Received: 19 March 2025 | Revised: 22 June 2025 | Accepted: 14 July 2025
DOI: 10.1111/jfb.70157

REGULAR ARTICLE

JOURNAL OF FISH BIOLOGY

The acute osmoregulatory effects of low pH and Cu, alone and in combination, on the dwarf cichlid (*Apistogramma agassizii*) in Rio Negro blackwater and Rio Solimões whitewater: Flux rates of ions, nitrogenous wastes and water

Carolyn Morris[1] | **Anne Crémazy**[2] | **Jhonatan Mota da Silva**[3] | **Ora E. Johannsson**[1] | **Colin J. Brauner**[1] | **Chris M. Wood**[3,4] | **Adalberto Luis Val**[3]

[1]Department of Zoology, University of British Columbia, Vancouver, British Columbia, Canada

[2]Eau Terre Environnement Research Centre, Institut National de la Recherche Scientifique, Québec City, Québec, Canada

[3]Laboratory of Ecophysiology and Molecular Evolution, Instituto Nacional de Pesquisas da Amazônia, Manaus, Brazil

[4]Department of Biology, McMaster University, Hamilton, Ontario, Canada

Correspondence
Carolyn Morris, Department of Zoology, University of British Columbia, Vancouver, BC V6T 1Z4, Canada.
Email: morris@zoology.ubc.ca

Funding information
Natural Sciences and Engineering Research Council of Canada, Grant/Award Numbers: RGPIN-2023-037, RGPIN-2019-04400, CGS-D Post Graduate; CNPq (Brazilian National Research Council), CAPES (Coordination of Superior Level Staff Improvement), and FAPEAM (Amazonas State Research Foundation) via funding for INCT ADAPTA, Grant/Award Number: 465540/2014-7

Abstract

Increases in anthropogenic activities in the Amazon have led to pollution from trace metals, including copper. Dissolved organic carbon (DOC) is known to protect against metal toxicity and ionoregulatory disturbances in Amazonian fish, particularly at low pH. However, little is known about the effects of DOC and trace metals, such as copper, on the branchial water transport pathways. Water moves across the gills of fish through two distinct pathways: paracellularly through tight junctions and transcellularly by diffusion through aquaporins. In the present study, we evaluated the effects of copper (nominally 200 $\mu g\ L^{-1}$) on diffusive water flux rate (transcellular water movement), paracellular permeability ([^{3}H]-polyethylene glycol-4000 clearance), ion balance (net sodium, potassium and chloride fluxes) and nitrogenous waste (ammonia and urea) excretion in the dwarf cichlid, *Apistogramma agassizii*. Exposures were conducted in control water (low ions, very low DOC), in filtered Rio Negro (RN) blackwater (low ions, high DOC) and in filtered Rio Solimões (RS) whitewater (higher ions, intermediate DOC) at pH 7 and pH 4. Copper increased ion losses in control water, especially at low pH; RN water protected against these effects, whereas RS water did not, reflecting greater complexation of free Cu^{2+} ions by RN DOC. Our results are the first to show that copper tends to inhibit urea-N excretion as well as ammonia excretion, and also decreases branchial water transport both transcellularly and through tight junctions. The protective effects of DOC against the disturbances caused by copper were dependent on the source of the DOC and the water pH.

KEYWORDS
Amazon basin, dissolved organic carbon, nitrogenous waste excretion, osmoregulation, water transport

1 | INTRODUCTION

Blackwater systems are found globally (Gandois et al., 2020; Robroek et al., 2017; Saabater et al., 1993) and have characteristically high concentrations of dissolved organic carbon (DOC) and low pH and are often very ion poor. The Rio Negro (RN) is an example of these habitats, one that has been relatively well studied. RN water chemistry is influenced by the decomposition of jungle vegetation, resulting in one of the most naturally DOC-rich ($\sim$7–35 mg C L^{-1}), acidic (pH 3–6) and ion-poor (<30 μmol L^{-1} of Na^{+}, Cl^{-}, Mg^{2+} and Ca^{2+}) (Duarte et al., 2013) watersheds globally (Furch, 1984; Val & de Almeida-Val, 1995; Walker & Henderson, 1996). This water chemistry is so extreme that it would likely be toxic to most other freshwater fish (Val & de Almeida-Val, 1995). Yet it is one of the most biodiverse watersheds in the world, home to about 8% of the global freshwater fish species (Morris et al., 2021; Val & de Almeida-Val, 1995; Val & Wood, 2022). This biodiversity may be associated with the quality and quantity of DOC as well as physiological adaptations of the organisms to this very challenging environment.

Indeed, DOCs are able to exert ionoregulatory effects on freshwater fish that are often beneficial and particularly important in low-pH blackwater. Particularly, beneficial DOC effects have been observed against acidity. DOC has been shown to protect against the inhibition of active ion uptake and the often-fatal increase in passive loss of major salt ions (Na^{+}, Cl^{-}) across the gills at acidic pH (200Al-Reasi et al., 2016; Duarte et al., 2016, 2018; Glover et al., 2005; Glover & Wood, 2005; Gonzalez et al., 1998, 2002, 2006; Matsuo et al., 2004; Wood et al., 2002, 2003, 2011). The presence of DOC also protects against disturbances in ammonia excretion in response to low pH exposure in fish. Generally, an increase in ammonia excretion rate is observed in the presence of RN DOC at low pH (Sadauskas-Henrique et al., 2019, 2021; Wood et al., 2003), which appears to help maintain Na^{+} coupling to ammonia excretion. Duarte et al. (2016) suggested that DOC supports this coupling via the Rh metabolon (Wright & Wood, 2009; Wright & Wood, 2012). However, overall, very little work has been done on the interactive effects of low pH and DOC (and metals) on ammonia excretion rate and none, to our knowledge, on urea-N excretion rates.

DOC is also known to protect fish against toxic trace metals, such as copper (Cu), a contaminant of interest in the Amazon (Braz-Mota et al., 2025; Duarte et al., 2024; Moulatlet et al., 2023). Similar to acidity, Cu impairs Na^{+} homeostasis in freshwater fish, leading to often-fatal plasma Na^{+} loss. This occurs through inhibition of branchial Na^{+} influx via direct competition with Na^{+} at the Na^{+} channels on the apical membranes of the branchial ionocytes, and promotion of Na^{+} diffusive loss, via an increase in branchial epithelium permeability (Grosell et al., 2002; Laurén & McDonald, 1985). Using the biotic ligand model, the severity of these effects is usually attributed to the concentration of the free Cu^{2+} ions in solution, which is greatly affected by the ambient water chemistry (Di Toro et al., 2001; Niyogi & Wood, 2004). Particularly, it is well established that DOCs have the ability to complex with metals and thereby to reduce the bioavailability of metal cations, leading to the mitigation of metal toxicity (Crémazy et al., 2016; Hollis et al., 2001; Matsuo et al., 2004; Playle et al., 1993; Wood et al., 2011).

Another osmoregulatory area in which DOC may be important is in altering the permeability of the gill epithelium to water. In freshwater fish, water moves across the gill barrier through two distinct pathways. Firstly, it can move paracellularly through tight junctions where it is thought to move by bulk flow (Wood et al., 2019). Secondly, it can move by diffusion across the entire gill surface but largely by the transcellular pathway through aquaporins (Wood et al., 2019). These diffusive water channels were first discovered by Preston et al. (1992) using in vitro expression in frog oocytes. It has been suggested that DOC may affect paracellular or diffusive permeability (Galvez et al., 2008), but there are no experimental data confirming this, to our knowledge. Increases in paracellular permeability at low environmental pH have been reported in zebrafish (Al-Reasi et al., 2016; Kwong & Perry, 2013), a species which is acidophilic but does not occur in the Amazon. We are aware of only two previous studies, both on the oscar (*Astronotus ocellatus*), that have measured either of the two water transport pathways in any Amazonian fish (Robertson et al., 2015; Wood et al., 2009), and neither of them addressed the effects of low pH, DOC or Cu.

The ability of DOCs to protect against metal toxicity and exert physiological effects is linked to their physicochemistry; therefore, some DOCs exhibit more effective protection than others (Al-Reasi et al., 2011; Al-Reasi et al., 2013; Morris et al., 2024; Sadauskas-Henrique et al., 2019). Particularly, DOC from the RN blackwater (pH 3-6, DOC > 7 mg C L^{-1}) may offer more protection than DOC from Rio Solimões (RS) whitewaters, another major tributary of the Amazon basin. This latter water has a higher silt burden, lower DOC concentration ($\sim$2–6 mg C L^{-1}), a higher natural pH (6.0–7.0) and ion concentrations that are at least threefold higher compared to RN water (Morris et al., 2021). Many of the physicochemical properties of DOC can be determined using optical techniques (Al-Reasi et al., 2011; Wood et al., 2011). Generally, allochthonous, aromatic DOCs that are optically dark and rich in humic acid are more protective against metal toxicity and physiological disturbances (Al-Reasi et al., 2013; Morris et al., 2024; Wood et al., 2011). Indeed, based on absorbance and fluorescence measurements, RN water has been reported to be more allochthonous and highly aromatic and to have a higher percentage of humic acid–like content compared to RS water (Duarte et al., 2016; Holland et al., 2017; Johannsson & Holland, 2025; Sadauskas-Henrique et al., 2025).

In the present study we investigated the effects of low pH (4 vs. 7) and Cu (0 vs. 200 μg L^{-1}) on the dwarf cichlid (*Apistogramma agassizii*) exposed in two freshly collected waters from the Amazon basin, 'blackwater' from Anavilhanas archipelago region of the RN and 'whitewater' from Janaucá region of the RS. The dwarf cichlid was chosen as it is one of a number of species that lives naturally in both the RN and RS, and its basic ionoregulatory physiology has been well characterized, as well as some of its responses to low pH and Cu (Gonzalez et al., 2002, 2006; Duarte et al., 2009, 2013, 2024; Crémazy et al., 2022; Val & Wood, 2022). As an ornamental fish favoured by aquarists, it has regional socio-economic importance.

To simplify the experiments, the natural waters were filtered to remove particles, so that only the dissolved water chemistry could influence the physiological responses of the fish. We used Instituto Nacional de Pesquisas da Amazônia groundwater (INPA), an ion-poor, DOC-poor groundwater, to which the fish had been acclimated as a reference control. We measured net Na^+, K^+ and Cl^- flux rates, ammonia and urea-N excretion rates, diffusive water flux rate (a proxy for transcellular permeability), [^{3}H]PEG-4000 clearance rates (a proxy for paracellular permeability) and drinking rate (which is a by-product of the [^{3}H]PEG-4000 clearance rate measurements). This experimental design allowed us to determine which of the two field-collected waters was more protective against the disturbing effects of low pH and Cu, and whether there were interactive effects between pH and Cu in each water type.

Our specific hypotheses were as follows. (i) Low pH would increase the net loss rate of major ions (Na^+, K^+, Cl^-), and RN water would offer the greatest protection against these effects due to the beneficial physiological effects of its highly allochthonous DOC on ionic homeostasis noted earlier. (ii) Cu would also increase the net loss rate of major ions, and the extent of these disturbances would be a function of the concentration of the free Cu^{2+} ions in the various test waters, as estimated by chemical speciation modelling. (iii) RN would also provide a greater protection than RS against these ionoregulatory disturbances of low pH and Cu, due to the higher concentration of very allochthonous DOC in the RN. The RS response patterns would look more like the control response pattern. (iv) Other osmoregulatory parameters (diffusive water flux, paracellular permeability, drinking rate – that is, ones which have never been looked at before with respect to low pH and Cu) would exhibit the same response patterns as the net ion flux rates. (v) Because of the likely linkage between Na^+ uptake and ammonia excretion, treatments that disturbed Na^+ balance would inhibit ammonia excretion. (vi) This would not be the case for urea-N excretion, for which there is no evidence of linkage to Na^+ uptake.

2 | METHODS

This study was a semi-field project where the fish were taken from the laboratory to the natural water sites. The work was performed in November–December 2023 on board the research vessel, the *Ana Clara*, near Manaus, Brazil. Two field locations within the Amazon river basin were visited, and fresh river water was collected from each. The first field location in the Anavilhanas Archipelago of the RN (~110 km upstream of Manaus; 02°43′10.3″ S, 60°45′19.6″ W) was visited between 22 and 29 November 2023. The second location, near the village of Janauacá on the RS (~40 km upstream of the mixing zone between the RN and the RS, 03°21′19.7″ S, 60°12′40.1″ W) was visited between 30 November and 5 December 2023.

2.1 | Animals

Dwarf cichlids (*A. agassizii*) ($N = 175$, 268.0 ± 1.3 mg) were obtained from a commercial dealer in Manaus, Brazil. These fish were wild-caught before being sold through the dealer, so it is most likely that these fish were of mixed sex. Fish were held at the Laboratory of Ecophysiology and Molecular Evolution at Instituto Nacional de Pesquisas da Amazônia (LEEM-INPA) in aerated control water (INPA groundwater; Table 1) at 29 ± 1°C. After 7 days they were transferred to the research vessel *Ana Clara* in the same water. Experiments commenced on board the vessel several days later. Fish were fed to satiation every day with commercial dry food (TetraMin, Tropical Flakes Fish Food, Spectrum Brands, Blacksburg, VA, USA) and fasted for at least 24 h before testing. All experimental procedures were approved by the Ethics Committee on Animal Experiments of INPA under registration number 004/2018, in conformity with the Brazilian Ethics Committee on Animal Care (CONCEA) guidelines (RN 61/2023). The experiment was also approved by the Brazilian Institute of Environment and Renewable Natural Resources (IBAMA) (SISBio number: 29837-24).

2.2 | Exposure waters

A total of 12 exposure solutions were tested: control (INPA), RN and RS water at pH 7 and pH 4, each in the presence and absence of nominally 200 μg L^{-1} of Cu. Fresh RN water from near Anavilhanas Archipelago (2°43′00″ S, 60°45′29″ W) and fresh RS water from near Janauacá (3°21′26″ S, 60°12′26″ W) were collected upstream of Manaus. Control INPA water was collected directly from the tap at LEEM-INPA and thoroughly aerated to remove CO_2. Its particulate concentration was extremely low, so it was used without filtration. RN and RS waters were filtered using 0.45-μm pore size (47 mm), hydrophilic, polyethersulfone polymer membrane filters (MS PES Membrane Solutions, Auburn, WA, USA), which were pre-rinsed with 500 mL of distilled water to remove any contaminants. All waters were stored in the dark in sealed bottles at 4°C until further analysis. The pH was adjusted to~7 or 4 using HNO_3 or NaOH and measured using a sympHony pH electrode (C03243) and meter (SP70P) (VWR International, Radnor, PA, USA). A Cu stock solution was prepared from $CuSO_4{\cdot}5H_2O$ (99% pure, Sigma-Aldrich, St. Louis, MO, USA) and was added to the exposure waters 24 h before each test to allow for equilibration. Prior to experiments, waters were warmed to the experimental temperature (29 ± 1°C) and thoroughly aerated. Additionally, at this time, the pH was checked and adjusted appropriately if it deviated by more than ±0.1 pH units for the intended value.

The DOC concentrations in the test waters were measured using a total organic carbon (TOC) analyser (Shimadzu TOC-VCSH, Canby, OR, USA), and aqueous Cl^- concentration was measured using the colorimetric assay of Zall et al. (1956) (Table 1). Certified commercial standards were used (ThermoFisher Scientific, Waltham, MA, USA). Cu and major cation concentrations were measured in the test waters using inductively coupled plasma atomic emission spectroscopy (5110 dual-view ICP-AES, Agilent, Santa Clara, CA, USA) using certified standards (SCP Science, Baie-D'Urfé, QC, Canada). For this ICP-AES analysis, samples were in a matrix of 0.5% HNO_3 (v/v) (trace metal grade, Fisher Scientific) with internal standard yttrium 5 mg L^{-1} (yttrium oxide 99.99%, pure fom Sigma-Aldrich) and caesium chloride 0.2% (99% pure from Fisher). Calibration was checked with certified reference water AQ0-213-985, COO-061-401 and 900-Q30-101 (SCP

TABLE 1 Water characterization summary of INPA control, RN and RS waters.

Parameter	Control	RN	RS
[DOC] (mg L^{-1})	0.60 ± 0.08 (*N*= 5)	9.3 ± 0.09 (*N* = 12)	5.6 ± 0.3 (*N* = 7)
[Na] (μmol L^{-1})	101.8 ± 12.8 (*N* = 4)	69.9 ± 18.6 (*N* = 4)	325.6 ± 14.5 (*N* = 4)
[K] (μmol L^{-1})	25.2 ± 3.9 (*N* = 4)	18.8 ± 1.5 (*N*= 4)	61.5 ± 11.7 (*N* = 4)
[Ca] (μmol L^{-1})	12.0 ± 3.9 (*N*= 4)	13.2 ± 2.4 (*N* = 4)	355.0 ± 3.9 (*N* = 4)
[Mg] (μmol L^{-1})	2.2 ± 0.8 (*N* = 4)	4.9 ± 0.6 (*N* = 4)	52.7 ± 3.4 (*N* = 4)
[Cl] (μmol L^{-1})	10.6 ± 1.9 (*N* = 4)	6.6 ± 2.5 (*N* = 4)	37.1 ± 4.8 (*N* = 4)
Background [Cu] (μg L^{-1})	4.0 ± 0.5 (*N* = 2)	5.0 ± 0.5 (*N* = 2)	6.0 ± 1.5 (*N* = 2)
Total [Cu] (μg L^{-1})	150 ± 26.6 (*N* = 2)	153 ± 15.3 (*N* = 2)	166 ± 39.4 (*N* = 2)

Note: Mean ± standard error of the mean (*n*).
Abbreviations: DOC, dissolved organic carbon; INPA, Instituto Nacional de Pesquisas da Amazônia groundwater; RN, Rio Negro; RS, Rio Solimões.

Science, percentage recovery was >90% for Cu). A blank, quality control, and calibration standard were reanalysed every 20 samples to check and correct for potential instrument drift.

2.3 | Diffusive water flux measurements

The procedure outlined by Onukwufor and Wood (2020) was followed for measuring diffusive water flux rates. Briefly, the loading period began 2 h before the experiment started. Fish (six to eight per treatment) were pooled and exposed to 100 μCi L^{-1} of ^3H_2O (Eckert & Ziegler, Valencia, CA, USA) in 100 mL of INPA control water for 2 h. After the loading period, each individual fish was quickly rinsed in clean control water, with care to minimize disturbance. Each fish was immediately placed in an individual enclosed, aerated, temperature-controlled chamber containing 45 mL of the appropriate control or experimental solution without ^3H_2O. These containers were purposefully narrow, allowing sequential removal of water samples without immersing these very small fish. A sample of the ^3H_2O water was taken at the end of the loading period. For each treatment/replicate, a series of water samples were collected for analysis: a 5-mL water sample was taken at time 0 min and every 5 min for 30 min thereafter, and a final sample was taken at 4 h, at the end of the wash-out period, when the external and internal ^3H_2O pools were once again in equilibrium. The 4-h sample was used to calculate the original amount of radioactivity in the fish, and the 0–30 min samples were used to determine the diffusive water flux rates. The concentration of ^3H_2O was measured by adding 10 mL of Optiphase 3 fluor (PerkinElmer, Waltham, MA, USA) to the 5-mL water sample, shaking vigorously and loading it into a scintillation counter (Hidex 300SL Automatic, Turku, Finland). Preliminary tests showed that quenching and chemiluminescence were negligible.

2.4 | Net ion flux and ammonia and urea-N excretion measurements

After the wash-out period for the diffusive water flux measurements, the experiments to determine the net ion flux and ammonia and urea-N excretion rates were started. Thus, by this time, the fish had been exposed to the experimental waters in the same chambers for 4 h. These experiments were run using this order of operations to minimize handling stress. A 5-mL water sample was taken from each individual experimental chamber marking the start of the ion flux and nitrogenous waste excretion period, and a final 5-mL sample was taken at 2 h marking the end of the measurement period. Preliminary experiments demonstrated that this 2-h interval was adequate to detect reliable changes in ion concentrations and ammonia and urea-N levels in the water. Na^+ and K^+ ion concentrations were measured using atomic absorption spectrometry (Varian AA240FS, Santa Clara, CA, USA). Water concentrations of Cl^- (Zall et al., 1956), total ammonia (salicylate hypochlorite assay, Verdouw et al., 1978) and total urea-N (diacetyl monoxime assay, Rahmatullah & Boyde, 1980) were measured colorimetrically. For each colorimetric assay, the three water types (i.e. control, RN and RS) were analysed against standard curves made with that water type to account for any colour DOC may impart to the water.

2.5 | Paracellular permeability and drinking rate

The methodology developed by Robertson and Wood (2014) and modified by Al-Reasi et al. (2016) and Wood et al. (2019) was followed. A different batch of fish from those used in Sections 2.3 and 2.4 was transferred to individual aerated, temperature-controlled experimental chambers containing 35 mL of either control or experimental waters and were allowed to rest for 15 min. Similar studies on trout by Robertson & Wood (2014) and zebrafish by Al-Reasi et al. (2016) used a 30-min resting period, but in preliminary experiments with dwarf cichlids we found no differences between 15 and 30 min. Then 50 μCi L^{-1} [^{3}H]PEG-4000 (PerkinElmer, Shelton, CT, USA) was added to the water, followed by a 15-min mixing period. Water samples (5 mL) were taken for [^{3}H]PEG-4000 radioactivity at 0.25 and at 2 h to mark the end of the experiment. Fish were killed with an overdose of tricaine methanesulfonate (MS-222) at a concentration of 0.5 g L^{-1} neutralized with NaOH. The gastrointestinal tract was carefully removed by dissection. Carcasses and gastrointestinal tracts were then weighed and individually digested in 2 volumes of 1 M nitric acid for 48 h at 65°C using sealed vials. [^{3}H]PEG-4000 radioactivity was measured using liquid scintillation counting. Ultima Gold AB (PerkinElmer) scintillation fluid was used with sample digest (2:1). Quench curves were constructed to correct digest radioactivity to the same counting efficiency as water samples.

2.6 | Calculations

The rate constant of ^3H_2O efflux (k) was calculated by employing the procedure outlined by Onukufor and Wood et al. (2020). Briefly, using the radioactivity of the final 2-h wash-out sample, the initial amount of ^3H_2O in the fish at time 0 min was determined. It was then possible to calculate the amount of ^3H_2O left in the fish at each sampling time from 0 to 30 min based on the measured amounts that appeared in the external solution at each time point and the known volume of the system. Next, the natural logarithm of the total ^3H_2O in the fish at each sample time was regressed against time in minutes on a linear scale. The fractional rate constant k for water turnover was the slope of this line. Multiplying the rate constant k by 60 yielded the fraction of the body water pool turned over per hour. Generally, the total exchangeable water pool of a fish is 800 mL kg^{-1} (Holmes & Donaldson, 1969; Isaia, 1984; Olson, 1992). To calculate the diffusive water flux rate in millilitre per kilogram per hour, the mass of the fish in kilograms was multiplied by 800 mL kg^{-1} and then by the fraction of the body water pool turned over per hour.

Net ion flux rates and ammonia-N and urea-N excretion rates (J_{net}, μmol kg^{-1} h^{-1}) were calculated as per Wood (1992) using the following equation:

$$J_{\text{net}} = \left(\frac{[X_1] - [X_2] \times V)}{m \times \Delta t} \right. \tag{1}$$

where $[X_1]$ and $[X_2]$, respectively, represent the concentrations of x (x: Na$^+$, K$^+$, Cl$^-$, ammonia-N or urea-N) at the beginning (i) and end (ii) of the flux periods (μmol L^{-1}), V is the volume of the exposure solution (L), m is the wet mass of the fish (kg) and Δt represents the total duration of the flux period (h). Note, there is one nitrogen atom per ammonia molecule and two nitrogen atoms per urea molecule.

The percentage of urea-N excretion was calculated from the total nitrogenous waste excretion (i.e. ammonia excretion + urea-N excretion) per fish in each water type:

$$\%\text{Urea} - \text{N excretion} = \frac{\text{urea N excretion}}{\text{urea} - \text{N excretion} + \text{ammonia N excretion}} \times 100 \tag{2}$$

As a proxy of paracellular permeability, gill [^{3}H]PEG-4000 influx rates (expressed as water clearance rates, mL kg^{-1} h^{-1}) were calculated as per Robertson and Wood (2014), modified by Al-Reasi et al. (2016) and Wood et al. (2019):

$$\text{Gill influx clearance rate} = \frac{\text{PEG cpm gain from water at the final sampling point}}{\text{mean water}\frac{\text{cpm}}{\text{mL}}\text{over sampling time}} \times \frac{1}{m} \times \frac{1}{\Delta t} \tag{3}$$

where PEG cpm gain from the water at final sampling point is the measured [^{3}H]PEG-4000 radioactivity in the total carcass digest. The mean water cpm mL^{-1} is the average measured [^{3}H]PEG-4000 radioactivity of the water samples over the sampling time. m is the mass of the whole fish (kg) and Δt represents the total duration of the flux period (h).

Drinking rate was calculated based on the total gut [^{3}H]PEG-4000 cpm measured in the removed gastrointestinal tract:

$$\text{Drinking rate} = \frac{\text{gut PEG cpm gain from water at the final sampling point}}{\text{mean water}\frac{\text{cpm}}{\text{mL}}\text{over sampling time}} \times \frac{1}{m} \times \frac{1}{\Delta t} \tag{4}$$

where gut PEG cpm gain from the water at the final sampling point is the measured [^{3}H]PEG-4000 radioactivity in the whole gut digest. The mean water cpm mL^{-1} is the average measured [^{3}H]PEG-4000 radioactivity of the water samples over the sampling time. m is mass of the whole fish (kg) including the gut, and Δt represents the total duration of the flux period (h).

2.7 | Cu speciation modelling

Cu speciation was modelled in the three waters as detailed in Crémazy et al. (2016), where the authors found good agreement between measured free Cu^{2+} ion concentrations, measured using a cupric ion-selective electrode, and modelled Cu^{2+} concentrations in RN water. Briefly, we used the Windermere Humic Aqueous Model (WHAM, version VII) (Lofts and Tipping, 2011) and measured temperature, pH, concentrations of Cu, DOC, major cations and chloride. Alkalinity for the three waters was nominal and taken from Crémazy et al. (2016). We assumed that DOM was 50% carbon in weight (Buffle, 1988), only 65% active in complexing Cu, and composed of only fulvic acid (Bryan et al., 2002). Due to the relative proportion of protein-like DOC in the test waters, control (67%), RN (2.7%) and RS (4.3%) (Holland et al., 2017), and the limitations of WHAM in modelling metal binding to protein-like DOC, each of the three test waters was treated as containing fulvic acid only. A sensitivity analysis of the WHAM free ion estimates to the fulvic acid to humic acid ratio was tested assuming the opposite scenario (100% humic acid): the differences in %Cu^{2+} estimates proved to be negligible. The default log $KCuHCO_3$ of 14.62 (Mattigod & Sposito, 1979) was replaced by 12.13 in the thermodynamic database, as recommended by the International Union of Pure and Applied Chemistry (Powell et al., 2007).

2.8 | Statistical analyses

Data were expressed as mean ± standard error of the mean (SEM, *N*), where *N* represents the number of animals sampled. Percentage data were analysed both with and without arc-sin transformation. All data passed normality and homogeneity of variance tests and were analysed using two-way analysis of variance (ANOVA) (factors, pH and Cu) with Tukey's post hoc test (multiple comparisons). Two-way ANOVA *p*-values and F (DFn, DFd) for pH, total Cu and the interaction effect are summarized in Table S1.

Simple linear regressions ($p < 0.05$) were used to assess the relationships between each of the physiological parameters and the %free copper ion (Cu^{2+}) for each water type. All statistical analyses and data plots were done using GraphPad Prism 10 (GraphPad Software, San Diego, CA, USA). A significance level of $p < 0.05$ (i.e. two tailed) was used throughout.

3 | RESULTS

3.1 | Water chemistry

In general, the control (INPA) and RN waters exhibited similar, very low ion concentrations but differed greatly in DOC concentration, which was about 15-fold greater in RN (9.3 mg L^{-1}) than in INPA water (0.6 mg L^{-1}) (Table 1). In contrast to both, RS water exhibited ion concentrations that were at least 3-fold higher (Na^+, K^+, Cl^-) and up to 30- to 50-fold higher (Ca^{2+}, Mg^{2+}) than in the other two waters. DOC concentration in RS water (5.6 mg L^{-1}) was intermediate between control and RN waters. In all three waters, measured Cu concentrations after addition of nominally 200 μg L^{-1} were actually about 75% of the intended values. Note that as part of our experimental design, suspended particles were removed from RN and RS water by 0.45-μm filtration, as they would otherwise bind and remove significant amounts of Cu out of solution (cf. Crémazy et al., 2019, 2025). There were no suspended particles in control water. Our aim was to achieve the same concentrations of dissolved Cu in the three waters.

3.2 | Cu speciation

The relative proportions of the chemical forms of dissolved copper in the experimental waters were modelled at pH 7 and pH 4 (Table 2). Copper bound to DOC accounted for the largest percentage in most but not all of the experimental solutions, generally followed by Cu^{2+} (i.e. free Cu^{2+} ions). Notably for RN at pH 7 and RS at pH 7, %Cu^{2+} values were negligible because almost all (>95%) of the Cu was bound to the DOC. The two exceptions to the dominance of Cu-DOC were control water at pH 4 and RS water at pH 4 where the free Cu^{2+} ion predominated. This reflected the general trend that for all waters the 1000-fold higher H^+ ion concentration at pH 4 versus 7 displaced much of the Cu from the DOC into the free ion form. Thus, in each experimental water there was a higher %Cu^{2+} and a lower %Cu-DOC at pH 4 compared to pH 7 (Table 2). At pH 7, control + Cu had the highest %Cu^{2+} ^{N}(27%), whereas RS + Cu and RN + Cu exhibited only very small %Cu^{2+} and the highest %Cu-DOC values (Table 2). At pH 4, control + Cu exhibited very high %Cu^{2+} (91%) whereas this decreased to 58% in RS + Cu and 33% in RN + Cu. Other copper species were generally of minor importance. For all solutions there was 0% $CuCl^+$ and 0% $Cu(CO_3)_2^{[2-]}$.

Speciation analyses were also performed for the low-background levels of Cu (4–6 μg L^{-1}) in the waters without added Cu (data not shown). In these waters, the vast majority of background Cu was bound up by DOC, with more than 94% Cu-DOC in all waters except control at pH 4 where it was 68% Cu-DOC and 32% Cu^{2+}.

3.3 | The effects of pH and Cu on net ion flux rates in the three types of water

In general, low pH, Cu and their combination tended to increase the net loss rates of major physiological ions in both control and RS waters, whereas these effects were not seen in RN water (Figure 1).

In control water at pH 7, the fish were in approximately zero net Na^+ balance (Figure 1a). There was a significant overall effect of pH ($p = 0.0078$) on net Na^+ flux rate, such that the net Na^+ loss rate tended to become greater at low pH. There was no overall effect of Cu ($p = 0.1586$) on net Na^+ flux rate, nor was there an interactive effect ($p = 0.7849$). The only significant difference was between pH 7 and pH 4 + Cu treatments, where the highly negative net Na^+ flux rate was 12-fold greater than the positive Na^+ flux rate in the former (Figure 1a). In RN water, the fish exhibited negative Na^+ balance at pH 7 (Figure 1b). There was no overall effect of pH ($p = 0.5532$) or Cu ($p = 0.2634$) and no interactive effect ($p = 0.0984$). There were also no significant differences in net Na^+ flux rate among any of the

TABLE 2 Relative percentages of the main chemical forms of Cu in the experimental waters calculated using WHAM, version VII.

	%Cu^{2+}	%$CuHCO_3^+$	%$CuOH^+$	%$Cu(OH)_2$	%$CuCO_3$	%Cu-DOC
Control 7 + Cu	27.35	0.26	10.88	0.29	9.84	51.38
Control 4 + Cu	90.7	1.31	0.005	0.00	0.07	7.88
RN 7 + Cu	0.05	0.00	0.02	0.00	0.02	99.91
RN 4 + Cu	33.07	0.46	0.02	0.00	0.02	66.43
RS 7 + Cu	1.57	0.06	0.68	0.02	2.70	94.96
RS 4 + Cu	58.54	2.60	0.03	0.00	0.12	38.70

Abbreviations: RN, Rio Negro; RS, Rio Solimões; WHAM, Windermere Humic Aqueous Model.

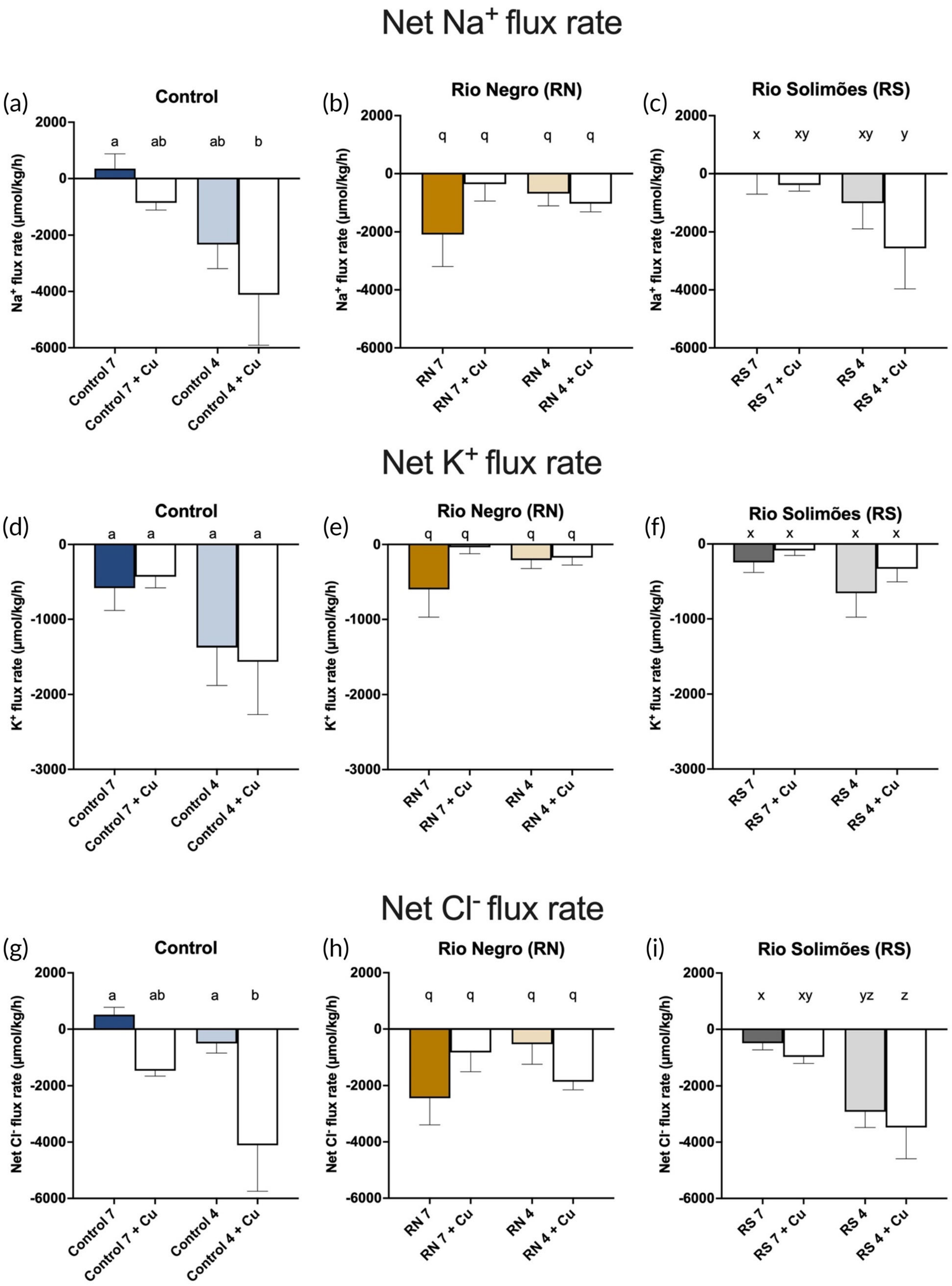


FIGURE 1 Legend on next page.

exposures (Figure 1b) in RN water. In RS water, the fish exhibited net zero Na^+ balance at pH 7 (Figure 1c). There were no overall effects of pH ($p = 0.0652$) or Cu ($p = 0.2552$) and no interactive effect ($p = 0.4765$). Similar to the pattern in control water, there was an observed trend of greater net Na^+ loss with the decrease in pH and addition of Cu; however, the only significant difference (101-fold more negative net Na^+ flux rate) was at pH 4 + Cu relative to pH 7 (Figure 1c).

In control water at pH 7, the fish were in negative K^+ balance (Figure 1d); the same was true in RN water (Figure 1e) and RS water (Figure 1f). In each of the three water types, there were no overall effects of pH on net K^+ flux rate (control $p = 0.0513$, RN $p = 0.4747$, RS $p = 0.0766$), though there were tendencies for greater loss rates with decreasing pH in control and RS waters. There were also no overall effects of Cu (control $p = 0.9686$, RN $p = 0.0990$, RS $p = 0.1836$) and no interactive pH and Cu effects (control $p = 0.7194$, RN $p = 0.1388$, RS $p = 0.6463$). There were no significant differences among mean net K^+ flux rates with the change in pH from 7 to 4 or with the addition of Cu (Figure 1d–f).

The response patterns of net Cl^- flux rates (Figure 1g–i) in the three water types were similar to those of net Na^+ flux rates (Figure 1a–c). At pH 7, net Cl^- balance was close to zero or slightly positive in control water and RS water but negative in RN water. The general trend was for increased Cl^- loss with decreased pH and the addition of Cu in control water (Figure 1g) and in RS water (Figure 1i) but not in RN water (Figure 1h). There were significant overall effects of both pH ($p = 0.0399$) and Cu ($p = 0.0027$) on net Cl^- flux rate in control water, but there was no interactive effect of pH and Cu ($p = 0.3488$). In control water there was a significant eightfold more negative net Cl^- flux rate at pH 4 + Cu relative to pH 7 (Figure 1g). In RN water, there was no overall effect of pH ($p = 0.5230$) or Cu ($p = 0.8311$), but there was a significant interactive effect between these two factors ($p = 0.0399$) on net Cl^- flux rate. There were no significant differences in net Cl^- flux rate in RN among any of the experimental treatments (Figure 1h). In RS water, there was an overall effect of pH ($p = 0.0004$) on net Cl^- flux rate, such that there was an increase in Cl^- loss with the decrease in pH (Figure 1i). There was no overall effect of Cu ($p = 0.3945$) and no interactive effect ($p = 0.9519$) between pH and Cu in RS water. There was a significant sevenfold increase in net Cl^- loss rate between pH 7 and pH 4 + Cu (Figure 1i).

3.4 | The effects of pH and Cu on nitrogenous waste excretion rates in the three types of water

The overall trend was for both ammonia and urea-N excretion rates to decrease in the presence of Cu, with negligible effects of pH (Figure 2).

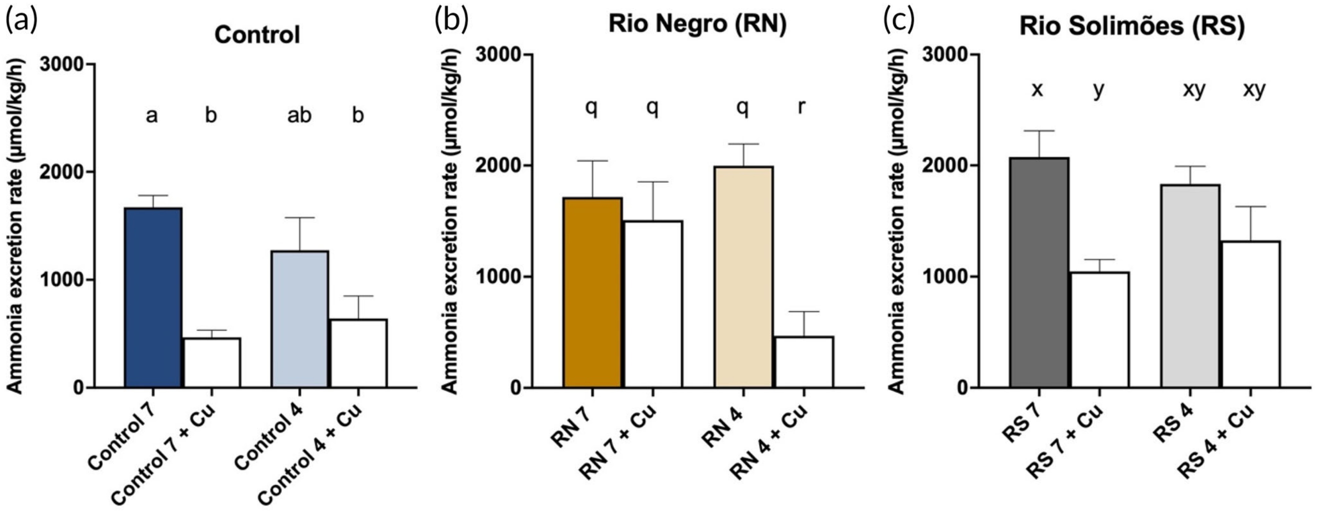
For ammonia excretion, in control water there was no overall effect of pH ($p = 0.5773$) but a significant overall inhibitory effect of Cu ($p < 0.0001$), with no interactive effect ($p = 0.1602$). Ammonia excretion rate decreased significantly in control water with the addition of Cu at both pH 7 and pH 4 by 30%–40% (Figure 2a). In RN water there was no overall effect of pH ($p = 0.1693$), but there was again a significant inhibitory effect of Cu ($p = 0.0034$) as well as a significant interaction effect ($p = 0.0215$). Copper addition significantly reduced ammonia excretion rate at pH 4 by about 70% (Figure 2b). In RS water, there was no overall effect of pH ($p = 0.9253$), but there was an overall inhibitory effect of Cu ($p = 0.0012$) and again no interactive effect ($p = 0.2303$) of pH and Cu on ammonia excretion rate. There was a significant 50% decrease in ammonia excretion rate between pH 7 and pH 7 + Cu (Figure 2c).

For urea-N excretion, in control water there was no overall pH effect ($p = 0.2823$) but a strong inhibitory effect of Cu ($p = 0.0006$) without significant interaction ($p = 0.9801$). In control water, urea-N excretion rate decreased significantly by 70%–80% upon the addition of Cu at both pH 7 and pH 4 (Figure 2d). In RN water, there was an overall inhibitory effect of Cu ($p = 0.0002$) on urea-N excretion rate but no overall effect of pH ($p = 0.2970$), and the interactive effect was not significant ($p = 0.0585$). There was a significant 80% decrease in urea-N excretion rate between pH 4 and pH 4 + Cu in RN water (Figure 2e). In RS water, there was no overall effect of pH ($p = 0.4385$) but a significant overall effect of Cu ($p = 0.0311$), with no interactive effect ($p = 0.6906$). There were no statistically significant differences in urea-N excretion rates in RS water among any of the tested experimental solutions (Figure 2f).

The mean %urea-N excretion rate (i.e. as a % of total ammonia-N + urea-N excretion rate; see Equation 2) was about 25% in control water and somewhat lower in RN and RS waters (Table 3). The data were analysed both with and without the generally recommended arc-sin transformation for percentage data. Both analyses yielded the same conclusion of no significant effects of pH and Cu within each of the experimental waters (Table 3).

FIGURE 1 The effects of copper (nominally 0 vs. 200 μg L^{-1} of Cu) and pH (pH 7 vs. pH 4) on net Na^+ flux rate (a) in control INPA (Instituto Nacional de Pesquisas da Amazônia) water (overall effect of pH: $p = 0.0078$, Cu: $p = 0.1586$, interaction: $p = 0.7849$), (b) in RN (Rio Negro) water (overall effect of pH: $p = 0.5532$, Cu: $p = 0.2634$, interaction: $p = 0.0984$) and (c) in RS (Rio Solimões) water (overall effect of pH: $p = 0.0652$, Cu: $p = 0.2552$, interaction: $p = 0.4765$); on net K^+ flux rate (d) in control water (overall effect of pH: $p = 0.0513$, Cu: $p = 0.9686$, interaction: $p = 0.7194$), (e) in RN water (overall effect of pH: $p = 0.4747$, Cu: $p = 0.0990$, interaction: $p = 0.1836$) and (f) in RS water (overall effect of pH: $p = 0.0766$, Cu: $p = 0.1836$, interaction: $p = 0.6463$); and on net Cl^- flux rate (g) in control water (overall effect of pH: $p = 0.0399$, Cu: $p = 0.0027$, interaction: $p = 0.3488$), (h) in RN water (overall effect of pH: $p = 0.5230$, Cu: $p = 0.8311$, interaction: $p = 0.0399$) and (i) in RS water (overall effect of pH $p = 0.0004$, Cu: $p = 0.3945$, interaction: $p = 0.9519$). Data are mean ± SEM (standard error of the mean), $n = 6–8$. Bars not sharing the same letters are significantly different ($p < 0.05$) (two-way analysis of variance with Tukey's test).

Ammonia-N excretion rate



Urea-N excretion rate

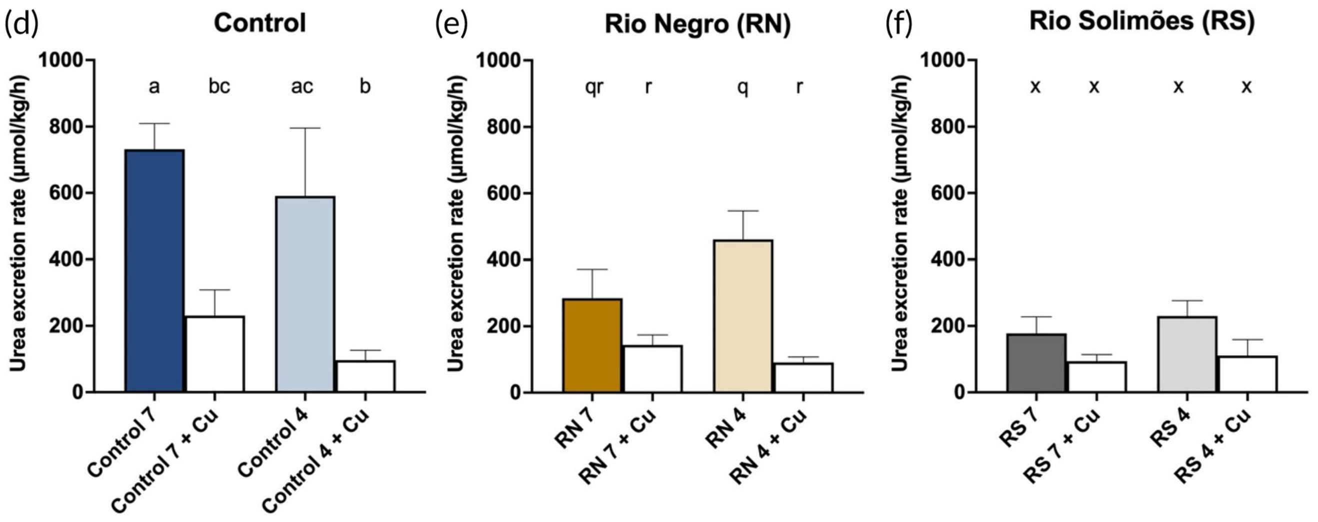


FIGURE 2 The effects of copper (nominally 0 vs. 200 µg L^{-1} of Cu) and pH (pH 7 vs. pH 4) on ammonia-N excretion rate (a) in control INPA (Instituto Nacional de Pesquisas da Amazônia) water (overall effect of pH: $p = 0.5773$, Cu: $p < 0.0001$, interaction: $p = 0.1602$), (b) in Rio Negro (RN) water (overall effect of pH: $p = 0.1693$, Cu: $p = 0.0034$, interaction: $p = 0.0215$) and (c) in RS (Rio Solimões) water (overall effect of pH: $p = 0.9253$, Cu: $p = 0.0012$, interaction: $p = 0.2303$), and on urea-N excretion rate (d) in control water (overall effect of pH: $p = 0.2823$, Cu: $p = 0.0006$, interaction: $p = 0.9801$), (e) in RN water (overall effect of pH: $p = 0.2970$, Cu: $p = 0.0002$, interaction: $p = 0.0585$) and (f) in RS water (overall effect of pH: $p = 0.4385$, Cu: $p = 0.0311$, interaction: $p = 0.6906$). Data are mean ± SEM (standard error of the mean), $n = 6–8$. Bars not sharing the same letters are significantly different ($p < 0.05$) (two-way analysis of variance with Tukey's test).

TABLE 3 %Urea-N excretion (mean ± SEM) at pH 7 and pH 4 with and without the addition of nominally 200 µg L^{-1} of Cu in INPA control, RN and RS waters.

	Control	RN	RS
pH 7	29.7 ± 2.8 ($N = 6$)	16.0 ± 4.8 ($N = 6$)	7.9 ± 2.1 ($N = 8$)
pH 7 + Cu	33.2 ± 9.6 ($N = 7$)	9.4 ± 1.76 ($N = 7$)	8.7 ± 2.0 ($N = 8$)
pH 4	26.6 ± 8.2 ($N = 7$)	17.9 ± 2.3 ($N = 7$)	11.1 ± 1.7 ($N = 7$)
pH 4 + Cu	20.0 ± 7.2 ($N = 6$)	31.9 ± 9.9 ($N = 8$)	13.2 ± 6.9 ($N = 7$)

Abbreviations: INPA, Instituto Nacional de Pesquisas da Amazônia groundwater; RN, Rio Negro; RS, Rio Solimões; SEM, standard error of the mean.

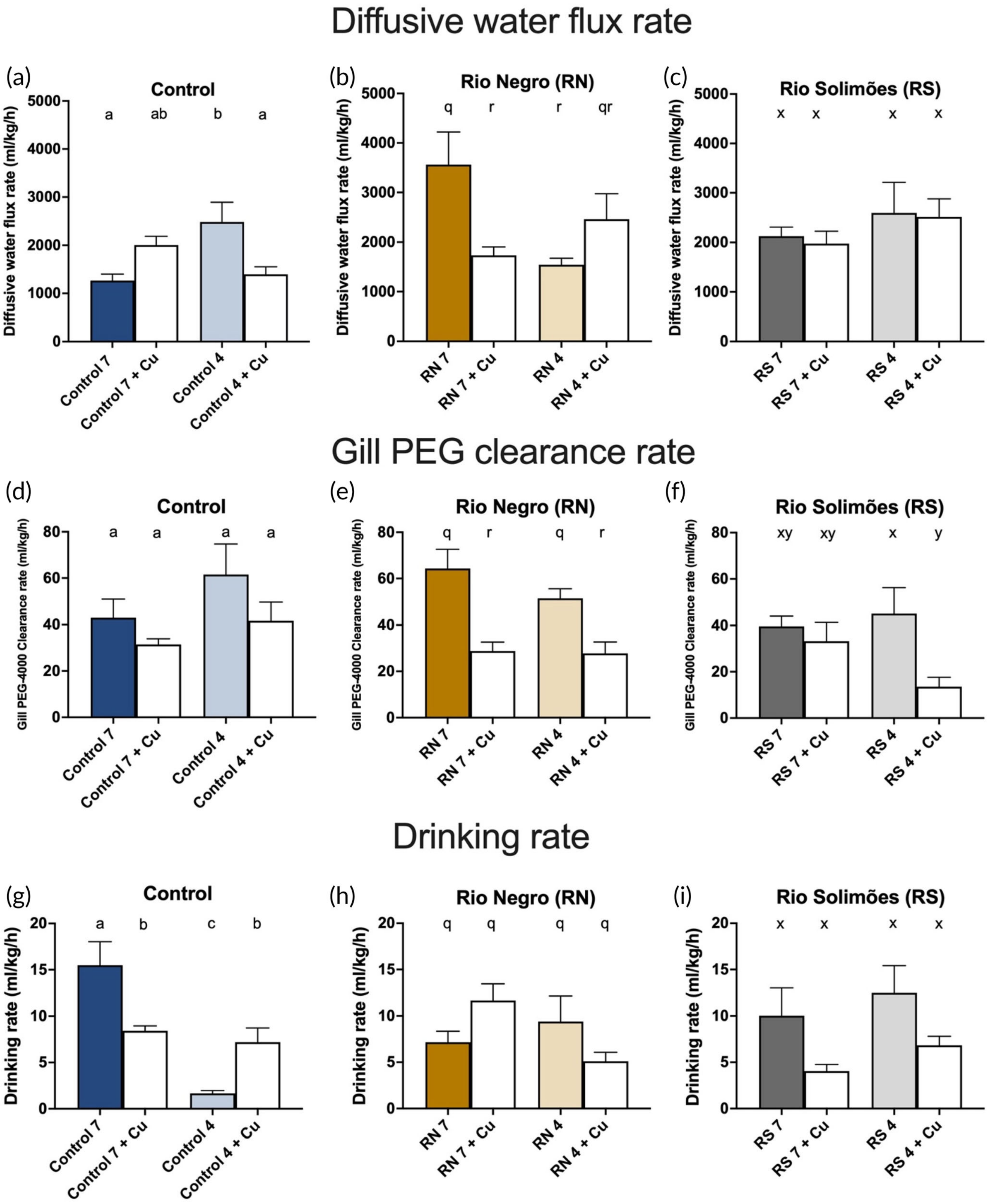


FIGURE 3 Legend on next page.

3.5 | The effects of pH and Cu on the water transport pathways and drinking rate in the three types of water

Diffusive water flux rates (a proxy for transcellular permeability) were very high in these small fish, generally >1000 mL kg $^{-1}$ h^{-1} (Figure 3a–c), meaning that the water turnover by diffusion was more than the entire water volume in the body each hour. In control water, there was no overall effect of pH ($p = 0.2315$) or Cu ($p = 0.4845$); however, there was a significant interactive effect ($p = 0.0011$). In the absence of added Cu, there was a significant twofold greater diffusive water flux rate at pH 4 relative to pH 7 ($p = 0.0052$). There was also a significant 40% decrease in the rate at pH 4 with the addition of Cu ($p = 0.0275$) (Figure 3a).

In RN water, again there was no overall effect of pH ($p = 0.1436$) or Cu ($p = 0.2950$), but there was an interactive effect ($p = 0.0039$). In RN water at pH 7, diffusive water flux rate was very high and decreased significantly by 55% with the addition of Cu (Figure 3b). In contrast, at pH 4 there was an increase in the rate with added Cu, although not statistically significant. In RS water, there were no overall effects of pH ($p = 0.1812$), Cu ($p = 0.7535$) or their interaction ($p = 0.9251$), and also no significant differences among any of the treatments (Figure 3c).

Gill [^{3}H]PEG-4000 clearance rates (a proxy for paracellular permeability) were generally <100 mL kg^{-1} h^{-1} (Figure 3d–f) – that is, more than one order of magnitude lower than diffusive water flux rates. The general trend was a decrease with the addition of Cu in all three waters at both pH values. In control water there were no overall effects of pH ($p = 0.1161$) or Cu ($p = 0.0873$), and no interactive effects ($p = 0.6411$) between pH and Cu. There were no significant differences between any of the experimental treatments in control water (Figure 3d). In RN water there was no overall effect of pH ($p = 0.2167$) but a very strong overall effect of Cu ($p < 0.0001$) to reduce [^{3}H]PEG-4000 clearance rate, with no interactive effect ($p = 0.2888$). There was no significant change in gill PEG clearance rate between pH 7 and pH 4. However, the addition of Cu resulted in significant 40%–50% reductions in the rates at both pH 7 and pH 4 (Figure 3e). In RS water, there was no overall effect of pH ($p = 0.3303$) but a significant overall inhibitory effect of Cu ($p = 0.0134$) and no interactive effect ($p = 0.0883$). There was a significant 70% reduction in gill PEG clearance rate at pH 4 with the addition of Cu in RS water (Figure 3f).

Drinking rates were in the general range of 5–15 mL kg^{-1} h^{-1} (Figure 3g–i). In control water, there was a strong overall effect of pH ($p < 0.0001$), no overall effect of Cu ($p = 0.5984$) and a significant interactive effect ($p = 0.001$). Drinking rate significantly decreased by almost 90% between pH 7 and pH 4 and also by 50% between pH 7 and pH 7 + Cu. In contrast, drinking rate significantly increased by 4.5-fold between pH 4 and pH 4 + Cu (Figure 3e). In RN water there was no overall effect of pH ($p = 0.2483$) or Cu ($p = 0.9593$), but there was a significant interaction effect (0.0233) on drinking rate. There were no significant differences among the treatment means (Figure 3f). In RS water, there was no overall effect of pH ($p = 0.2825$), but there was an effect of Cu ($p = 0.022$) such that drinking rate decreased in the presence of Cu. There was no interactive effect ($p = 0.9448$). There were no significant differences in drinking rate among the treatments (Figure 3g).

4 | DISCUSSION

4.1 | Overview

Both low pH and elevated Cu caused osmoregulatory disturbances. The protective effects of DOC against the disturbances caused by Cu were dependent on the source of the DOC and the water pH. Some factors in support of our first three original hypotheses are as follows. (i) Low pH increased net loss rates of Na$^+$ and Cl$^-$ in all water types, except RN where ion loss rates were actually lower at pH 4 than at pH 7 (Figure 1). (ii) Cu also increased the net loss rates of two of the major ions (Na$^+$ and Cl$^-$), effects which were associated with the concentration of free Cu^{2+} ions in solution, as estimated by speciation modelling (Figure 1; Table 2). This was further confirmed by regression analyses where there were significant negative correlations between all net ion flux rates (Na$^+$, K$^+$, Cl$^-$) and %free copper ion (Cu^{2+}) such that as %free Cu^{2+} increased, net flux rate became more negative (Figure 4). There were no other significant relationships between %free Cu^{2+} ions and any of the other physiological parameters (Figure S1). The highest ion loss rates were all at pH 4, with the greatest loss rates in control water (91% Cu^{2+}), intermediate loss rates in RS water (58% Cu^{2+}) and smallest loss rates in RN water (33% Cu^{2+}). (iii) RN water did provide much better protection than RS water against these ionoregulatory disturbances of low pH and Cu, reflecting the high concentration of very allochthonous DOC in the RN. The RS response pattern generally resembled the control response pattern

FIGURE 3 The effects of copper (nominally 0 vs. 200 μg L^{-1} of Cu) and pH (pH 7 vs. pH 4) on diffusive water flux rate (a) in control INPA (Instituto Nacional de Pesquisas da Amazônia) water (overall effect of pH: $p = 0.2315$, Cu: $p = 0.4845$, interaction: $p = 0.0011$), (b) in RN (Rio Negro) water (overall effect of pH: $p = 0.1436$, Cu: $p = 0.2950$, interaction: $p = 0.0039$) and (c) in RS (Rio Solimões) water (overall effect of pH: $p = 0.1812$, Cu: $p = 0.7535$, interaction: $p = 0.9251$); on gill PEG clearance rate (d) in control water (overall effect of pH: $p = 0.1161$, Cu: $p = 0.0873$, interaction: $p = 0.6411$), (e) in RN water (overall effect of pH: $p = 0.2167$, Cu: $p = <0.0001$, interaction: $p = 0.2888$) and (f) in RS water (overall effect of pH: $p = 0.3303$, Cu: $p = 0.0134$, interaction: $p = 0.0883$); and on drinking rate (g) in control water (overall effect of pH: $p < 0.0001$, Cu: $p = 0.5984$, interaction: $p = 0.001$), (h) in RN water (overall effect of pH: $p = 0.2483$, Cu: $p = 0.9593$, interaction: $p = 0.0233$) and (i) in RS water (overall effect of pH: $p = 0.2825$, Cu: $p = 0.022$, interaction: $p = 0.9448$). Data are mean ± SEM (standard error of the mean), NN= 6–8. Bars not sharing the same letters are significantly different ($p < 0.05$) (two-way analysis of variance with Tukey's test).

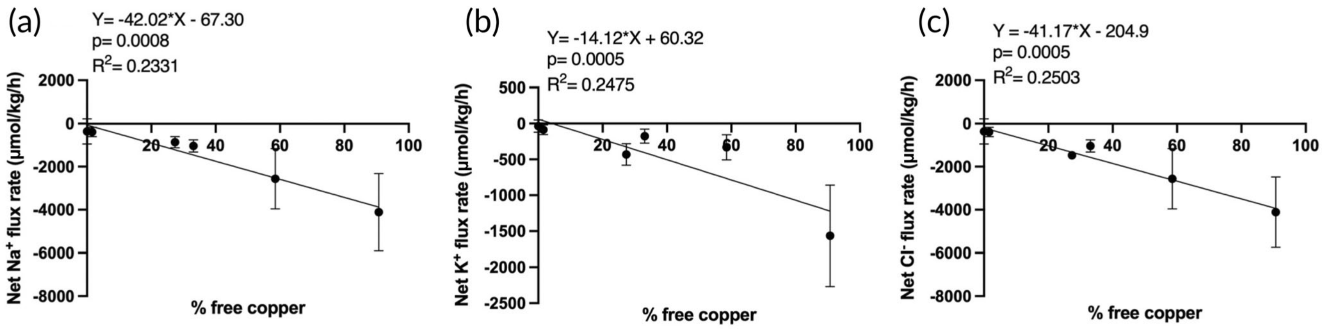


FIGURE 4 The effect of %free copper ion (Cu^{2+}) on net (a) Na^+, (b) K^+ and (c) Cl^- flux rates. Data at nominal total Cu concentrations of 200 µg L^{-1} in control water, RN (Rio Negro) water and RS (Rio Solimões) water at both pH 7 and pH 4 have been combined in these relationships. Values are mean ± SEM (standard error of the mean), N= 6–8. Relationships were assessed using simple linear regression ($p < 0.05$ is considered significant).

(Figure 1). We had hypothesized (iv) that other osmoregulatory parameters that have not been previously evaluated with respect to low pH and elevated Cu (diffusive water flux, paracellular permeability, drinking rate) would exhibit the same response patterns as the net ion flux rates. Our data did not support this. With low pH and with the addition of Cu, we observed an increase in net ion loss rates (Figure 1). In contrast, in many cases there was an inhibition of water transport and drinking rates (Figure 3), suggesting that different mechanisms were involved. (v) Given the previously described coupling between Na^+ uptake and ammonia excretion via Rh-mediated Na^+/NH_4^+ exchange, we had hypothesized that experimental solutions that disturbed Na^+ balance and exacerbated net loss would likely inhibit ammonia excretion. Our results were generally supportive of this idea: at low pH and with the addition of Cu, net Na^+ loss rate increased in most experimental waters (Figure 1a), whereas ammonia excretion rate decreased in all water types (Figure 2a). Our final hypothesis (vi) was not confirmed. In contrast to prediction, urea-N excretion rate followed the response pattern of ammonia excretion, also decreasing in experimental conditions that disturbed Na^+ balance (Figure 3), suggesting some commonality in the mechanism(s) involved.

4.2 | Water chemistry greatly affects the speciation of Cu

Speciation modelling showed that, in most of the experimental waters, the most important Cu form was Cu bound to DOC, which was most often followed by $\%Cu^{2+}$ as the second-largest component. There was a higher $\%Cu^{2+}$ and a lower %Cu-DOC at pH 4 compared to pH 7 (Table 2). Control + Cu had the highest $\%Cu^{2+}$, followed by RS + Cu. RN + Cu had the lowest $\%Cu^{2+}$ and the highest %Cu-DOC at both pH values, reflecting differences in absolute DOC concentrations (Table 2). As mentioned in the 'Methods' section, an important caveat with speciation modelling is that it treats all DOCs the same. Although we did run two scenarios, considering all DOC as 100% fulvic acid or as 100% humic acid, it had only minor effects on the outcome. From previous optical data (Holland et al., 2017; Sadauskas-Henrique et al., 2025), it is clear that the compositions of RN and RS DOCs are quite different. As the composition varies, so does the protective nature and metal-binding capacity of the DOC (Al-Reasi et al., 2011; Al-Reasi et al., 2013; Morris et al., 2024; Sadauskas-Henrique et al., 2019). This is mirrored in the differences in the physiological responses to RN and RS DOCs in the presence and absence of Cu at neutral and acidic pH, in this study and in previous work (Crémazy et al., 2016, 2019, 2022). Although speciation modelling is valuable in predicting metal toxicity, it is important to recall that the differences in DOCs, which certainly influence the physiological response and metal binding capacity, are not reflected in the model.

4.3 | RN DOC provides protection against ionoregulatory disturbances caused by low pH or Cu

Fish species in the order Cichliformes, including dwarf cichlids, have a unique, pH-sensitive, low-affinity ion transport system. This system allows for generally low rates of Na^+ uptake and greatly limits but not eliminatesa net ion loss at low pH (Duarte et al., 2013; Gonzalez et al., 2002, 2006; Val & Wood, 2022; Wood et al., 2003). This results in an energetically economical ionoregulatory strategy (Gonzalez and Dunson, 1987). Our results (Figure 1) agreed with the response pattern shown in discus angelfish (Duarte et al., 2013) and other RN cichlids (Gonzalez et al., 2002, 2006).

In control water, we observed an increase in net Na^+ and Cl^- loss rates with the addition of Cu; however, this response was much less in RN water (Figure 1). A similar trend was reported by Crémazy et al. (2022) in the dwarf cichlid. Generally, for each measured ion, there was a predictable response trend for control water and RS water whereby both low pH and the addition of Cu increased net ion loss (Figure 1). However, with RN water the response trend differed from that of control and RS waters, and there were no significant changes in net ion flux rate for any of the ions measured. It is possible that RN DOC mitigated the negative effect of both low pH (by physiological actions) and added Cu (by complexation of Cu^{2+}).

4.4 | Cu decreases water transport through tight junctions and transcellularly as a function of water chemistry

Diffusive water flux was used as a proxy for transcellular water movement (Figure 3a) and gill PEG clearance rate (Figure 3b) as a proxy for water movement through tight junctions. These data provide only the second measurements of both water transport pathways in the same Amazonian fish. As in that study on the Amazonian oscar (*A. ocellatus*, Agassiz, 1831) (Wood et al., 2009) where severe hypoxia greatly decreased diffusive water permeability whereas PEG permeability remained unchanged, the current data show inconsistencies in pH and Cu responses between the two metrics of water flux. Nevertheless, in both studies, the diffusive permeability was more than an order of magnitude greater than PEG permeability, and the patterns did not coincide with those observed for ion fluxes (cf. Figure 1). It would appear that water fluxes are uncoupled from ion fluxes in response to the environmental challenges of the present experiments. One caveat in the interpretation of our results is that to the best of our knowledge, there is no information on whether Cu and/or low pH can differentially affect gill ^{3}[H]PEG-4000 permeability relative to true gill paracellular water permeability.

Generally, DOCs that are rich in humic acids have long aliphatic chains, which contribute to the surfactant properties of the molecule (Thurman, 1985). As mentioned in the 'Introduction', previous optical data have shown that RN DOC has more relative %humic-acid-like fluorescent signatures compared to RS (Holland et al., 2017). In a prior study on marine flatfish, DOCs that were more rich in humic acid increased the diffusive water flux rate (Morris et al., 2024). Similarly, in the present study we observed a significant increase in diffusive water flux rate between pH 7 for control water and pH 7 for RN water (Figure 3a), likely due to humic acid concentration.

In both control water at pH 4 and in RN water at pH 7, there were significant decreases in diffusive water flux rate with the addition of Cu (Figure 3a). The same also occurred with PEG permeability in RN water at both pH 7 and pH 4 with the addition of Cu. This suggests that Cu may reduce both transcellular and paracellular permeabilities to water. This conclusion fits with the well-known ability of copper to block aquaporins (i.e. diffusive water permeability) (Zelenina et al., 2004) but is inconsistent with the equally well-known ability of Cu to disrupt tight junctions (Ferruzza et al., 1999), thereby increasing paracellular permeability. On the contrary, Cu has been shown to decrease the relative expression of important messenger RNA tight-junction markers in the gill, including occludin, claudin b, claudin 3 and claudin 12 (Wang et al., 2015). Because our data are the first showing the effect of Cu on both water transport pathways in any fish species, further investigation is required.

The drinking rates of dwarf cichlids were relatively high (5–15 mL kg^{-1} h^{-1}, Figure 3g–i). Although it is often thought that freshwater fish do not drink, this is not true, especially in small fish, as reviewed by Al-Reasi et al. (2016) who reported drinking rates of 2–4 mL kg^{-1} h^{-1} in zebrafish of similar size at slightly lower temperature than the present study. High drinking rates in the present dwarf cichlids were likely due to both the high temperature (29°C) and very small size (mean: 268 mg). Using a standard allometric scaling coefficient of 0.75, a drinking rate of 15 mL kg^{-1} h^{-1} in a typical 268-mg dwarf cichlid would scale to a rate of only 1.9 mL kg^{-1} h^{-1} in a 1-kg fish. Particularly, in partial agreement with the present study, Al-Reasi et al. (2016) reported no effects of different DOC types in combination with low pH on drinking rate in zebrafish, though they did not see an inhibitory effect of pH alone in control water.

4.5 | Cu tends to inhibit nitrogenous waste excretion rates

An increase in ammonia excretion rate at low pH in the presence of RN DOC has been previously reported in stingrays (Wood et al., 2003), tambaqui (Sadauskas-Henrique et al., 2019) and zebrafish (Sadauskas-Henrique et al., 2021). Our data show a slight increase in mean ammonia excretion rate between RN at pH 7 and RN at pH 4 although not statistically significant (Figure 2b). It has been suggested that DOC helps maintain Na$^+$ coupling to ammonia excretion (Duarte et al., 2016) via the Rh metabolon (Wright & Wood, 2009; Wright & Wood, 2012). Our data support this as we observed no statistically significant changes in net Na$^+$ flux rate between RN pH 7 and RN pH 4 but noted a trend of decreased net Na$^+$ loss at pH 4 (Figure 1b). Indeed, between pH 7 and pH 4 in both control water and RS water there was a trend of increased net Na$^+$ loss that corresponded to a slight decrease in ammonia excretion rate (Figures 1a,c and 2a,c). It has been suggested,that the composition of the DOCs will influence both the ammonia and urea-N excretion rates, at least in sea water (Morris et al., 2024). DOCs with a higher amino acid concentration resulted in a higher ammonia excretion rate (Morris et al., 2024). Indeed, RS DOC has previously been reported to have a higher % amino acid-like content (tyrosine and tryptophan) compared to RN DOC (Sadauskas-Henrique et al., 2025). Coincidently we observed a slight increase in the mean ammonia excretion rates between control water and RS water.

In both freshwater and seawater fish, a common response to Cu exposure is inhibition of ammonia excretion (Laurén and McDonald, 1985; Grosell et al., 2003, 2004; Blanchard & Grosell, 2006, Zimmer et al., 2012). Indeed, Lim et al. (2015) reported that Cu inhibits both ammonia uptake and excretion in juvenile rainbow trout at the same Cu concentration used in the present study. This implicated bidirectional Rh glycoproteins as a target for Cu toxicity (Lim et al., 2015). In agreement, our study showed that the addition of Cu to each water type at both pH values resulted in a trend of inhibition of ammonia excretion (Figure 2a–c).

Although the extent of urea movement through the transcellular pathway in freshwater fish remains unclear, there is evidence that many species have urea transporters (UT) that facilitate diffusive transport of urea through the gills (McDonald et al., 2006, 2012). It has been suggested that amino-acid-rich DOCs not only increase ammonia excretion rates as described earlier but also decrease urea-N excretion rates (Morris et al., 2024). There was a statistically significant ($p < 0.0001$) reduction in urea-N excretion rate between control pH 7 and RS pH 7 and a significant trend between control pH 4 and RS pH 4 (Figure 2d,f).

With regard to the effect of Cu, the urea-N excretion rate response pattern (Figure 2d–f) mirrored that of ammonia exertion rate (Figure 2a--c). Cu consistently, although not always significantly, inhibited urea-N excretion. This was contrary to what we had predicted. This inhibitory effect of Cu on both ammonia and urea-N excretion does not seem to relate to the %Cu^{2+} (free ion), as the inhibitions of excretion rates do not become greater when there was a higher %Cu^{2+}, at low pH (Table 2; Figure 2). It is possible that Cu is acting directly to inhibit the UTs as well as the Rh proteins, as suggested earlier. Alternately, or additionally, Cu inhibition of aquaporins may be involved, as argued earlier for the inhibitory effects of Cu on diffusive water fluxes. It has been reported that some aquaporin subtypes found in teleost gills have the ability to transport both ammonia and urea-N in vitro (Chen et al., 2010; Cutler et al., 2007; Ip et al., 2013; Kolarevic et al., 2012; Tipsmark et al., 2010). However, these measurements showing the effect of Cu on urea-N excretion are the first to the best of our knowledge, so further studies will be necessary.

4.6 | The properties of DOC influence the effect of pH and Cu on osmoregulation

The present work shows that the detrimental effects of Cu on osmoregulatory processes in dwarf cichlids depend on the water composition and pH. The present study focused on the effects of pH, Cu and DOCs within natural waters. It is important to recognize that, in an effort to truly represent the response differences induced by the natural waters, we used intact waters and did not experimentally manipulate the individual aspects of their composition such as the concentrations of DOC and individual ions. These could also be important in the physiological responses observed and should be evaluated in future studies. Increases in anthropogenic activities, including mining, agriculture and industrial practices, have led to elevation in metal pollutants in Amazonian water, including Cu (Moulatlet et al., 2023; Braz-Mota et al., 2024; Echevarría et al. 2024). Based on our findings, increases in Cu in the RN, at the natural low pH of this river, would not be too problematic for the fish species used in this study. However, the same concentration of dissolved Cu in the RS, particularly at low pH, would be a greater threat to this fish and possibly other local species. In that respect, the particles that are normally abundant in RS water (but removed in our experiments) provide protection by greatly reducing the dissolved Cu concentration (Crémazy et al., 2016; Crémazy et al., 2019; Crémazy et al., 2025). The greater protection provided by RN against dissolved Cu is likely due to its higher concentration of DOC (Table 1) as well as the different DOC compositions (Holland et al., 2017). The Cu speciation results can, in some cases, explain the physiological effects of Cu, simply based on the bioavailability of the free Cu^{2+} ions. However, there were also physiological functions for which the addition of Cu had pronounced inhibitory effects even when there was negligible free Cu^{2+} present. Indeed, the regression analyses showed significant negative correlation between net Na^+, K^+ and Cl^- flux rates against %free Cu^{2+} ions (Figure 4) but no significant relationships between any of the other physiological parameters measured and %free Cu^{2+} ions (Figure S1). Because our study is the first to provide data on these negative actions of Cu on urea-N excretion rates and on both water transport pathways through the gills, additional mechanistic studies are necessary. Further knowledge of the mechanism(s) by which Cu disturbs the osmoregulatory processes and the role of water chemistry may help inform guidelines for Cu pollution levels in different areas across the Amazon basin.

AUTHOR CONTRIBUTIONS

Carolyn Morris: conceptualization, resources, formal analysis, investigation, methodology, validation, visualization, writing – original draft, writing – review and editing. Anne Crémazy: conceptualization, investigation, methodology, writing – review and editing. Jhonatan Mota da Silva: investigation, writing – review and editing. Colin J. Brauner: conceptualization advice, writing – review and editing. Ora E. Johannsson: investigation, writing – review and editing. Chris M. Wood: conceptualization, methodology, resources, writing – review and editing. Adalberto Luis Val: project administration, funding acquisition, resources, writing – review.

ACKNOWLEDGEMENTS

We thank two anonymous reviewers for their constructive comments that improved the manuscript.

FUNDING INFORMATION

Funded by the Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery Grants to Chris M. Wood (RGPIN-2023-03714) and Anne Crémazy (RGPIN-2019-04400). Carolyn Morris also received an NSERC CGS-D Post-graduate Scholarship. This study was partially funded by CNPq (Brazilian National Research Council), CAPES (Coordination of Superior Level Staff Improvement) and FAPEAM (Amazonas State Research Foundation) via funding for INCT ADAPTA (CNPQ process no. 465540/2014-7, CAPES – finance code 001, and FAPEAM process 062.01187/2017) to Adalberto Luis Val. Adalberto Luis Val is the recipient of a research fellowship from CNPq.

ORCID

Carolyn Morris https://orcid.org/0000-0002-7254-8433
Anne Crémazy https://orcid.org/0000-0002-0918-2336
Jhonatan Mota da Silva https://orcid.org/0000-0001-6907-0507
Ora E. Johannsson https://orcid.org/0000-0002-7944-379X
Colin J. Brauner https://orcid.org/0000-0002-3695-7707
Chris M. Wood https://orcid.org/0000-0002-9542-2219
Adalberto Luis Val https://orcid.org/0000-0002-3823-3868

REFERENCES

Al-Reasi, H. A., Smith, D. S., & Wood, C. M. (2016). The influence of dissolved organic matter (DOM) on sodium regulation and nitrogenous waste excretion in the zebrafish (*Danio rerio*). *Journal of Experimental Biology*, *219*, 2289–2299.

Al-Reasi, H. A., Wood, C. M., & Smith, D. S. (2011). Physicochemical and spectroscopic properties of natural organic matter (NOM) from various sources and implications for ameliorative effects on metal toxicity to aquatic biota. *Aquatic Toxicology*, *103*(3–4), 179–190.

Al-Reasi, H. A., Wood, C. M., & Smith, D. S. (2013). Characterization of freshwater natural dissolved organic matter (DOM): Mechanistic explanations for protective effects against metal toxicity and direct effects on organisms. *Environment International*, *59*, 201–207.

Blanchard, J., & Grosell, M. (2006). Copper toxicity across salinities from freshwater to seawater in the euryhaline fish *Fundulus heteroclitus*: Is copper an ionoregulatory toxicant in high salinities? *Aquatic Toxicology*, *80*, 131–139.

Braz-Mota, S., Duarte, R. M., & Val, A. L. (2024). Toxicological impacts of pesticides and heavy metals on amazon fish: Trends and future perspectives. In S. S. de Souza, S. Braz-Mota, & A. L. Val (Eds.), *The future of Amazonian aquatic biota*. Springer.

Braz-Mota, S., Val, A. L., & Duarte, R. (2025). Toxicological impacts of pesticides and heavy metals on Amazon fish: Trends and future perspectives. In S. S. de Souza, S. Braz-Mota, & A. L. Val (Eds.), *The future of Amazonian aquatic biota* (pp. 313–343). Springer.

Bryan, S. E., Tipping, E., & Hamilton-Taylor, J. (2002). Comparison of measured and modelled copper binding by natural organic matter in freshwaters. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, *133*(1), 37–49.

Buffle, J. (1988). *Complexation reactions in aquatic systems: An analytical approach*. Ellis Horwood.

Chen, L. M., Zhao, J., Musa-Aziz, R., Pelletier, M. F., Drummond, I. A., & Boron, W. F. (2010). Cloning and characterization of zebrafish homologue of human AQP1: A bifunctional water and gas channel. *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology*, *299*(5), R1163–R1174.

Crémazy, A., Braz-Mota, S., Brix, K. V., Duarte, R. M., Val, A. L., & Wood, C. M. (2022). Investigating the mechanisms of dissolved organic matter protection against copper toxicity in fish of Amazon's black waters. *The Science of the Total Environment*, *843*, 157032.

Crémazy, A., Morris, C., Braz-Mota, S., Wood, C. M., Duarte, R. M., Johannsson, O. E., & Val, A. L. (2025). *Joint effects of elevated copper and temperature in juvenile Tambaqui exposed in black and white waters of the Amazon*. Submitted.

Crémazy, A., Wood, C. M., Smith, D. S., Ferreira, M. S., Johannsson, O. E., Giacomin, M., & Val, A. L. (2016). Investigating cooper toxicity in the tropical fish cardinal tetra (*Paracheirodon axelrodi*) in natural Amazonian waters: Measurements, modeling, and reality. *Aquatic Toxicology*, *180*, 353–363.

Crémazy, A., Wood, C. M., Smith, D. S., & Val, A. L. (2019). The effects of natural suspended solids on copper toxicity to the cardinal tetra in Amazonian river waters. *Environmental Toxicology and Chemistry*, *38*(12), 2708–2718.

Cutler, C. P., Martinez, A. S., & Cramb, G. (2007). The role of aquaporin 3 in teleost fish. *Comparative Biochemistry and Physiology A*, *148*, 82–91.

Di Toro, D. M., Allen, H. E., Bergman, H. L., Meyer, J. S., Paquin, P. R., & Santore, R. C. (2001). Biotic ligand model of the acute toxicity of metals. 1. Technical basis. *Environmental Toxicology and Chemistry*, *20*(10), 2383–2396.

Duarte, R. M., Cremazy, A., Wood, C. M., Almeida-Val, V. M. F., & Val, A. L. (2024). The biotic ligand model as a promising tool to predict Cu toxicity in Amazon blackwaters. *Environmental Pollution*, *341*, 122988.

Duarte, R. M., Ferreira, M. S., Wood, C. M., & Val, A. L. (2013). Effect of low pH exposure on Na^+ regulation in two cichlid fish species of the Amazon. *Comparative Biochemistry and Physiology A*, *166*, 441–448.

Duarte, R. M., Menezes, A. C. L., da Silveira Rodrigues, L., de Almeida-Val, V. M. F., & Val, A. L. (2009). Copper sensitivity of wild ornamental fish of the Amazon. *Ecotoxicology and Environmental Safety*, *72*, 693–698.

Duarte, R. M., Smith, D. S., Val, A. L., & Wood, C. M. (2016). Dissolved organic carbon from the upper Rio Negro protects zebrafish (*Danio rerio*) against ionoregulatory disturbances caused by low pH exposure. *Scientific Reports*, *6*, 20377.

Duarte, R. M., Wood, C. M., Val, A. L., & Smith, D. S. (2018). Physiological protective action of dissolved organic carbon on ion regulation and nitrogenous waste excretion of zebrafish (*Danio rerio*) exposed to low pH in ion poor water. *Journal of Comparative Physiology B*, *188*, 793–807.

Echevarría, G., Lujan, N. K., Montoya, J., Granda-Albuja, M. G., Valdiviezo-Rivera, J., Sánchez, F., Cuesta, F., & Ríos-Touma, B. (2024). Abiotic and biotic factors influencing heavy metals pollution in fisheries of the Western Amazon. *Science of The Total Environment*, *908*, 168506. https://doi.org/10.1016/j.scitotenv.2023.168506

Ferruzza, S., Scarino, M. L., Rotilio, G., Ciriolo, M. R., Santaroni, P., Muda, A. O., & Sambuy, Y. (1999). Copper treatment alters the permeability of tight junctions in cultured human intestinal Caco-2 cells. *American Journal of Physiology. Gastrointestinal and Liver Physiology*, *277*(6), G1138–G1148.

Furch, K. (1984). Water chemistry of the Amazon basin: The distribution of chemical elements among freshwaters. In H. Sioli (Ed.), *The Amazon: Limnology and landscape ecology of a mighty Tropical River and its basin* (pp. 167–199). Junk.

Galvez, F., Donini, A., Playle, R. C., Smith, D. S., O'Donnell, M. J., & Wood, C. M. (2008). A matter of potential concern: Natural organic matter alters the electrical properties of fish gills. *Environmental Science and Technology*, *42*, 9385–9390.

Gandois, L., Hoyt, A., Mounier, S., Le Roux, G., Harvey, C., Claustres, A., Mohammed Nuriman, M., & Anshari, G. (2020). From canals to the coast: Dissolved organic matter and trace metal composition in rivers draining degraded tropical peatlands in Indonesia. *Biogeosciences*, *17*, 1897–1909.

Glover, C. N., Pane, E. F., & Wood, C. M. (2005). Humic substances influence sodium metabolism in the freshwater crustacean *Daphnia magna*. *Physiological and Biochemical Zoology*, *78*(3), 405–416.

Glover, C. N., & Wood, C. M. (2005). Physiological interactions of silver and humic substances in *Daphnia magna*: Effects on reproduction and silver accumulation following an acute silver challenge. *Comparative Biochemistry and Physiology, Part C: Toxicology & Pharmacology*, *139*, 273–280.

Gonzalez, R. J., & Dunson, W. A. (1987). Adaptations of sodium balance to low pH in a sunfish (Enneacanthus obesus) from naturally acidic waters. *Journal of Comparative Physiology B*, *157*(5), 555–566. https://doi.org/10.1007/bf00700975

Gonzalez, R. J., Wilson, R. W., & Wood, C. M. (2006). Ionoregulation in tropical fish from ion-poor, acidic blackwaters. In A. L. Val, V. M. Almeida-Val, & D. J. Randall (Eds.), *The physiology of tropical fish, fish physiology* (Vol. 22, pp. 397–437). Academic Press.

Gonzalez, R. J., Wood, C. M., Patrick, M. L., & Val, A. L. (2002). Diverse strategies for ionregulation in fish collected from the ion-poor, acidic Rio Negro. *Physiological and Biochemical Zoology*, *75*(1), 37–47.

Gonzalez, R. J., Wood, C. M., Wilson, R. W., Patrick, M. L., Bergman, H. L., Narahara, A., & Val, A. L. (1998). Effects of water pH and Ca^{2+} concentration on ion balance in fish of the Rio Negro, Amazon. *Physiological Zoology*, *71*, 15–22.

Grosell, M., McDonald, M. D., Walsh, P. J., & Wood, C. M. (2004). Effects of prolonged copper exposure in the marine gulf toadfish (*Ospanus *beta*) II: Copper accumulation, drinking rate and Na^+/K^+-ATPase activity in osmoregulatory tissues. *Aquatic Toxicology*, *68*, 263–275.

Grosell, M., Nielsen, C., & Bianchini, A. (2002). Sodium turnover rate determines sensitivity to acute copper and silver exposure in freshwater animals. *Comparative Biochemistry and Physiology*. *C*, *133*, 287–303.

Grosell, M., Wood, C. M., & Walsh, P. J. (2003). Copper homeostasis and toxicity in the elasmobranch *Raja erinacea* and the teleost *Myoxecephalus octodecemspinosus* during exposure to elevated water-borne copper. *Comparative Biochemistry and Physiology*. *C*, *135*, 179–190.

Holland, A., Wood, C. M., Smith, D. S., Gabriel, T., & Val, A. L. (2017). Nickel toxicity to cardinal tetra (*Paracheirodon axelrodi*) differs seasonally and among the black, white and clear river waters of the Amazon Basin. *Water Research*, *123*, 21–29.

Hollis, L., Hogstrand, C., & Wood, C. M. (2001). Tissue-specific cadmium accumulation, metallothionein induction, and tissue zinc and copper levels during chronic sublethal cadmium exposure in juvenile rainbow trout. *Archives of Environmental Contamination and Toxicology*, *41*(4), 468–474.

Holmes, W. N., & Donaldson, E. M. (1969). Body compartments and distribution of electrolytes. In W. S. Hoar & D. J. Randall (Eds.), *Fish physiology*, *vol 1* (pp. 1–89). Academic.

Ip, Y. K., Soh, M. M. L. X. L., Chen, K., Ong, J. L. Y., Chng, Y. R., Ching, B., Wong, W. P., Lam, S. H., & Chew, S. F. (2013). Molecular characterization of branchial aquaporin 1aa and effects of seawater acclimation emersion or ammonia exposure on its mRNA expression in the gills, gut, kidney and skin of freshwater climbing perch. *Anabas Testudineus*. *Plos*, *8*(4), 61162.

Isaia, J. (1984). Water and nonelectrolyte permeability. In W. S. Hoar & D. J. Randall (Eds.), *Fish physiology*, *vol 10*, *Part B* (pp. 1–38). Academic.

Johannsson, O. E., & Holland, A. (2025). Dissolved organic carbon (DOC) of the Rio Negro, Amazonia: Importance, processes and potential anthropogenic impacts. In S. S. de Souza, S. Braz-Mota, & A. L. Val (Eds.), *The future of Amazonian aquatic biota* (pp. 49–75). Springer.

Kolarevic, J., Takle, H., Felip, O., Ytteborg, E., Selset, R., Good, C. M., Baeverfjord, G. T., Asgard, T., & Terjes, B. F. (2012). Molecular and physiological responses to long term sublethal ammonia exposure in Atlantic salmon (*Salmo salar*). *Aquatic Toxicology*, *124*(125), 48–57.

Kwong, R. W., & Perry, S. F. (2013). The tight junction protein claudin-b regulates epithelial permeability and sodium handling in larval zebrafish, *Danio rerio*. *American Journal of Physiology*. *Regulatory*, *Integrative and Comparative Physiology*, *304*(7), R504–R513.

Laurén, D. J., & McDonald, D. G. (1985). Effects of copper on branchial ionoregulation in the rainbow trout, Salmo gairdneri Richardson. *Journal of Comparative Physiology B*, *155*(5), 635–644. https://doi.org/10.1007/bf00694455

Lim, M., Zimmer, A. M., & Wood, C. M. (2015). Acute exposure to waterborne copper inhibits both the excretion and uptake of ammonia in freshwater rainbow trout (*Oncorhynchus mykiss*). *Comparative Biochemistry and Physiology*. *C*, *168*, 48–54.

Lofts, S., & Tipping, E. (2011). Assessing WHAM/Model VII against field measurements of free metal ion concentrations: Model performance and the role of uncertainty in parameters and inputs. *Environmental Chemistry*, *8*(5), 501. https://doi.org/10.1071/en11049

Matsuo, A. Y. O., Playle, R. C., Val, A. L., & Wood, C. M. (2004). Physiological action of dissolved organic matter in rainbow trout in the presence and absence of copper: Sodium uptake kinetics and unidirectional flux rates in hard and soft water. *Aquatic Toxicology*, *70*(1), 63–81.

Mattigod, S., & Sposito, G. (1979). *Chemical Modeling of Trace Metal Equilibria in Contaminated Soil Solutions Using the Computer Program Geochem* (Technical Report HEEP/80/03271; Numéro HEEP/80/03271).

McDonald, M. D., Gilmour, K. M., & Walsh, P. J. (2012). New insights into the mechanisms controlling urea excretion in fish gills. *Respiratory Physiology & Neurobiology*, *184*(3), 241–248.

McDonald, M. D., Smith, C. P., & Walsh, P. J. (2006). The physiology and evolution of urea transport in fishes. *Journal of Membrane Biology*, *212*(2), 93–107.

Morris, C., Val, A. L., Brauner, C. J., & Wood, C. M. (2021). The physiology of fish in acidic waters rich in dissolved organic carbon, with specific reference to the Amazon basin:I onoregulation, acid–base regulation, ammonia excretion, and metal toxicity. *Journal of Experimental Zoology Part A*, *Ecological and Integrative Physiology*, *335*(9–10), 843–863.

Morris, C., Zulian, S., Brauner, C. J., Smith, D. S., & Wood, C. M. (2024). A critical assessment of physicochemical indices used to characterize natural dissolved organic carbon, their inter-relationships, and the effects of pH. *Environmental Chemistry*, *21*, EN24021.

Moulatlet, G. M., Yacelga, N., Rico, A., Mora, A., Hauser-Davis, R. A., Cabrera, M., & Capparelli, M. V. (2023). A systematic review on metal contamination due to mining activities in the Amazon basin and associated environmental hazards. *Chemosphere*, *339*(139), 700.

Niyogi, S., & Wood, C. M. (2004). Biotic ligand model, a flexible tool for developing site-specific water quality guidelines for metals. *Environmental Science & Technology*, *38*(23), 6177–6192.

Olson, K. R. (1992). Blood and extracellular fluid volume regulation. In W. S. Hoar, D. J. Randall, & A. P. Farrell (Eds.), *Fish physiology*, *vol 12*. *Part B* (pp. 135–254). Academic.

Onukwufor, J. O., & Wood, C. M. (2020). Osmorespiratory compromise in zebrafish (*Danio rerio*): Effects of hypoxia and acute thermal stress on oxygen consumption, diffusive water flux, and sodium net loss rates. *Zebrafish*, *17*(6), 400–411.

Playle, R. C., Dixon, D. G., & Burnison, B. K. (1993). Copper and cadmium binding to fish gills: Modification by dissolved organic carbon and synthetic ligands. *Canadian Journal of Fisheries and Aquatic Sciences*, *50*, 2667–2677.

Powell, K. J., Brown, P. L., Byrne, R. H., Gajda, T., Hefter, G., Sjöberg, S., & Wanner, H. (2007). Chemical speciation of environmentally significant metals with inorganic ligands part 2: The Cu^{2+}-OH^-, Cl^-, CO_3^{2-}, SO_4^{2-}, and PO_4^{3-} systems (IUPAC technical report). *Pure and Applied Chemistry*, *79*(5), 5.

Preston, G. M., Carroll, T. P., Guggino, W. B., & Agre, P. (1992). Appearance of water channels in *Xenopus* oocytes expressing red cell CHIP28 protein. *Science*, *256*, 385–387.

Rahmatullah, M., & Boyde, T. R. (1980). Improvements in the determination of urea using diacetyl monoxime; methods with and without deproteinisation. *Clinca Chim Acta*, *107*(1–2), 3–9.

Robertson, L. M., Kochhann, D., Bianchini, A., Matey, V., Almeida-Val, V. F., Val, D., & Wood, C. M. (2015). Gill paracellular permeability and the osmorespiratory compromise during exercise in the hypoxia-tolerant Amazonian oscar (*Astronotus ocellatus*). *Comparative Biochemistry and Physiology*. *B*, *185*, 741–754.

Robertson, L. M., & Wood, C. M. (2014). Measuring gill paracellular permeability with polyethylene glycol-4000 in freely swimming trout: Proof of principle. *The Journal of Experimental Biology*, *217*(9), 1425–1429.

Robroek, B. J. M., Jassey, V. E. J., Payne, R. J., Martí, M., Bragazza, L., Bleeker, A., Buttler, A., Caporn, S. J., Dise, N. B., Kattge, J., & Zając, K. (2017). Taxonomic and functional turnover are decoupled in European peat bogs. *Nature Communications*, *8*, 1161.

Saabater, F., Meyer, J., & Edwards, R. (1993). Longitudinal patterns of dissolved organic carbon concentration and suspended bacterial density along a blackwater river. *Biogeochemistry*, *21*, 73–93.

Sadauskas-Henrique, H., de Souza-Bastos, L. R., Smith, D. S., Val, A. L., & Wood, C. M. (2025). Dissolved organic carbon from “white” waters in Amazon basin (Brazil) and the osmoregulatory responses of tambaqui (*Colossoma macropomum*) in neutral and acidic environments'. *Journal of Fish Biology*, 1–12.

Sadauskas-Henrique, H., Smith, D. S., Val, A. L., & Wood, C. M. (2021). Physicochemical properties of the dissolved organic carbon can lead to different physiological responses to zebrafish (*Danio rerio*) when challenged by acidic water: Effects of the acclimation under circumneutral pH. *Journal of Experimental Zoology A*, *35*(9–10), 864–878.

Sadauskas-Henrique, H., Wood, C. M., Souza-Bastos, L. R., Duarte, R., Smith, D., & Val, A. L. (2019). Does dissolved organic carbon from Amazon black water (Brazil) help a native species, the tambaqui *Colossoma macropomum* to maintain ionic homeostasis in acidic water? *Journal of Fish Biology*, *94*, 595–605.

Thurman, E. (1985). Geochemistry of natural waters. In N. Martinus & W. Junk (Eds.), *Boston*. Dordrecht.

Tipsmark, C. K., Sorensen, K. J., & Madsen, S. S. (2010). Aquaporin expression dynamics in osmoregulatory tissues of Atlantic salmon during smoltification and seawater acclimation. *Journal of Experimental Biology*, *213*, 368–379.

Val, A. L., & de Almeida-Val, V. M. F. (1995). *Fishes of the Amazon and their environment: Physiological and biochemical aspects*. Springer-Verlag.

Val, A. L., & Wood, C. M. (2022). Global change and physiological challenges for fish of the Amazon today and in the near future. *Journal of Experimental Biology*, *225*(10), jeb216440.

Verdouw, H., Echteld, C., & Dekkers, E. (1978). Ammonia determination based on indophenol formation with sodium salicylate. *Water Research*, *12*, 399–402.

Walker, I., & Henderson, P. A. (1996). Ecophysiological aspects of Amazonian blackwater. Litterbank fish communities. In A. L. Val, V. M. F. Almeida-Val, & D. J. Randall (Eds.), *Physiology and biochemistry of fishes of the Amazon* (pp. 7–22). Instituto Nacional de Pesquisas da Amazonia (INPA).

Wang, B., Feng, L., Jiang, W. D., Wu, P., Kuang, S., Jiang, J., Tang, L., Tang, W., Zhang, Y.-A., Liu, W., & Zhou, X. (2015). Copper-induced tight junction mRNA expression changes, apoptosis and antioxidant responses via NF-κB, TOR and Nrf2 signaling molecules in the gills of fish: Preventive role of arginine. *Aquatic Toxicology*, *158*, 125–137.

Wood, C. M. (1992). Flux measurements as indices of H^+ and metal effects on freshwater fish. *Aquatic Toxicology*, *22*, 239–264.

Wood, C. M., Al-Reasi, H. A., & Smith, D. S. (2011). The two faces of DOC. *Aquatic Toxicology*, *105*, 3–8.

Wood, C. M., Iftikar, F. I., Scott, G. R., De Boeck, G., Sloman, K. A., Matey, V., Valdez Domingos, F. A., Mendonça Duarte, R., Almeida-Val, V. M. F., & Val, A. L. (2009). Regulation of gill transcellular permeability and renal function during acute hypoxia in the amazonian oscar (*Astronotus ocellatus*): New angles to the osmo-respiratory compromise. *Journal of Experimental Biology*, *212*, 1949–1964.

Wood, C. M., Matsuo, A. Y. O., Gonzalez, R. J., Wilson, R. W., Patrick, M. L., & Val, A. L. (2002). Mechanisms of ion transport in *Potamotrygon*, a stenohaline freshwater elasmobranch native to the ion-poor blackwaters of the Rio Negro. *The Journal of Experimental Biology*, *205*(19), 3039–3054.

Wood, C. M., Matsuo, A. Y. O., Wilson, R. W., Gonzalez, R. J., Patrick, M. L., Playle, R. C., & Val, A. L. (2003). Protection by natural blackwater against disturbances in ion fluxes. Caused by low pH exposure in freshwater stingrays endemic to the Rio Negro. *Physiological and Biochemical Zoology*, *76*(1), 12–27.

Wood, C. M., McDonald, M. D., Grosell, M., Mount, D. R., Adams, W. J., Po, B. H., & Brix, K. V. (2020). The potential for salt toxicity: Can the trans-epithelial potential (TEP) across the gills serve as a metric for major ion toxicity in fish? *Aquatic Toxicology*, *95*, 105–568.

Wood, C. M., Ruhr, I. M., Schauer, K. L., Wang, Y., Mager, E. M., McDonald, M. D., Stanton, B., & Grosell, M. (2019). The osmorespiratory compromise in the euryhaline killifish: Water regulation during hypoxia. *Journal of Experimental Biology*, *222*(18), jeb204818.

Wright, P. A., & Wood, C. M. (2009). A new paradigm for ammonia excretion in aquatic. Animals: Role of rhesus (RH) glycoproteins. *Journal of Experimental Biology*, *212*(15), 2303–2312.

Wright, P. A., & Wood, C. M. (2012). Seven things fish know about ammonia and we don't. *Respiratory Physiology & Neurobiology*, *184*, 231–240.

Zall, D. M., Fisher, D., & Garner, M. Q. (1956). Photometric determination of chlorides in water. *Analytical Chemistry*, *28*(11), 1665–1668.

Zelenina, M., Tritto, S., Bondar, A. A., Zelenin, S., & Aperia, A. (2004). Copper inhibits the water and glycerol permeability of aquaporin-3. *Journal of Biological Chemistry*, *279*(50), 51939–51943.

Zimmer, A. M., Barcarolli, I. F., Wood, C. M., & Bianchini, A. (2012). Waterborne copper exposure inhibits ammonia excretion and branchial carbonic anhydrase activity in euryhaline guppies acclimated to both fresh water and sea water. *Aquatic Toxicology*, *122–123*, 172–180.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Morris, C., Crémazy, A., Mota da Silva, J., Johannsson, O. E., Brauner, C. J., Wood, C. M., & Val, A. L. (2025). The acute osmoregulatory effects of low pH and Cu, alone and in combination, on the dwarf cichlid (*Apistogramma agassizii*) in Rio Negro blackwater and Rio Solimões whitewater: Flux rates of ions, nitrogenous wastes and water. *Journal of Fish Biology*, 1–17. https://doi.org/10.1111/jfb.70157