershed is one of the most biodiverse in the world and represents about 8% of the global freshwater fish species. Clearly, this biodiversity must be associated with a range of physiological adaptations to this otherwise very challenging environment, as discussed below.

## 5 | ACTIVE ION UPTAKE AND PASSIVE ION LOSS AT CIRCUMNEUTRAL AND ACIDIC PHS

Animals living in hypo-osmotic environments face the osmotic influx of water and diffusive loss of salts to the external environment (Aladin & Potts, 1995; Evans et al., 2005; Harris & Aladin, 1997; Kirschner, 2004; Larsen et al., 2014). Freshwater fish maintain ionic balance by employing active ion uptake through specialized branchial transport mechanisms, having low permeability of the body surface to ions and tight gill epithelia to limit diffusive ion loss through paracellular tight junctions (Duarte et al., 2013; Evans et al., 2005; Lockwood, 1997; Potts & Parry, 1964; Rudy, 1967). Dietary salt uptake may also be important (Wood & Bucking, 2011), especially when branchial ion transport is reduced by low environmental pH (D'Cruz & Wood, 1998). The physiological response of nonacidophilic fish to environmentally relevant acidity (e.g., pH 3.5–5.0) is generally inhibition of active $Na^+$ and $Cl^-$ uptake and elevation of passive ion loss resulting in reduced plasma $Na^+$ and $Cl^-$ levels, lowered blood volume, haemoconcentration, and elevated plasma protein concentrations (McDonald, 1983a; McDonald et al., 1980; Wood, 1989; Wood & McDonald, 1982). Mortality eventually occurs due to circulatory failure (Milligan & Wood, 1982). At extremely low pH (2.5–3.5), suffocation and rapid death are due to the breakdown of the gill structure and branchial mucus production (Daye & Garside, 1976; Ultsch & Gros, 1979). Increased ambient $Ca^{2+}$ concentrations generally mitigate the damaging effects of low pH because of the competitive binding between $Ca^{2+}$ versus $H^+$ at sites on the gills that normally maintain epithelial integrity of the tight junctions (Hunn, 1985; McDonald, 1983a; McDonald & Rogano, 1986; McWilliams, 1982; McWilliams & Potts, 1978). As discussed subsequently, elevated environmental $Ca^{2+}$ also changes the nature of the physiological response away from ionoregulatory disturbance towards acid–base disturbance (Wood, 1989).

In most "standard" freshwater teleosts (e.g., the nonacidophilic rainbow trout [*Oncorhynchus mykiss*], the acidophilic zebrafish [*Danio rerio*]), $Na^+$ uptake through the gills occurs via an electroneutral ion exchanger ($Na^+/H^+$ antiporter, NHE) which exports $H^+$ and/or an electrogenic apical V- $H^+$ ATPase which also exports $H^+$, the latter energizing $Na^+$ entry via an apical channel (Kirschner, 2004; Randall & Lin, 1993) (Figure 2a). The $Na^+$ channel may be an "acid-sensing ion channel" (Dymowska et al., 2014, 2015). Over the past 15 years, it has become clear that these mechanisms are coupled to ammonia excretion through apical Rh glycoproteins (Nawata et al., 2007; Wright & Wood, 2009, 2012), channels that facilitate $NH_3$ movement across the gill membranes (Nawata et al., 2010). By this model (Figure 2b), $NH_4^+$, the dominant form at physiological pH, is deprotonated as it enters the Rh channel, the $NH_3$ moves through the channel, and the $H^+$ is transported outwards by the V- $H^+$ ATPase or NHE, acidifying the external boundary layer, and immediately reprotonating the $NH_3$ back to $NH_4^+$, effectively trapping it in the exhalent water (Wright & Wood, 2009, 2012). The various components (NHE, V- $H^+$ ATPase, Rh glycoprotein, and carbonic anhydrase as a source of additional protons) may be organized into a functional metabolon (Ito et al., 2013). Based on assumed electrochemical gradients, it was originally thought that $Na^+$ uptake in low pH environments would rely on the V- $H^+$ ATPase/$Na^+$ channel mechanism because NHE could not accomplish $Na^+$ uptake (Avella & Bornancin, 1989; Parks et al., 2008). However, NHE appears to be quite important in $Na^+$ uptake at low pH (3.5–4.0) in several species (e.g., zebrafish—Kumai, Bahubeshi et al., 2011; Kumai & Perry, 2011; Lake Osorezan dace—Hirata et al., 2003). It has been argued that upregulation of the Rh-glycoprotein metabolon removes the assumed electrochemical constraints on NHE function, such that $Na^+$ uptake via NHE can function at low pH, and also that "active" ammonia excretion against apparent ammonia gradients can occur (Kwong et al., 2014; Wright & Wood, 2012). The exact energetics powering these mechanisms remain to be determined.

At the basolateral membrane of the gill cells, $Na^+$ is exported into the blood via a $Na^+/K^+$-ATPase contributing to the electrochemical gradient driving the inward movement of $Na^+$ across the

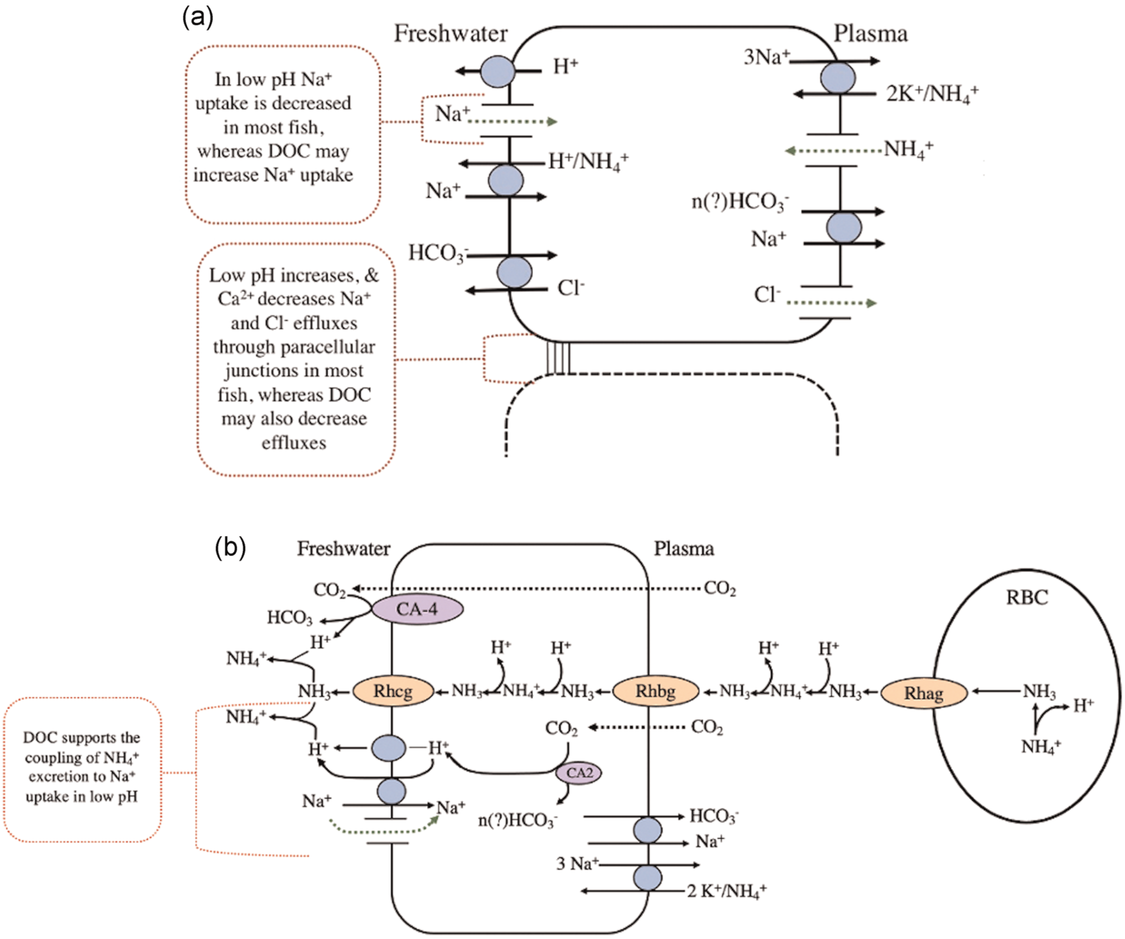


**FIGURE 2** Models of transport processes in ionocytes of the freshwater fish gill. (a) proposed model of ion uptake pathways in the freshwater fish gill epithelium noting the effects of DOC, $Ca^{2+}$, and low pH on ion transporters (b) proposed model of ammonia transport through the freshwater gill epithelium, noting the effects of DOC at low pH. DOC, dissolved organic carbon [Color figure can be viewed at wileyonlinelibrary.com]

apical membrane (Figure 2a; Evans, 2011; Kirschner, 2004). An electrogenic $Na^+$-$HCO_3^-$ cotransporter (NBC) of uncertain stoichiometry may also contribute to the basolateral export of $Na^+$ (Parks et al., 2007). In some fish, including at least one Rio Negro native, the characid cardinal tetra (*Paracheirodon axelrodi*; Wood et al., 2014), the $Na^+/K^+$-ATPase can also act as a $Na^+/NH_4^+$-ATPase with $NH_4^+$ at equal potency to $K^+$ at concentrations (1 mmol/L) that are physiologically relevant to plasma $NH_4^+$ levels, providing an alternate route for ammonia entry at the basolateral membrane (Figure 2a,b). Uptake of $Cl^-$ through the gill is coupled to $HCO_3^-$ (base) excretion via an apical $Cl^-/HCO_3^-$ exchanger (Figure 2a), but there is little knowledge of the details or on the basolateral pathways (Evans, 2011).

Many fish species native to ion-poor, acidic waters show particular ion transport mechanisms that differ from nonnative species, allowing them to thrive in these harsh environments (Gonzalez et al., 2006; Nelson, 2015; Yanagitsuru et al., 2019). This likely involves unique adaptations that contrast with the general teleost models described above (Gonzalez & Preest, 1999; Gonzalez et al., 2002). The lack of these mechanisms may explain why nonacidophilic fish do not readily acclimate to low pH (Audet et al., 1988; Audet & Wood, 1988, 1993).

Amazonian blackwaters are rich in allochthonous DOC that is the very cause of the acidity. Many of the Rio Negro fish species have an exceptionally high acid tolerance, surviving at or below pH 4.0, by avoiding imbalances in net $Na^+$ and $Cl^-$ flux rates. While the degree of tolerance to extremely dilute, low pH waters in Rio Negro natives is species-specific (Duarte et al., 2013; Gonzalez et al., 2002, 1998; Wilson et al., 1999), one common feature may be the maintenance of generally lower plasma $Na^+$ levels, a strategy to minimize energy expenditure in the low $Na^+$ waters of the Rio Negro (Mangum et al., 1977, 1978). Another may be a general resistance to the stimulation of gill diffusive ion loss caused by low pH. This stimulated efflux, associated with a leaching away of $Ca^{2+}$ ions, critical to maintaining the "tightness" of paracellular junctions, is the major cause of net ion loss and subsequent death at low pH in most fish (Wood, 1989).

There appear to be two basic ionoregulatory strategies of Rio Negro fish native to ion-poor, acidic blackwaters. A high-affinity (= low $K_m$) ion transport system that supports generally high rates of

$Na^+$ uptake is characteristic of the Order Characiformes and a low-affinity (= high $K_m$) ion transport system that achieves generally low rates of $Na^+$ uptake is characteristic of the Order Cichliformes, more specifically the Family Cichlidae (Gonzalez et al., 2018; Gonzalez, Hsu et al., 2020; Gonzalez et al., 2002, 2006). By examining representatives of these two groups that are not native to the Amazon, Gonzalez et al. (2017) concluded that the strategies were likely linked to phylogeny. While there have not been any correlations described between the two basic ionoregulatory strategies and ecophysiological traits (e.g., ion and DOC concentration, diet, or hypoxia tolerance) it is certainly an interesting area for future research.

Teleosts in the Order Characiformes, the most abundant order in Rio Negro, are generally insensitive to low pH and have high maximum transport capacity ($J_{max}$), high-affinity (= low $K_m$) ion transport systems which continue to provide high rates of unidirectional $Na^+$ uptake (i.e., influx) at low pH; rates may even increase under very acidic conditions (Duarte et al., 2013; Gonzalez et al., 2018; Gonzalez, Patrick et al., 2020; Gonzalez & Preest, 1999; Gonzalez et al., 2002; Gonzalez et al., 1997; Wood et al., 2014). Unidirectional $Na^+$ efflux rates are also quite resistant to low water pH. Indeed, ionoregulation in neon tetras (*Paracheirodon innesi*) and tambaqui (*Colossoma macropomum)* appears to be unaffected down to a water pH of 3.5 (Gonzalez et al., 2020; Gonzalez & Preest, 1999).

Notably, $Na^+$ influx rates in characid species exhibiting this strategy are also generally insensitive to most drugs that inhibit $Na^+$ transport, including those (e.g., 100 μmol/L each of amiloride, 5-N, N dimethyl amiloride, 5-N-methyl-N-isopropyl amiloride, 5-N, N-hexamethylene amiloride, benzamil, and vanadate [$Na_3VO_4$], 10–100 μmol/L of phenamil, and 50 μmol/L of 5-N-ethyl-N-isopropyl amiloride]), that are known to block the standard NHE and V- $H^+$ ATPase/$Na^+$-channel systems described earlier (Figure 2; Gonzalez & Preest, 1999; Preest et al., 2005; Wood et al., 2014). On the one hand, this could indicate that $H^+$ may not be necessary as the counter-ion for $Na^+$ uptake. On the other hand, the carbonic anhydrase inhibitor acetazolamide (100 μmol/L) reduced $Na^+$ uptake in some studies but not others (Gonzalez et al., 2018; Preest et al., 2005; Wood et al., 2014), whereas 0.06–0.10 μmol/L $AgNO_3$ (a less specific, but more potent, rapidly acting carbonic anhydrase blocker and a more slowly acting NKA inhibitor; Morgan et al., 2004) quickly eliminated both $Na^+$ and $Cl^-$ uptake in two studies (Preest et al., 2005; Wood et al., 2014). These results suggest that both processes are dependent on $H^+$ and $HCO_3^-$ provision by carbonic anhydrase. Therefore, whether $H^+$ is required as a counter-ion for $Na^+$ uptake remains unclear (Wood et al., 2014). These authors also found that branchial $Na^+$, $K^+$ ATPase could be activated by $NH_4^+$ just as effectively as by $K^+$ (Figure 2a,b). Based on this and other evidence, Wood et al. (2014) proposed that $Na^+$ uptake was dependent on ammonia excretion, but that ammonia excretion was not dependent on $Na^+$ uptake, but Gonzalez et al. (2018) argued against this interpretation. Regardless, the studies described above suggest that the characid transport mechanism appears to be unique, and does not fit current transport models.

The other ionoregulatory strategy, employed by members of another abundant teleost Order in the Rio Negro, the Cichliformes, as well as the elasmobranch Rajiformes (freshwater stingrays) is to greatly limit but not eliminate net ion loss at low pH. The diet may be used to make up the deficit. These species employ a pH-sensitive, low-affinity (= high $K_m$) ion transport system; transport capacity is variable but often low (Duarte et al., 2013; Gonzalez, Hsu et al., 2020; Gonzalez et al., 2002, 2006; Wood et al., 2002).

Recent reports indicate there may be some variability within the cichlid strategy. For example, responses to a $Na^+$ channel blocker (phenamil) varied (Gonzalez et al., 2017), as did kinetic characteristics and the degree of resistance of $Na^+$ uptake to inhibition by low pH (Gonzalez, Hsu et al., 2020; Gonzalez et al., 2017). While most Rio Negro species in the Order Cichliformes have a low-affinity ion transport system (= high $K_m$) as described above, Tapajos cichlids (*Geophagus* sp) and keyhole cichlids (*Cleithracara maronii*), both native to ion-poor water similar to that of Rio-Negro but endemic to neighboring regions, were found to have high-affinity (= low $K_m$) $Na^+$ transporters (Gonzalez, Hsu et al., 2020). The Tapajos cichlid in particular was an outlier, in being similar to members of the Order Characiformes, (but unlike keyhole cichlids), in having both a high-affinity (= low $K_m$) and high maximum transport capacity ($J_{max}$) $Na^+$ transport system. The Tapojos cichlid is the first cichlid described to display this transport pattern (Gonzalez, Hsu et al., 2020).

Overall a low-affinity system is more energetically economical, compared to a high-affinity, high-capacity system so long as efflux remains low (Gonzalez & Dunson, 1987). Indeed, in Amazonian species showing this strategy, efflux rates are extremely resistant to stimulation by low pH, which may be a unique adaptation (Duarte et al., 2013; Gonzalez et al., 2002, 2006; Wood et al., 2003). As outlined below, DOC may play a role. The $Na^+$ and $Cl^-$ uptake systems in this group have been pharmacologically characterized with a wide range of antagonists only in the freshwater stingrays (*Potamotrygonidae* sp.), where they appear to be similar to those of standard freshwater teleosts (Wood et al., 2002). At low pH, $Na^+$ uptake in angelfish (*Pterophyllum scalare)* was inhibited both competitively and noncompetitively (Duarte et al., 2013). Non-competitive inhibition may be due to the observed acute reduction in N-ethylmaleimide (NEM)-sensitive branchial ATPase activity, but not in $Na^+/K^+$-ATPase activity (Duarte et al., 2013). The specificity of NEM has been challenged (Forgac, 1989), but Nawata et al. (2007) directly tested the same NEM concentration against 10 μM bafilomycin on trout gill, and found the same results with the two inhibitors. This has been interpreted as evidence for the involvement of $H^+$-ATPase; however, NEM is not a specific inhibitor of this enzyme so other ATPases cannot be ruled out.

## 6 | THE PROTECTIVE AND SUPPORTIVE EFFECTS OF NATURAL DOC ON IONOREGULATION

DOC can alter or stabilize biological membranes such as the gills and appears to be particularly effective in this regard at low pH, thereby mitigating the negative impacts of ambient acidity (Freda et al., 1989;

Gonzalez et al., 1998; Karns, 1983; Nelson, 2015; Wood et al., 2011). Given the complexity of the composition of DOC, it is extremely difficult to determine the mechanism(s) of these interactions. However as noted earlier, allochthonous, darkly colored DOCs with a high aromatic content of humic substances and high PBI and $SAC_{340}$ values seem to exert the greatest protective effects on ionoregulation (Al-Reasi, Wood et al., 2013; Wood et al., 2011). Studies to date have suggested that paracellular tight junctions, apical ion transporters, and basolateral $Na^+/K^+$-ATPase may be the direct or indirect targets of these DOC effects (Gonzalez et al., 2006).

With respect to paracellular junctions, the presence of DOC (6–15.5 mg/L) has been shown to reduce the efflux rates of $Na^+$ and $Cl^-$ across the gills in a variety of species, both at circumneutral and acidic pHs, but often with more pronounced effects in the latter (Al-Reasi et al., 2016; Duarte et al., 2016, 2018; Gonzalez et al., 2002, 2006; Wood et al., 2002, 2003). In freshwater stingrays, the protective effects of the natural DOC of Rio Negro blackwater at pH 4.0 were mimicked by a 10-fold increase in environmental $Ca^{2+}$ concentration (in the absence of DOC), suggesting that DOC had the same "tightening" actions on the paracellular junctions as those of $Ca^{2+}$ discussed earlier (Wood et al., 2003). Thus, the normal displacement of $Ca^{2+}$ from tight junctions by high $H^+$ concentration may be prevented by DOC, or DOC may somehow replace $Ca^{2+}$. However, in some other native Rio Negro species, $Ca^{2+}$ was not protective, suggesting that other mechanisms must occur in other species (Gonzalez & Preest, 1999; Gonzalez et al., 1998). As discussed below, the hyperpolarizing effects of DOC on the TEP across the gills (Figure 3; Galvez et al., 2008) also suggest action on the paracellular pathway, but one that is different from the depolarizing effect of $Ca^{2+}$ (McWilliams & Potts, 1978; Wood et al., 1998).

In addition to reducing ion loss, DOC may minimize or reverse the inhibitory effects of low pH on active ion uptake, possibly by acting on apical and basolateral membranes. A consistent theme appears to be an increase in maximum transport rate ($J_{max}$) for $Na^+$, as well as for $Cl^-$ and $Ca^{2+}$, as revealed by concentration–kinetic analysis (Al-Reasi et al., 2016; Glover, Pane, 2005; Glover & Wood, 2005; Matsuo et al., 2004, 2005; Wood et al., 2003). This stimulation of $J_{max}$ could be explained by the increased total gill $Na^+$, $K^+$-ATPase activity, which could secondarily drive $Cl^-$ uptake. McGeer et al. (2002) reported that chronic exposure of trout to elevated DOC resulted in greater branchial $Na^+/K^+$-ATPase activity, and a similar phenomenon was recently reported in zebrafish (Sadauskas-Henrique et al., 2021). It could also be explained by a higher abundance or more efficient functioning of apical transport sites. For example, if DOC actually binds to the gill surface, it might increase the local concentration of ions such as $Na^+$, $Ca^{2+}$, and $Cl^-$ available to apical transporters as has been proposed for gill and body mucus (Handy, 1989; Handy, Eddy, et al., 1989). DOC may also act as a buffer in the external microenvironment of the gills so as to minimize the inhibitory effects of low pH on apical $Na^+$ uptake transporters (Al-Reasi et al., 2016; Wood et al., 2003). Finally, in a related explanation to be discussed in a subsequent section, through its ability to facilitate ammonia excretion, DOC might improve the local electrochemical gradient for $Na^+$ uptake, thereby promoting the coupling of $Na^+$ influx to ammonia excretion.

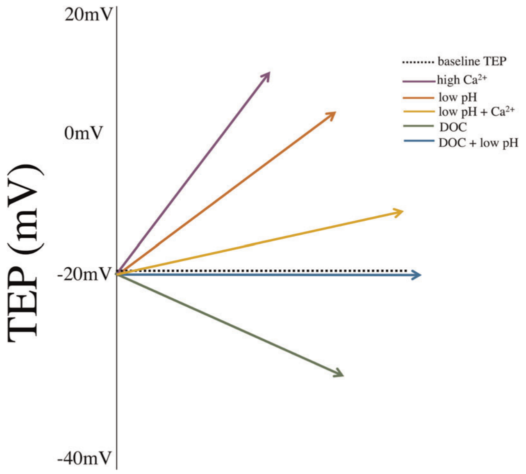


**FIGURE 3** The interactive effects of dissolved organic carbon (DOC), pH, and $Ca^{2+}$ on gill transepithelial potential (TEP) in freshwater fish [Color figure can be viewed at wileyonlinelibrary.com]

Most of the above explanations assume that DOC directly interacts and binds with gill membranes (Figure 4). Physiological evidence supporting this idea is that protective effects on ionoregulation appear to increase with prior acclimation to DOC (Matsuo et al., 2005). In zebrafish (*D. rerio*), after 7 days of pre-exposure to natural allochthonous DOCs, the protective effects on $Na^+$ influx and efflux during low pH exposure persisted even when the DOC was removed during the 3-h period of low pH challenge (Duarte et al., 2016, 2018). Furthermore, the protective effects of high DOCs against copper (Cu) and cadmium (Cd) binding to the gills of tambaqui were consistent with the effects of high $Ca^{2+}$ levels, and in both cases persisted even when DOC or $Ca^{2+}$ was not present during the metal exposures (Matsuo et al., 2005).

DOC molecules generally bear net negative charge at circumneutral pH, so a priori, it is not intuitive how they interact with the freshwater fish gill. The latter is usually considered to bear net negative charge, explaining its high binding affinity for metallic cations (Reid & McDonald, 1991). However, DOC molecules are also generally amphiphilic, with hydrophilic and hydrophobic components (Figure 1). Hydrophobic groups contribute to lipophilicity, and these parts of the molecule could conceivably bind to or dissolve in the lipoprotein gill cell membranes. Al-Reasi et al. (2011) found no relationship between DOC lipophilicity and the ability of DOC to bind to and detoxify cationic metals. This is perhaps not surprising, because metal binding would occur at anionic (hydrophilic) sites. However, at low pH, where DOC molecules are most effective in supporting ionoregulation, much of the negative charge on both the gills and the DOC molecules will be removed by $H^+$ titration, resulting in ligands that on an overall basis would be more hydrophobic and lipophilic, thereby promoting interaction. Campbell et al. (1997) demonstrated by transmission electron microscopy that DOC

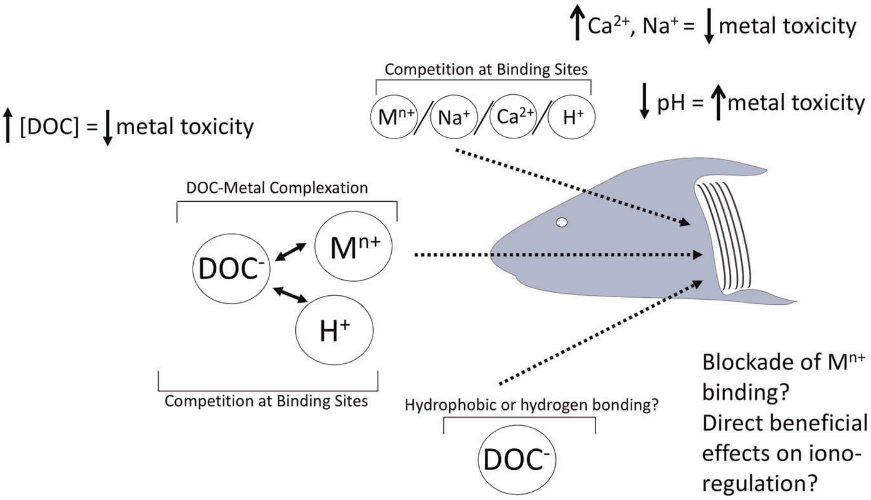


**FIGURE 4** A conceptual model of the interactive effects of DOC, low pH, and water $Ca^{2+}$ and $Na^+$ concentrations with respect to ionoregulation and metal toxicity at the gills of freshwater fish. $M^{n+}$ represents metallic cations. DOC, dissolved organic carbon [Color figure can be viewed at wileyonlinelibrary.com]

molecules can bind directly to the surface of living membranes of algae, and proposed that the same would occur with fish gill cells. They presented electrophysical evidence that this phenomenon increased at ambient low pH (4.0 vs. 7.0) in both fish gills and algal cells. These workers proposed that binding was either a hydrogen-bonding sorption mechanism, whereby electronegative functional groups of DOC form hydrogen bonds with the biological surfaces or due to the formation of hydrophobic bonds between the hydrophobic domain of DOC and the plasma membrane (Figure 4). The direct binding of DOC molecules may alter the total charge or stabilize tight junctions in the paracellular channels of the gills which in turn may alter the permeability ratio between $Na^+$ and $Cl^-$, thereby altering the TEP (Galvez et al., 2008). Alternately or additionally, the hydrophobic domain of DOC could indirectly alter the activity of $Na^+$ transport sites by changing the fluidity of the lipoprotein bilayers in the transcellular pathway (Wood et al 2011). Indeed, Vigneault et al. (2000) demonstrated that DOC could alter the permeability of algal cell membranes in a pH-dependent fashion.

Finally, as noted earlier for commercial humic acid, not all highly allochthonous, highly aromatic DOCs are necessarily protective, and indeed even natural DOCs may lose their protectiveness during storage. A highly colored DOC, freshly., collected and isolated from the upper Rio Negro at São Gabriel da Cachoeira (SGC), with a very high $SAC_{340}$, was extremely protective against ionoregulatory disturbance in nonnative zebrafish caused by acute low environmental pH exposure (Duarte et al., 2016). However, after 2 years of refrigerator storage, it had lost about half of its color as indicated by the decline in $SAC_{340,}$ and the mean MW had also declined, though the relative distribution (quantified by PARAFAC) of humic acid-, fulvic acid-, and amino acid-like components remained unchanged. When tested at this time, SGC DOC exacerbated rather than protected against ionoregulatory disturbance in a native species, the tambaqui, even though it still exerted hyperpolarizing effects on TEP (Sadauskas-Henrique et al., 2019). Unfortunately, species differences and the potential degradation of the DOC confounded interpretation in that investigation. However, in a very recent study, Sadauskas-Henrique et al. (2021), reported that this stored SGC DOC, now even older, did not protect against (and indeed exacerbated) ionoregulatory disturbance in zebrafish acutely exposed to pH 4.0. This result, now on the same species, strongly suggests that some or all the protective effects of DOC on ionoregulation may be lost during long-term storage, even if other effects (e.g., stimulation of $Na^+/K^+$-ATPase activity, hyperpolarization of TEP, positive effects on ammonia excretion) persist.

## 7 | ACID–BASE BALANCE AT CIRCUMNEUTRAL AND ACIDIC pHs

In "standard" freshwater fish, ventilatory control of acid–base status is minimal, so acid–base homeostasis between the external environment and internal extracellular fluid is maintained mainly by the branchial exchange of acid–base equivalents for $Na^+$ or $Cl^-$ respectively, thus linking acid–base balance to ion transport (Goss & Wood, 1990; Wood, 1991). Acid–base exchange and ion uptake occur simultaneously through the gills where $H^+$ ("acid") is exported in exchange for $Na^+$ and $HCO_3^-$ ("base") is exported in exchange for $Cl^-$, as described above (Figure 2). In addition, differential rates of net $Na^+$ versus net $Cl^-$ loss, or net $Na^+$ versus net $Cl^-$ gain, will constrain net acid or base fluxes. This is because overall acid–base balance must conform to the constraints of electrical neutrality, as recognized by strong ion difference (SID) theory (Stewart, 1983). In simple terms, if net fluxes of the major strong cation (i.e., $Na^+$) and strong anion (i.e., $Cl^-$) are equal, there can be no net acid–base flux. If net $Na^+$ loss exceeds net $Cl^-$ loss, net acid uptake (= base loss) must occur. If net $Cl^-$ loss exceeds net $Na^+$ loss, net acid excretion (= base gain) must occur. The diversity in ionoregulatory mechanisms described above may be reflective of acid–base demands due to the activity and habitat of a particular species. Increased activity as well as inhabiting anoxic or acidic waters disturbs internal acid–base balance and requires greater ion transport capacity to restore balance. Species that inhabit moderate environments (i.e., circumneutral pH) do not have as large an environmental threat to acid–base status and can afford to have lower rates of acid–base relevant ion flux (Gonzalez et al., 2002).

As reviewed by Wood and McDonald (1982) and Wood (1989), during the acid-rain crisis of the 1970s–1980s in the northern hemisphere, blood acidosis was initially thought to be an important part of the toxic response in fish. Studies on rainbow trout demonstrated that net acid uptake occurred at the gills due to differential inhibition of $Na^+$ and $Cl^-$ uptake and stimulation of efflux processes, such that net $Na^+$ loss exceeded net $Cl^-$ losses. Blood pH dropped during low pH exposure, and the trout were only able to partially compensate by increasing net acid excretion in the urine via the kidney (McDonald, 1983a, 1983b; McDonald et al., 1980, 1983; McDonald & Wood, 1981; Wood & McDonald, 1982). However, it later became apparent that this clear acid–base response occurred only when the fish were acclimated to relatively hard water, high in $Ca^{2+}$ concentration (mmol/L range). When experiments were performed in soft water with low $Ca^{2+}$ levels (<250 μmol/L range), $Na^+$ and $Cl^-$ losses were even greater, but there was little uptake of acid at the gills and blood acid–base disturbance was negligible, because $Na^+$ and $Cl^-$ losses were approximately equal, in accord with SID theory (Stewart, 1983). The low water $Ca^{2+}$ situation seems to be the environmentally relevant one in nature, because these waters are generally also low in alkalinity and buffer capacity, and are therefore the ones that are easily acidified. Certainly, this is the situation in most Amazonian waters ($Ca^{2+}$ <250 μmol/L), and particularly in the Rio Negro watershed (Table 1) where, as noted above, $Ca^{2+}$ may be less than 10 μmol/L (Val & Almeida-Val, 1995).

There appear to be few investigations of the acid–base responses of native fish to low pH exposure under natural soft water conditions. A study by Wilson et al. (1999) demonstrated that net acid fluxes were altered in only one of the three tested freshwater Rio Negro native fish species upon exposure to low pH (3.5–5.0). Net acid fluxes were positive (acid uptake or base excretion) in all three species under control conditions (pH 6.0); however, when exposed to low pH, net acid uptake decreased in matrinchã (*Brycon erythropterus*), and did not change in either tamoatá (*Hoplosternum littorale*) (which died) or tambaqui (which survived), though all three suffered ion loss to the water, which was greatest in tamoatá and least in tambaqui. In all three, the net acid fluxes were relatively small. A parallel study on tambaqui revealed only a small disturbance of internal acid–base status, plasma ions, and associated blood parameters down to a water pH of 4.0, with some changes at pH 3.0. The physiological pH limit of tambaqui appears to be 3.0–3.5 at which point permanent damage occurs to ionoregulatory mechanisms (Wood et al., 1998; Gonzalez et al., 2020). Therefore, this species is 10–100-fold more $H^+$-tolerant than the rainbow trout, but still appears to conform to the same pattern of negligible acid–base disturbance in low $Ca^{2+}$ waters. Unfortunately, all of these studies were performed in soft ground waters almost devoid of DOC, and there is a need to repeat them in natural soft water high in natural allochthonous DOC.

## 8 | ELECTRICAL PROPERTIES OF THE GILL (TEP) AT CIRCUMNEUTRAL AND ACIDIC PHS

TEP across freshwater fish gills is almost entirely a diffusion potential between the external medium and the blood plasma (Potts, 1984). While some authors have suggested that an electrogenic component may also contribute to TEP (e.g., Evans, 1980), there is little evidence supporting this (Kirschner, 2004; Po & Wood, 2021; Wood et al., 2020). At circumneutral pH this diffusion potential arises predominantly from the differential permeability of the gills to $Na^+$versus $Cl^-$, with ion movements generally believed to occur via the paracellular pathway. The resulting TEP is inside negative as the $Na^+$ permeability is higher than that of $Cl^-$ (Potts, 1984). Transepithelial diffusion is dependent on the concentration gradient, TEP, branchial surface area, and epithelial permeabilities to the various ions (Gonzalez et al., 2002). Therefore, knowledge of fish gill electrophysiology is essential in the interpretation of ion movement across the gills, as has become apparent from recent studies on salt toxicity (Po & Wood, 2021; Wood et al., 2020). DOC, much like other water quality parameters (e.g., hardness, pH and salinity; Potts, 1984) may have a large impact on the electrical properties of the gill (Galvez et al., 2008; Sadauskas-Henrique et al., 2019).

Wood et al. (1998) demonstrated that TEP in tambaqui increased from negative values in pH 6.5 water to positive values in pH 4.0 water. This depolarization in response to relatively low pH was similar to that observed earlier in brown trout and rainbow trout (McWilliams & Potts, 1978; Ye et al., 1991). Elevated ambient $Ca^{2+}$ concentration at circumneutral pH shifted TEP to become more positive in tambaqui and brown trout but slightly moderated the hyperpolarizing effect of low pH exposure (McWilliams & Potts, 1978; Wood et al., 1998). Thus, as discussed earlier, while both DOC and $Ca^{2+}$ may help to reduce the ion permeability of the gill, especially at low pH, they have opposite effects on TEP (Figure 3), suggesting dissimilar mechanisms of action.

DOC appears to directly influence gill TEP in rainbow trout both in vitro and in vivo (Galvez et al., 2008). Exposure of cultured gill epithelia from rainbow trout in vitro at circumneutral pH to various DOCs collected in Ontario, Canada resulted in hyperpolarization (Galvez et al., 2008). $Ca^{2+}$ activity measurements eliminated the removal of $Ca^{2+}$ from solution by DOC-complexation as a possible indirect cause of the hyperpolarization. The magnitude of this hyperpolarization was strongly correlated to the $SAC_{340}$ values of the various DOCs, suggesting that the aromaticity of the molecules directly influences the electrophysiology of the fish gill (Galvez et al., 2008). Allochthonous isolates had large effects, while autochthonous isolates had only small effects. This study showed that similar, but slightly reduced hyperpolarization also occurred in response to DOC exposures in vivo (Galvez et al., 2008). These effects were reversible and likewise correlated to the aromaticity of the DOC molecules. Galvez et al. (2008) offered two explanations for the attenuated hyperpolarization reported in vivo. DOC may have affected the paracellular permeability of the cultured epithelia to a greater extent compared to the whole animal, or diffusive permeability was counteracted by the electrogenic uptake of ions initiated by DOC in vivo (Galvez et al., 2008). To date, there are no data to support the latter possibility. Nevertheless, the more negative internal TEP would certainly favor the uptake of all cations, including $Na^+$. Regardless, differentially altered membrane permeability to $Na^+$ versus $Cl^-$ upon exposure to DOC may be due to the abundance of charged functional groups in DOC that may influence the electrical properties of the paracellular pathway, or the entire gill epithelium (Campbell et al., 1997; Galvez et al., 2008).

While both low pH and DOC individually alter TEP, DOC may offer protection over changes in the electrical properties of the gill in response to low pH. Consistent with previously described TEP results in salmonids at low pH (McWilliams & Potts, 1978; Ye et al., 1991), exposure of tambaqui to low pH caused depolarization (Sadauskas-Henrique et al., 2019; Wood et al., 1998). However, exposure to DOC from SGC at circumneutral pH caused hyperpolarization, and the DOC completely mitigated the depolarization of TEP at low pH (Sadauskas-Henrique et al., 2019). This occurred despite the inability of this DOC to protect against net ion loss at low pH, perhaps because it had degraded during storage, as discussed earlier. Mean measured losses of $Na^+$ and $Cl^-$ were about equal, in accord with no net acid–base disturbance, as would be expected in the low $[Ca^{2+}]$ water in which the study was performed. It appears as though the discrepancy of relative permeabilities of $Na^+$ and $Cl^-$ seen at circumneutral pH is diminished in low pH as indicated by depolarization in acidic water (Sadauskas-Henrique et al., 2019).

## 9 | AMMONIA TRANSPORT

Ammonia, derived from the degradation of proteins, nucleic acids, purines, and pyrimidines, is the primary waste product of nitrogen metabolism in teleost fish (Wilkie, 1997; Wood, 1993). Ammonia production is less metabolically demanding than urea and is promptly excreted via the gills to prevent toxicity. Overall, Amazonian fish native to acidic waters are more sensitive to ammonia toxicity than "standard" fish endemic to circumneutral waters in North America, when compared at a common pH 7.0 (Souza-Bastos et al., 2017). In other words, toxicity occurs at lower total ammonia and lower $NH_3$ concentrations. This may be because Amazonian species both evolved and presently live at low pH, where the $NH_3$ fraction is lower. Given that $NH_3$ is the more toxic form of ammonia relative to $NH_4^+$ and is the form that diffuses into the fish across the gills, thereby setting the blood levels (Chew et al., 2006; Randall & Tsui, 2002; Walsh et al., 2007), and Amazonian species have had less exposure to $NH_3$, they are in turn less resistant to it.

While ammonia transport across the gills has been extensively investigated, the specific mechanisms involved have been historically controversial (Wilkie, 1997; Wood, 1993; Wright & Wood, 2009). Our understanding was revolutionized by the relatively recent discovery of Rh glycoproteins in the gills and their involvement in facilitating ammonia excretion (Nakada et al., 2007; Nawata et al., 2007, 2010). This "Rh-mediated $Na^+/NH_4^+$ exchange" model has been reviewed by Wright and Wood (2009, 2012), where apical transport has been described as a metabolon involving several membrane transporters (apical Rhcg, V-type $H^+$- ATPase, $Na^+/H^+$ exchanger NHE-2 and/or NHE-3, $Na^+$ channel, carbonic anhydrase) creating a $Na^+/NH_4^+$ exchange complex which accounts for the variable linkage of $Na^+$ uptake and net ammonia efflux. The overall model (Figure 2b) suggests that Rhag moves $NH_3$ out of the erythrocyte into the plasma, and Rhbg facilitates the transit of $NH_3$ across the basolateral membrane of the branchial ionocyte. Then the multiple membrane transporters (including Rhcg) working as an apical $Na^+/NH_4^+$ exchange complex provides an acid trapping mechanism for apical export, as described earlier. The already complex mechanism(s) of ammonia excretion in freshwater fish is certainly influenced by low ambient pH and elevated DOC concentrations. To our knowledge, there have been no molecular investigations of the "Rh-mediated $Na^+/NH_4^+$" exchange complex in fish endemic to acidic, DOC-rich tropical waters, but as outlined below, some physiological investigations cast light on this issue.

In the oscar (*Astronotus ocellatus*), a member of the Cichliformes that would likely have the "standard" $Na^+$ uptake systems, $Na^+$ influx and ammonia excretion appeared to be coupled under normoxic conditions at circumneutral pH, but this coupling disappeared under acute hypoxia (Wood et al., 2007). The tambaqui, a member of the Characiformes that exhibits the unique $H^+$-resistant $Na^+$ uptake system, was able to maintain ammonia excretion against a high external ammonia concentration (2.5 mmol/L), but it remains unclear whether the observed ammonia excretion was active (Wood et al., 2017). These findings are compatible with the "Rh-mediated $Na^+/NH_4^+$" model. However, based on pharmacological tests on three endemic members ("tetras") of the Characiformes, in which ammonia excretion appeared to be independent of $Na^+$-uptake and persisted in its absence (Wood et al., 2014), it was suggested that the model did not apply. High ambient $NH_4^+$ resulted in increased $Na^+$ influx in the cardinal tetra (Wood et al., 2014) and the black neon tetra, as well as in an African characiform, the Congo tetra, *Phenacogrammus interruptus* (Gonzalez et al., 2017), in contrast to the general inhibition of $Na^+$ uptake caused by this treatment in cichlids, including the oscar (Gonzalez, Hsu et al., 2020; Gonzalez et al., 2017). However, not all species of Characiformes showed an increase in $Na^+$ uptake in HEA, in fact most tetras showed no stimulation, including the black neon tetra, *Hyphessobrycon herbertaxelrodi*, (Gonzalez et al., 2018, 2017). Based on an earlier model originally proposed by Randall et al. (1996), Wood et al. (2014) proposed that $Na^+$ uptake was nevertheless dependent on ammonia excretion in the cardinal tetra, driven by active $NH_4^+$ entry via a competition on the $K^+$ site of basolateral $Na^+/K^+$-ATPase, where it was equally or more effective than $K^+$ in enzyme activation (Figure 2a,b).

With respect to responses to low environmental pH, Wood et al. (1998) demonstrated that internal total ammonia levels in tambaqui remained constant upon exposure to values as low as pH 4.0 in very soft water, but doubled from control levels at pH 3.0. Recovery was incomplete when water pH was returned to 6.5. This trend was likewise described in a parallel study by Wilson et al. (1999) in tambaqui as well as two other Amazonian teleosts, matrinchã and tamoatá: only modest changes in branchial ammonia excretion occurred down to pH 4.0, but marked stimulation at pH 3.5. The mirrored response of plasma cortisol in tambaqui was suggested to be an important contributor to elevated plasma ammonia in low pH due to the known effect of cortisol in promoting proteolysis (Wood et al., 1998). A later study confirmed unchanged ammonia excretion down to pH 4.0 in this species (Wood et al., 2018). However, all the above-mentioned investigations (except Wood et al., 2014) were performed in ion-poor water essentially lacking DOC.

The presence of DOC influences the ammonia excretion response of fish to low pH exposure. Sadauskas-Henrique et al. (2019) found increased ammonia excretion at pH 4.0 in tambaqui when SGC DOC was present, whereas there was no change in the absence of DOC. A similar response with SGC DOC was seen in zebrafish (Sadauskas-Henrique et al., 2021), even though both studies used long-term stored DOC. Ammonia excretion was similarly increased by 60% in stingrays exposed to pH 4.0 in fresh DOC-rich natural Rio Negro water, but not in matching reference water lacking DOC (Wood et al., 2003). Amiloride and amiloride analogs inhibited both $Na^+$ uptake and ammonia excretion in the stingrays in the DOC-rich water at circumneutral pH (Wood et al., 2002). These stingray results, before the discovery of the “Rh-mediated $Na^+/NH_4^+$ exchange” model (Wright & Wood, 2009), were interpreted as inhibition of “diffusion trapping” of $NH_3$ in the gill boundary layer caused by the blockade of $Na^+$-coupled $H^+$ efflux. In fact, they fit the model well. DOC would act by partially protecting $Na^+$ influx in low ambient pH so it may also influence $H^+$ extrusion and in turn lead to elevated ammonia excretion (Wood et al., 2003), though other explanations are possible. Freshly collected Rio Negro SGC DOC also appeared to maintain $Na^+$ coupling to ammonia excretion in zebrafish exposed to low pH in ion-poor water suggesting that DOC supports this coupling via the Rh metabolon (Duarte et al., 2016). Increased ammonia excretion can therefore drive elevated $Na^+$ uptake in acidic water (Duarte et al., 2016). Another allochthonous DOC from Luther Marsh provided similar protection to zebrafish at low pH (Duarte et al., 2018), and in both studies this persisted after prior acclimation to DOC, even if the DOC was removed during the period of low pH challenge. This again suggests that the binding of the amphiphilic DOC molecules to the gill surfaces (Figure 4) influences ionocyte physiology, including the persistence of the coupling of $Na^+$ uptake to ammonia excretion at low pH. Interestingly in daphnia, natural DOCs had exactly the opposite effect, reducing ammonia excretion at low pH without affecting $Na^+$ flux rates (Al-Reasi, Yusuf et al., 2013).

## 10 | AMELIORATION OF METAL TOXICITY

Increasing pollution by metals is one of the many anthropogenic pressures currently threatening the entire Amazon watershed (Duarte & Val, 2020). In “non-polluted” Rio Negro waters, the concentrations of major metals are low (e.g., 0–2 ug/L; Holland et al., 2017; Wood et al., 2003), generally below the levels used in toxicity studies (10-fold or more greater), but the situation is different where there are anthropogenic inputs. For example, Pinto et al. (2008) reported waterborne Cu and Cd concentrations of 10–50 and 8–90 μg/L, respectively in several tributaries in “natural areas” of the Rio Negro in the region of Manaus. In areas with heavy industrial inputs, the measured water Cu was up to 2100 μg/L and Cd was up to 590 μg/L (Geissler, 1999; Pinto et al., 2009, 2008). All of these elevated concentrations are in the range of potential acute toxicity.

DOC is a well-recognized mitigating factor of metal toxicity, however, the mechanistic details of its protective nature remain poorly understood because of the structural irregularity, heterogeneity, and complexity of DOC (Al-Reasi et al., 2011). Nevertheless, as noted earlier, there is a general relationship whereby DOCs with high $SAC_{340}$ values also generally have high PBI values, and both of these indices correlate with a high protective ability against metal toxicity, as well as a high capacity to exert direct biological effects on organisms (Al-Reasi, Wood et al., 2013). The latter is important because it has been argued that only part of the protective actions of DOC against metal toxicity is explained by its ability to complex metal cations so as to reduce their bioavailability; the other part is due to the direct beneficial effects of DOC on ionoregulation due to its actions on gill membranes as discussed earlier (Crémazy et al., 2016; Wood et al., 2011; Figure 4). DOC with high $SAC_{340}$ and PBI values are typically darkly colored, highly aromatic, allochthonous DOCs with high humic acid content. Rio Negro DOCs, especially those from the upper part of the river such as SGC samples (Duarte et al., 2016) have these characteristics.

Metal toxicity is alleviated by DOC in a concentration-dependent manner (e.g., Crémazy et al., 2017) as DOC decreases the bioavailability of metal cations by chelating, sequestering, and thereby altering their speciation (Figure 4; Al-Reasi et al., 2011; Hollis et al., 2001; Matsuo et al., 2004; Playle et al., 1993). In accord with their lower PBI and $SAC_{340}$ values (Al-Reasi, Wood et al., 2013; Duarte et al., 2016), autochthonous DOCs are less effective against metal toxicity than terrigenous (allochthonous) DOCs (Luider et al., 2004; Richards et al., 2001; Schwartz et al., 2004). This is true for copper (Cu) and lead (Pb) toxicity and may influence the toxicity of silver (Ag), cadmium (Cd), and inorganic mercury (Hg), but there are insufficient data to draw definitive correlations between these latter metals and the effect of DOC with regard to quality and source (Al-Reasi et al., 2011).

Most cationic metals act to disrupt ionoregulatory processes at the gills (Niyogi & Wood, 2004; Wood, 2001). Therefore, very soft, acidic ion-poor waters such as Rio Negro blackwaters, are expected to exacerbate metal toxicity as there is a low concentration of ions for uptake, including a low concentration of protective cations (e.g., $Ca^{2+}$) to compete with metals for important binding sites on fish gills such as those that regulate tight junction permeability. Additionally, the low pH ensures that dissolved metals will occur mainly as free cations, generally considered to be the most toxic aqueous species, and will tend to liberate metallic cations ($M^{n+}$) from DOC by

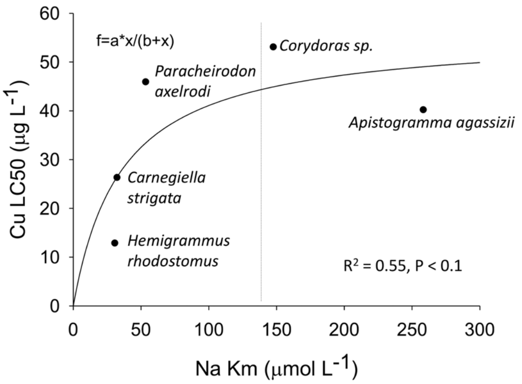


**FIGURE 5** The relationship between the affinity ($K_m$ values from Gonzalez et al., 2002) for $Na^+$ uptake and the toxicity of Cu (96-h $LC_{50}$ values from Duarte et al., 2009) in five species of Rio Negro fish. The species to the left of the line are members of the Characiformes, whereas Corydoras is a catfish, and Apistogramma is a cichlid

$H^+$ competition (Figure 4). In Amazonian fish, residing in extremely soft water, responses to only three metals have been studied in detail so far—copper (Cu), cadmium (Cd), and nickel (Ni). A fourth, Ag, has been used pharmacologically as a very effective inhibitor of $Na^+$ and $Cl^-$ uptake in both characid and cichlid fishes (Gonzalez, Hsu et al., 2020; Preest et al., 2005; Wood et al., 2014), probably reflecting its ability to block both carbonic anhydrase quickly and $Na^+$, $K^+$ ATPase more slowly (Morgan et al., 2004).

In most fish, Cu increases diffusive permeability of the gills, thereby elevating ion efflux rates, inhibits active ion uptake, and decreases the density of mitochondrial-rich cells within the gills causing ionoregulatory disruption leading to toxicity; $Na^+$ regulation is particularly affected (Lauren & McDonald, 1985). The eventual cause of death appears to be circulatory failure induced by high net losses of ions, similar to that caused by low pH (Wood, 2001). Additionally, at sublethal levels in soft water, Cu is a potent inducer of oxidative stress (Craig et al., 2007). In accord with expectation, Cu toxicity was found to be generally high (i.e., very low $LC_{50}$ values) in 10 native ornamental species tested in a typically ion-poor Amazonian softwater, and this was alleviated when $Na^+$ and $Ca^{2+}$ levels were raised (Duarte et al., 2009; Figure 4). Characiformes were the most sensitive to Cu, suggesting that the unique nature of their $Na^+$ uptake mechanisms could be a contributory factor. In a very recent study (Duarte et al., 2021), Characiformes were again the most sensitive Order to Cu, relative to Cichliformes and Siluriformes (catfish). Therefore, in Figure 5 we have plotted the affinity ($K_m$) for $Na^+$ uptake determined by Gonzalez et al. (2002) against the $LC_{50}$ values reported by Duarte et al. (2009) for five of the same species. There appears to be at least a weak positive hyperbolic relationship ($p < .10$) in which the Characiformes with the unique $Na^+$ uptake system with high affinity (low $K_m$) tend to exhibit greater sensitivity to Cu. Perhaps greater affinity for $Na^+$ translates to a greater affinity for $Cu^{2+}$, as the two cations may share an uptake mechanism (Grosell & Wood, 2002). There were no significant relationships with $J_{max}$ or efflux rates of $Na^+$. Interestingly, when exposed to the same relatively low concentrations of Cu (~20 µg/L = half of the $LC_{50}$s), oxidative stress dominated in the dwarf cichlid (*Apistogramma agassizii*), a member of the Cichliformes (Braz-Mota et al., 2018), whereas ionoregulatory disruption dominated in the cardinal tetra (*P. axelrodi*), a member of the Characiformes (Braz-Mota et al., 2018; Cremazy et al., 2016). Interestingly, $Na^+$ uptake in the oscar, another cichlid with a relatively high $K_m$ (Wood et al., 2007), was also very resistant to inhibition by Cu (Gonzalez, Hsu et al., 2020).

However, it should be noted that another member of the Characiformes, the tambaqui, appeared to be extremely tolerant of Cu with $LC_{50}$s 10–100-fold higher than those in Figure 5 (Tavares-Dias et al., 2011; Oliveira, 2003). In accord with this high Cu tolerance, very high levels (up to 400 µg/L) had only modest effects to either inhibit $Na^+$ uptake or stimulate $Na^+$ efflux in the tambaqui (Matsuo et al., 2005). In this same study, prior acclimation to either added $Ca^{2+}$ or added “foreign” DOC before Cu exposure was effective at decreasing Cu accumulation at the gills, even when these agents were no longer present during the Cu exposure. Taylor et al. (2002) suggested that both the number of binding sites and binding affinity for Cu on the gills are regulated by $Ca^{2+}$ acclimation. DOC may have similar effects on the binding properties of the gill. High levels of dietary Cu also had negligible effects on the physiology of the tambaqui (Giacomin et al., 2018), again indicating that this species is unusually tolerant to Cu.

One complication with all the above cited Cu tests is that they were performed in Amazonian groundwater with an ionic composition very similar to that of Rio Negro blackwaters, but essentially devoid of native DOC. More recent tests with some of these same species have demonstrated that the presence of Rio Negro DOC at natural levels greatly ameliorated toxicity, raising $LC_{50}$ levels up to 20-fold (Cremazy et al., 2016; Duarte et al., 2021). Indeed, Duarte et al. (2021) concluded that DOC concentration was by far the most important component of water chemistry protecting against Cu toxicity in multiple Rio Negro species. In the case of cardinal tetra, DOC greatly ameliorated ionoregulatory disruption (Cremazy et al., 2016). A detailed analysis in the cardinal tetra indicated that only part of the protective effect of Rio Negro DOC could be attributed to the complexation of Cu by DOC, with the other part attributed to the direct beneficial effects of DOC on the ionoregulatory physiology of the gills (Cremazy et al., 2016; Figure 4). In this same species, the presence of suspended particles, as would occur when whitewater flows into blackwater, also greatly alleviated toxicity by complexation of Cu, though the particles themselves were mildly disruptive to ionoregulation, perhaps by physical damage to the gills (Crémazy et al., 2019).

In most fish, Cd disrupts calcium homeostasis, specifically by blocking the active uptake of $Ca^{2+}$ at the gills (Niyogi & Wood, 2004; Wood, 2001), specifically by competition at apical channels and by inhibiting basolateral $Ca^{2+}$-ATPase in branchial ionocytes (Verbost et al., 1987, 1989). Additionally, it has been suggested that soft water causes ionocyte proliferation (reviewed by Perry, 1997) potentially contributing to increased susceptibility to Cd toxicity

(Matsuo et al., 2005). Therefore, Cd contamination may present a particular threat to fish in the Amazon basin due to the low $Ca^{2+}$ concentration available to outcompete its binding to key gill sites (Playle et al., 1993). Mechanistic studies with Cd have been performed only with tambaqui. In contrast to the high tolerance of this species to Cu, the tambaqui appears to be very sensitive to waterborne Cd. While $LC_{50}$ data are not available, in DOC-free soft water, moderate levels of Cd exposure (10–80 µg/L) for 24 h caused 50%–90% inhibition of $Ca^{2+}$ uptake (Matsuo et al., 2005). Acclimation to added $Ca^{2+}$ or added foreign DOC before Cd exposure was effective in decreasing Cd accumulation at the gills, even when these agents were no longer present, suggesting persistent protective changes in gill properties, as similarly seen with Cu exposure (Matsuo et al., 2005; Figure 4). In contrast to dietary Cu, dietary Cd, at the same level as used for Cu ($500\,\mu g\,Cd\,g^{-1}$), resulted in much greater internal accumulation, and marked effects on the internal metabolic physiology of tambaqui, again indicating its greater potency in this species (Giacomin et al., 2018).

The toxic mechanism of Ni to fish is not well understood, in contrast to Cu and Cd. There is evidence of respiratory disruption, oxidative stress, and disturbances in the regulation of $Ca^{2+}$, $Mg^{2+}$, and iron ($Fe^{2+/3+}$) (Brix et al., 2017). In general Ni toxicity occurs at concentrations several orders of magnitude greater than those for Cu and Cd (high µg/L–low mg/L levels). Ni toxicity is known to be influenced by pH, DOC concentration and water hardness, making Amazonian waters ideal study sites, but to date, only the cardinal tetra has been investigated. Ni toxicity in cardinal tetra differed seasonally in DOC-rich water with greater toxicity at pH 7 relative to pH 4, except during the wet season (Holland et al., 2017). Surprisingly Ni was especially toxic in water at pH 4 with high concentrations of allochthonous Rio Negro DOC. At pH 4, Ni toxicity was negatively related to FI and positively related to DOC concentration and the amount of humic and fulvic-like components. Clearly DOC quality and pH interact in a complex manner to influence Ni toxicity. These interactions differ from those for other metals, and much more work is needed to understand their nature in acidic, DOC-rich softwaters.

## 11 | FUTURE DIRECTIONS

Although there has been an increasing number of studies over the past two decades on the specific effects of DOC, pH, and ionic composition on ionoregulation, acid–base regulation, TEP, ammonia excretion, and metal toxicity in native fish, there remain large knowledge gaps. DOC-rich acidic softwaters comprise ecosystems that are hotspots of biodiversity, but they are critically threatened by climate change, industrial discharges, population growth, and deforestation, especially in the Amazon. Key areas for future research include the following:

- The unusual ion uptake and ammonia excretion mechanisms in Characiformes that are so resistant to low pH defy current models and should be investigated with molecular techniques. Members of the other native Orders should also be surveyed to see if this is a widespread strategy, or whether additional strategies for ionoregulation at low pH exist.
- Virtually nothing is known about the possible structural changes in the gill morphology that may occur when fish residing in these waters are faced with ionoregulatory challenges. Comparison with the vast amount of morphological information on nonnative fish will prove instructive.
- The mechanistic basis of the apparent positive effects of DOC on ionoregulation and associated functions needs to be much better understood. Studies to date have been correlational rather than mechanistic, and many have used inappropriate DOCs. The use of native, freshly collected DOCs should be encouraged, as well as the use of well-characterized “model” DOC compounds as experimental probes. It is quite possible that gill function in acidic DOC-rich waters may be specific to the properties and quality of the native DOC. Given that DOC appears to interact with the gill boundary layer and/or surface, it may directly induce structural and molecular changes of the gills.
- The effects of DOC on gill TEP, and its interactions with pH and $Ca^{2+}$, remain particularly mysterious. A good starting point will be to determine whether these actions of DOC on gill TEP are concentration-dependent, as they are for $H^+$ and $Ca^{2+}$. This should be investigated in several DOCs with different physicochemical properties, during exposure to different pH and $Ca^{2+}$ levels.
- With increased mining and industrial discharges (Duarte & Val, 2020), as well as precipitation-borne inputs of a variety of metals, there is an urgent need to better understand metal effects in tropical blackwaters. DOC is a valuable water quality parameter that clearly influences metal toxicity, and it has been incorporated into regulatory tools such as the biotic ligand model (BLM) (Niyogi & Wood, 2004). However, recent BLM analyses of Rio Negro Cu toxicity data have highlighted some uncertainties (Cremazy et al., 2016; Duarte et al., 2021). It remains unclear whether the DOCs of tropical acidic blackwaters behave in the same ways as those of the northern hemisphere. It would be of special interest to understand whether the DOC functional groups that bind metals are the same as those that provide the beneficial ionoregulatory effects that have been described. Likewise, only three metals (Cu, Cd, and Ni) have been investigated in Amazonian fish from an environmental viewpoint. Of these only Cu has been studied in any detail, and results with Ni do not fit expected patterns. More extensive studies with a wider range of metals are needed. Exploring these mechanisms may aid in the creation of effective environmental regulations.

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## CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

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