Investigating the mechanisms of dissolved organic matter protection against copper toxicity in fish of Amazon's black waters

Anne Crémazy [a,*], Susana Braz-Mota [b], Kevin V. Brix [c,d], Rafael M. Duarte [e], Adalberto L. Val [b], Chris M. Wood [f]

[a] Institut National de la Recherche Scientifique, Centre Eau Terre Environnement, Quebec City, QC G1K 9A9, Canada
[b] Laboratory of Ecophysiology and Molecular Evolution, Brazilian National Institute for Research of the Amazon (INPA), Manaus, AM 69067-375, Brazil
[c] EcoTox LLC, Miami, FL, United States
[d] University of Miami, RSMAS, Miami, FL, United States
[e] Biosciences Institute, São Paulo State University - UNESP, Coastal Campus, São Vicente, SP, Brazil
[f] Department of Zoology, University of British Columbia, Vancouver, BC V6T 1Z4, Canada

HIGHLIGHTS

- Dissolved organic matter (DOM) from Rio Negro protected fish against acute Cu toxicity.
- There was no evidence of physiological protection from DOM against Cu-induced Na^+ loss.
- The nature of DOM protection seemed purely geochemical (from Cu-DOM complexation).
- Copper exposure led to similar Na^+ loss in the cardinal tetra and the dwarf cichlid.
- Copper effects on unidirectional Na^+ fluxes reflected fish distinct ion regulation strategies.

GRAPHICAL ABSTRACT
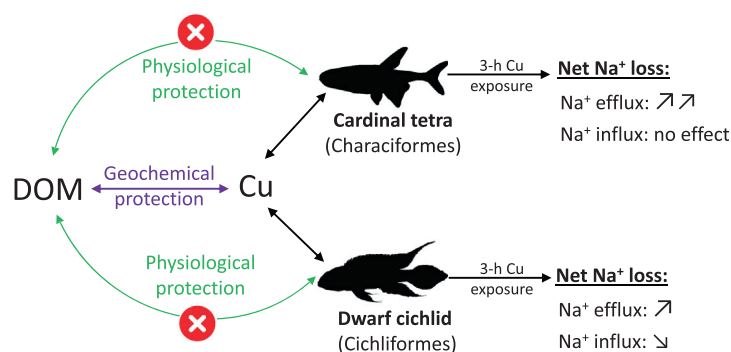
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ABSTRACT

We investigated how natural dissolved organic matter (DOM) of the Rio Negro (Amazon) affects acute copper (Cu) toxicity to local fish: the cardinal tetra (*Paracheirodon axelrodi*) and the dwarf cichlid (*Apistogramma agassizii*). It is established that Cu^{2+} complexation with DOM decreases Cu bioavailability (and thus toxicity) to aquatic organisms, as conceptualized by the Biotic Ligand Model (BLM). However, we also know that Rio Negro's DOM can interact with fish gills and have a beneficial effect on Na^+ homeostasis, the main target of acute Cu toxicity in freshwater animals. We aimed to tease apart these potential protective effects of DOM against Cu-induced Na^+ imbalances in fish. In the laboratory, we acclimated fish to Rio Negro water (10 mg L^{-1} DOC) and to a low-DOM water (1.4 mg L^{-1} DOC) with similar ion composition and pH (5.9). We measured 3-h Cu uptake in gills and unidirectional and net Na^+ physiological fluxes across a range of Cu concentrations in both waters. Various DOM pre-acclimation times (0, 1 and 5 days) were evaluated in experiments with *P. axelrodi*. Copper exposure led to similar levels of net Na^+ loss in the two fish, but with distinct effects on Na^+ influx and efflux rates reflecting their different ionoregulation strategies. Rio Negro DOM protected against Cu uptake and toxicity in the two fish species. Both Cu uptake in fish gills and Na^+ regulation disturbances were relatively well predicted by the modelled aqueous free Cu^{2+} ion concentration. These findings suggest that protection by DOM occurs mainly from Cu complexation under the tested conditions. The prevalence of this geochemical-type protection over a physiological-type protection agrees with the BLM conceptual framework, supporting the use of the BLM to assess the risk of Cu in these Amazonian waters.

* Corresponding author.
  E-mail address: anne.cremazy@inrs.ca (A. Crémazy).

1. Introduction

Dissolved organic matter (DOM) decreases metal bioavailability and thus toxicity to aquatic organisms, by complexing free metal ions in natural waters (Campbell, 1995; Playle et al., 1993; Tipping, 2002). Thus, DOM concentration is a critical input parameter in bioavailability-based models used to assess the environmental risk of metals under local water chemistry conditions (e.g. the Biotic Ligand Model, BLM) (Di Toro et al., 2001; Niyogi and Wood, 2004; Paquin et al., 2002). Notably, a copper (Cu) BLM is currently used to generate site-specific water quality guidelines in various jurisdictions (e.g. USEPA, EU Water Framework Directive) (Campbell et al., 2006; USEPA, 2007). This modulating effect of DOM is particularly important for Cu, since Cu-DOM complexes typically dominate Cu speciation in natural fresh waters (Bourgeault et al., 2013).

A growing body of evidence suggests that the ameliorative role of DOM against metal toxicity might not always be strictly limited to geochemical effects, and that physiological effects may also be important. Indeed, about two decades ago, studies demonstrated that DOM molecules can directly interact with the external surface of aquatic organisms (e.g. fish gills and algal cell walls), likely via hydrogen bonding and/or hydrophobic interactions (Campbell et al., 1997; Vigneault et al., 2000). This binding of DOM onto biological surfaces appears to affect the physiology of freshwater organisms, as evidenced by various studies showing DOM helping ion regulation under acute exposure to challenging low pH and ion levels (Duarte et al., 2016, 2018; Gonzalez et al., 2005; Morris et al., 2021; Wood et al., 2003, 2011). Many of these studies have been conducted on tropical fish with natural DOM from the Rio Negro, a major tributary of the Amazon River. The Rio Negro is a typical example of blackwaters and is characterized by a low pH (typically 4–6), extremely low ion levels (major cations ≤ 40 μmol L^{-1}) and abundant optically dark DOM (measured as dissolved organic carbon (DOC) = 8–15 mg L^{-1}) (Sioli, 1968; Val and Almeida-Val, 1995). This DOM has been shown to protect Na$^+$ homeostasis in fish at pH 4 in ion-poor water by increasing Na$^+$ transport capacity, sometimes accompanied by a change in Na$^+$ affinity, and usually more importantly by reducing Na$^+$ efflux via decreased gill permeability (Duarte et al., 2016; Matsuo and Val, 2007; Morris et al., 2021; Wood et al., 2003). Exposure to Cu also impairs Na$^+$ homeostasis in freshwater fish, leading to a potentially lethal plasma Na$^+$ loss (Grosell et al., 2002). Indeed, Cu has been shown to inhibit Na$^+$ branchial influx (notably via direct competition with Na$^+$ at the apical Na$^+$ membrane channels) and to promote Na$^+$ diffusive loss (via an increase of the branchial epithelium permeability) (Grosell et al., 2002; Laurén and McDonald, 1995). In a previous study, we hypothesized that DOM in Rio Negro water could offer a physiological protection against these Cu-induced effects on ionic balance, as we observed a surprisingly low acute Cu toxicity in cardinal tetra when exposed in this water (Crémazy et al., 2016). This physiological protection might be attributed to beneficial effects on Na$^+$ branchial fluxes (Matsuo et al., 2004), but may also be the result of changes in gill-metal binding properties (Matsuo et al., 2005).

The goal of the present study was to tease apart the geochemical and the physiological components of DOM protection against Cu-induced ionoregulatory imbalance in freshwater fish, using the DOM of the Rio Negro. At the Brazilian National Institute for Amazonian Research (INPA, Manaus, Amazonas State, Brazil), we conducted a series of lab experiments to determine if Cu uptake and ionoregulatory disturbances in fish could be explained on the basis of metal bioavailability alone. For this study, we selected two Amazonian fish with comparable acute Cu sensitivities (Duarte et al., 2009) but different Na$^+$ regulation strategies (Gonzalez et al., 2002; Morris et al., 2021; Val and Wood, 2022) that may affect their responses to both Cu and DOM: the cardinal tetra (*Paracheirodon axelrodi*; Characiformes), and the dwarf cichlid (*Apistogramma agassizii*; Cichliformes). The former has high Na$^+$ affinity and high unidirectional influx and efflux rates, and the latter has low Na$^+$ affinity and low unidirectional influx and efflux rates (Val and Wood, 2022). Experiments were conducted in either freshly collected Rio Negro water (high-DOM water) or in well water from INPA (low-DOM water). Ion levels were adjusted so as to be similar in the two waters. Copper was

spiked into both waters at concentrations that are known to be acutely toxic to both fish, and the corresponding free Cu^{2+} concentrations were modelled with the Windermere Humic Aqueous Model (WHAM). We measured Cu-gill binding and unidirectional Na$^+$ fluxes in the two species exposed for 3 h to varying dissolved Cu concentrations in the two waters. We also evaluated the effects of various DOM pre-acclimation times on metal gill binding and Na$^+$ fluxes in the cardinal tetra.

2. Materials and methods

2.1. Acclimation and experimental waters

In mid-October 2018, Rio Negro water was collected in the area of Praia de Paricatuba, upstream of Manaus (S 3°5′41.5″, W 60°21′19.6″). The water was collected about 100 m from the shore, in 20-L clean plastic containers, then was brought to the Laboratory of Ecophysiology and Molecular Evolution at INPA (LEEM-INPA). The water was kept refrigerated and in the dark until 24 h prior to use, which was within 10 days of collection. The measured composition of the collected water was: pH = 5.7 ± 0.1, [DOC] = 10.1 ± 0.9 mg of C L^{-1}, (in μM): [Ca] = 6.0 ± 2.4, [Mg] = 5.3 ± 1.2, [Na] = 29 ± 3.8 and [K] = 13 ± 0.3 (mean ± SD, $n = 6$). The well water at INPA had the following composition: pH = 5.7 ± 0.1, [DOC] = 3.1 ± 0.5 mg of C L^{-1}, (in μM): [Ca] = 65 ± 3.7, [Mg] = 13 ± 0.13, [Na] = 78 ± 4.7 and [K] = 39 ± 3.2 (mean ± SD, $n = 6$). This latter composition shows that INPA well water was out of its typical levels in October 2018, since this water normally has a much lower (10-fold) DOC concentration and lower ion concentrations (2-fold for Na, 3-fold for K and Mg, 10-fold for Ca) which closely match the Rio Negro ionic composition (Crémazy et al., 2016). Thus, for the purpose of our study, the compositions of both the INPA well water and the Rio Negro water were adjusted for our tests, to i) approximately balance their ionic compositions and ii) increase the DOC concentration difference. To these ends, the INPA well water was diluted by 50 % with ultrapure water (resistivity ≥18 Mohms-cm) and the Rio Negro water was spiked with CaCl$_2$ (ACS grade, Fisher Scientific) and NaCl (ACS grade, Fisher Scientific). Hereafter, these two test waters are referred to as RN (for Rio Negro) and IW (for INPA well) waters and their compositions are given in Table 1.

2.2. Fish housing and acclimation

Cardinal tetra (*Paracheirodon axelrodi*, 150 ± 38 mg wet weight) and dwarf cichlid (*Apistogramma agassizii*, 150 ± 10 mg wet weight) were purchased from an ornamental fish store in Manaus and transferred to 310 L fiber glass tanks at LEEM-INPA. They were gradually acclimated to aerated INPA well water at 28 ± 1 °C (see non-adjusted composition in Section 2.1) for two weeks prior to experiments. The fish were fed daily with commercial dry pellet food and food was withdrawn 24 h prior to the experiments. About 80 % of the water was renewed daily. Experimental work was approved by the Ethics Committee on Animal Experiments of INPA under registration number 004/2018 and conformed to national animal care regulations.

Table 1
Measured water chemistry in experimental waters RN and IW. Values are given as mean ± SD (n). Chloride and alkalinity levels are nominal values.

	RN water	IW water
Background Cu (μg L^{-1})	3.3 ± 1.5 (13)	4.4 ± 1.5 (13)
T (°C)	28 ± 1 (5)	28 ± 1 (4)
pH	5.90 ± 0.22 (23)	5.95 ± 0.24 (20)
DOC (mg L^{-1})	10.1 ± 0.5 (9)	1.41 ± 0.3 (8)
DIC (mg L^{-1})	0.25 ± 0.11 (4)	0.32 ± 0.05 (4)
Ca (μM)	30.9 ± 0.5 (4)	37.1 ± 0.6 (6)
Mg (μM)	5.31 ± 1.12 (6)	6.36 ± 0.15 (6)
Na (μM)	42.5 ± 14.6 (6)	39.7 ± 2.2 (6)
K (μM)	9.3 ± 0.5 (6)	19.2 ± 0.1 (6)
Cl (μM)	65	57.5
Alkalinity (mEq L^{-1})	0.12	0.14

Different pre-acclimations to RN and IW waters (Table 1) were carried out with *P. axelrodi*, to assess the speed at which potential physiological effects of DOM (on Cu-gill binding and Na$^+$ physiological impairments) may appear then disappear in the fish. For fish tested in RN water, we performed a 0-d (i.e. no acclimation), a 1-d and a 5-d pre-acclimation to RN water. For fish tested in IW water, we performed a 0-d (i.e. no acclimation) and a 6-d pre-acclimation to RN water. During acclimation, fish were fed and the water was changed at the same rate as described above.

2.3. Copper gill binding experiments

We measured Cu uptake in the gills of both fish species after 3-h exposures to Cu in both IW and RN water. A stable isotope enrichment approach was used to discriminate the newly accumulated Cu from the background Cu in the fish gills, as described by Croteau et al. (2004). A stock solution was prepared with stable isotope ^{65}Cu (enrichment >99 % ^{65}Cu, native state, American Elements, Los Angeles, CA, USA), by dissolving the ^{65}Cu in concentrated nitric acid (HNO$_3$, Trace Metal grade, Fisher Scientific), then diluting with ultrapure water to 0.5 % HNO$_3$ (v/v). For each test water, in addition to controls (i.e. no Cu addition), six test solutions were prepared from this stock solution, with nominal Cu concentrations of 50, 100, 200, 500, 1000 and 3000 µg L^{-1} (i.e. 0.79, 1.6, 3.1, 7.9, 15 and 47 µM) in RN water and of 2.5, 5, 10, 25, 50 and 150 µg L^{-1} (i.e. 0.039, 0.079, 0.16, 0.39, 0.79 and 2.4 µM) in IW water. These ranges encompass the Cu concentration ranges where Na$^+$ imbalance have been observed in the cardinal tetra and in the dwarf cichlid (Braz-Mota et al., 2018; Crémazy et al., 2016; Duarte et al., 2009). Each treatment was replicated five times (i.e., $n = 5$ fish per treatment) using the same bulk test water, which was prepared the day prior to the experiment.

Fish were exposed to the different treatments individually in 100-mL plastic containers where aeration was provided with polyethylene capillary tubing and an air pump. About 2 h prior to the start of the experiment, the fish were transferred into these containers filled with Cu-free test water and allowed to recover from the handling stress. At $t = 0$ h, the water was quickly replaced by 50 mL of exposure solution. After 3 h of exposure, the test water was replaced by 20 mL of 1 M ethylenediaminetetraacetic acid (EDTA, ACS grade, Fisher Scientific) prepared in Cu-free test water. This rinsing step was performed to remove surface-bound Cu, so that only the internalized Cu was measured in the gills. After 5 min of rinsing, fish were euthanized with a solution of 250 mg L^{-1} of tricaine methanesulfonate (MS222) buffered with sodium bicarbonate. Death occurred within 2 min, then fish were blotted dry and kept in individual bullet tubes placed on ice until gill dissection. Fish were weighed and their gills were dissected and weighed within 2 h of the end of the Cu exposure. Gills were digested in 100 µL of 4 N HNO$_3$ (trace metal grade, Fisher Scientific) in sealed tubes at 40 °C for one week, with occasional vortexing. During the Cu exposure, filtered water samples (0.45 µm membrane filter, polyethersulfone, Fisher Scientific) were collected for the analyses of Cu, Na, Mg, K, Ca and DOC concentrations. Unfiltered water samples were also collected for immediate measurement of the pH. The Cu and pH analyses were done on water collected at the beginning and at the end of the tests, while the Na, Mg, K, Ca and DOC analyses were performed on surplus test waters.

2.4. Sodium flux experiments

Another set of 3-h Cu exposures was conducted to measure Na$^+$ flux rates. In these experiments, we used the radioactive isotope ^{22}Na to measure unidirectional Na$^+$ fluxes. A stock solution of Cu was prepared from CuSO$_4$·5H$_2$O (99 % pure, Sigma Aldrich) in ultrapure water. This stock solution was spiked in each water type to obtain five Cu treatments (in addition to Cu-free controls): 50, 100, 200, 500, 1000 µg L^{-1} (i.e. 0.79, 1.6, 3.1, 7.9 and 15 µM) in RN water and 5, 10, 25, 50 and 100 µg L^{-1} (i.e. 0.079, 0.16, 0.39, 0.79 and 1.6 µM) in IW water. Each treatment was replicated six times (i.e., $n = 6$ fish per treatment) using the same bulk test solution, which was prepared the day prior to the experiment. About 15 min prior to the

experiment, 0.002 µCi/mL of ^{22}Na (as NaCl in water, PerkinElmer, Waltham, MA, USA) was spiked into the test solutions.

As for the Cu-gill binding experiments, fish were exposed in aerated individual 100-mL plastic containers. They were transferred to these containers 2 h prior to the start of Cu exposure, then the acclimation water was changed for precisely 40 mL of test water. We collected 4 mL of water 5 min after this water change (flux start time), then again 3 h later (flux end time). At the end of the experiment, fish were euthanized with an overdose of buffered MS-222, blotted dry, then weighed. Total Na and ^{22}Na concentrations were measured in collected water samples to determine the Na$^+$ fluxes (see Sections 2.5 and 2.6). Filtered water samples (0.45 µm membrane filter, polyethersulfone) were also taken in surplus test solutions for analyses of pH and concentrations of Cu and DOC.

2.5. Chemical analyses

We used inductively coupled plasma mass spectrometry (quadrupole ICP-MS, Agilent 7700×, Santa Clara, CA, USA) to measure the concentrations of the naturally occurring stable isotopes of Cu (Cu^{65} and ^{63}Cu) in the gills and waters collected from the Cu-gill binding experiment. Standards (SCP Science, Baie-DUrfe, QC, Canada) and samples were in a matrix of 1 % HNO$_3$ (v/v) (Trace Metal grade, Fisher Scientific) with 10 µg L^{-1} Indium (SCP Science). Calibration was checked with a certified reference water (TM-25.4, Natural Resources Canada, Ottawa, Canada). We used atomic absorption spectrometry (AAS) with a graphite furnace (Perkin Elmer AAnalyst 800 AA spectrophotometer, Norwalk, CT, USA) to measure the total Cu concentration in the waters collected from the flux tests. Standards (PE N9300224, Calibration Solution, Perkin Elmer) and samples were acidified to 1 % HNO$_3$ (v/v) (Trace Metal grade, Fisher Scientific). The ^{22}Na in water samples from the flux test was measured by liquid scintillation counting (Triathler LSC, Hidex, Mississauga, Canada). Each 2-mL sample was added to 5 mL of scintillation cocktail (Optiphase, PerkinElmer) and counted 3 times for 1 min between 100 and 900 keV. Total Na, Mg, Ca and K concentrations were determined by AAS with a flame (Perkin Elmer AAnalyst 800 AA spectrophotometer, Norwalk, CT, USA). Dissolved organic carbon concentrations in the waters were measured with a Total Organic Carbon (TOC) Analyzer (Apollo 9000, Teledine Tekmar, Mason, OH, USA), using potassium phthalate (Shimadzu, Kyoto, Japan) for the standards. A pH meter (PG1800, Gehaka Inc., Sao Paulo, Brazil) was used to measure water samples pH after increasing their ionic strength with KCl (Fisher Scientific) to a final concentration of 0.01 M.

2.6. Data analyses

2.6.1. Aqueous free Cu^{2+} concentrations modeling

The Windermere Humic Aqueous Model (WHAM) software version 7 (Tipping et al., 2011) was used to calculate the free Cu^{2+} concentration in the test waters, based on the measured water chemistry (Table 1). For this modeling, the default log K$_{CuHCO3}$ of 14.62 (Mattigod and 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). For metal complexation with organic matter, we assumed that DOM contained 50 % carbon by weight (i.e. DOM = 2 × DOC) (Buffle, 1988), that 65 % of the DOM was chemically "active" (i.e., with binding properties), and that this active fraction was represented by fulvic acid (Bryan et al., 2002). Finally, the hydroxides Al(OH)$_3$ and Fe(OH)$_3$ were allowed to precipitate. These modeling conditions were used to predict Cu^{2+} concentration in Rio Negro water in a previous study where we observed a good agreement between modelled Cu^{2+} concentrations and measured Cu^{2+} concentrations (with a cupric ion-selective electrode) (Crémazy et al., 2016).

2.6.2. Calculations of newly accumulated Cu in the gills

Newly accumulated Cu in the fish gills (Cu_{gill}, in nmol g^{-1} wet wt) was calculated with Eq. (1):

$$Cu_{gill} = \frac{[^{65}Cu] \cdot V_{digest}}{m_{gill}} \qquad (1)$$

where V_{digest} is the volume of the analyzed gill digestion (in L), m_{gill} is the wet mass of digested gills (in g) and $[^{65}Cu]$ is the ^{65}Cu concentration in the gill digestion (in nmol L^{-1}), after correction for the natural ^{65}Cu background gill concentration. These $[^{65}Cu]$ values were obtained with Eq. (2), as detailed in Croteau et al. (2004):

$$[^{65}Cu] = p^{65} \times \left([T^{65}Cu] - [T^{63}Cu]\right) \qquad (2)$$

where the value p^{65} is the relative abundance of ^{65}Cu measured by the ICPMS and [T^{65}Cu] and [T^{63}Cu] are the total Cu concentrations from the ICPMS.

2.6.3. Unidirectional Na$^+$ flux calculations

Eqs. (3), (4) and (5) were respectively used to calculate the Na$^+$ net flux rates ($J_{Na,\ net}$), influx rates ($J_{Na,\ in}$) and efflux rates ($J_{Na,\ out}$) in the fish over the 3-h flux period (in nmol g^{-1} h^{-1}):

$$J_{Na,net} = \frac{\left([Na]_i - [Na]_f\right) \cdot V_{flux}}{t_{flux} \cdot m_{fish}} \qquad (3)$$

$$J_{Na,in} = \frac{\left([^{22}Na]_i - [^{22}Na]_f\right) \cdot V_{flux}}{SA \cdot t_{flux} \cdot m_{fish}} \qquad (4)$$

$$J_{Na,out} = J_{Na,net} - J_{Na,in} \qquad (5)$$

In Eq. (3), $[Na]_i$ and $[Na]_f$ are respectively the initial and the final total Na concentration in the water (in nmol L^{-1}). In Eq. (4), $[^{22}Na]_i$ and $[^{22}Na]_f$ are respectively the initial and the final ^{22}Na concentration in the water (in CPM L^{-1}) and SA is the mean specific activity of ^{22}Na in the water (in CPM nmol^{-1} of total Na). In Eqs. (3) and (4), V_{flux} is the volume of water in the experimental chamber (in L), t_{flux} is the flux period (in h) and m_{fish} is the wet mass of fish (in g).

2.6.4. Statistical analyses

Data analysis was performed in GraphPad Prism (version 6), using a significance level of 0.05 for all comparisons. Data are expressed as means ± SE (n) where n = number of fish. We used a regression approach to analyze the Cu-gill uptake data as a function of measured dissolved Cu concentration ([Cu]). Ambiguous fits were obtained when using a traditional ligand binding equation (Y = a·X/(b + X)) to model these data. Instead, the Cu uptake data were fitted against [Cu] using a linear model on the log-transformed Y and X values (Y = a·X + b, with Y = log Cu_{gill} and X = log [Cu]). Then, the effects of DOM pre-acclimation time and of water type (IW vs. RN) were tested using extra-sum-of-squares F-tests. The effect of water type was again evaluated after accounting for Cu bioavailability differences in both waters. To do so, the same method was used, but with Cu_{gill} data plotted as a function of modelled free Cu^{2+} concentration ([Cu^{2+}]) in the water. For the Na$^+$ flux experiments, we firstly evaluated the effects of pre-acclimation to RN's DOM on *P. axelrodi* Na$^+$ unidirectional ($J_{Na,in}$, $J_{Na,out}$) and net flux rates ($J_{Na,net}$) across the tested Cu concentration ranges, using two-way ANOVAs with Tukey or Sidak post-hoc tests. Note that, as these experiments showed no effects of pre-exposure time on $J_{Na,in}$, $J_{Na,out}$ and $J_{Na,net}$ values in *P. axelrodi*, the data from the various flux experiments were pooled together for this fish. Note that no acclimation test was conducted with *A. agassizii*. The effect of water type (IW vs RN) on Na$^+$ unidirectional fluxes was then tested for each species in the absence of Cu (i.e. in the control treatments), using unpaired *t*-tests. Finally, the effect of water type on unidirectional and net Na$^+$ fluxes was tested for each fish in the presence of Cu

using a regression approach. First, $J_{Na,in}$, $J_{Na,out}$ and $J_{Na,net}$ were modelled as a function of dissolved Cu concentration measured in the water. An exponential model (Y = Y$_0$·exp$^{k·X}$) was used for both $J_{Na,net}$ and $J_{Na,out}$ and a log dose-response and inhibitor model (Y = min + (max-min)/(1 + 10$^{X ·log\ IC50}$)) was used for $J_{Na,in}$. Second, extra-sum-of-squares F-tests were used to assess the effect of water type. The same comparison was done with the flux data as a function of modelled Cu^{2+} concentration in the water, to account for Cu bioavailability differences in both waters.

3. Results and discussion

3.1. Test water composition and copper speciation

The chemical compositions of RN and IW test waters are given in Table 1. Neither water was filtered before use in experiments, as a previous study assessed that the levels of total suspended solids were low (below detection limit in IW and about 8 mg L^{-1} in RN) and did not significantly affect Cu speciation (Crémazy et al., 2019). The RN water in this study corresponded to Rio Negro water, in which the Ca and Na concentrations were increased by a factor of 5 for Ca (from 6 to 30 μM) and by a factor of 1.5 for Na (from 30 to 44 μM) to match the ionic composition in IW water. The DOC concentration of 10 mg L^{-1} was typical of the Rio Negro water, while the pH of 5.9 was in the upper end of the typical river pH range (pH 4–6) (Sioli, 1968; Val and Almeida-Val, 1995). The chemical composition of IW water corresponded to INPA well water diluted by a factor 2 with ultrapure water, in order to decrease the DOC and major ion concentrations. Indeed, while the historical composition of INPA well water typically corresponds to DOC-free Rio Negro water, INPA well water had unusually high DOC and ion concentrations in October 2018. This unusual water composition might have been caused by a recent cleaning of the INPA well. While the dilution brought the ion composition closer to its usual levels (and Rio Negro levels), the DOC concentration (1.4 mg L^{-1}) was still about 4-fold higher than its typical concentrations (Crémazy et al., 2016). Overall, the two test water compositions only differed in the 7-fold difference of their respective DOC concentrations.

Copper concentrations measured in these two test waters were slightly lower than the nominal Cu concentrations selected for the Cu-gill binding and flux experiments (% recoveries of 82 ± 7 % in IW and 84 ± 17 % in RN). Copper speciation in these waters was dominated by Cu-DOC complexes (>95 % of all Cu species) at dissolved Cu concentrations <10 μg L^{-1} in IW and <200 μg L^{-1} in RN water. As dissolved Cu concentrations increased past these levels, the relative contribution of free Cu^{2+} became important. In IW, Cu^{2+} comprised 30 % and 10 % at the maximum tested measured Cu concentrations of 140 and 70 μg L^{-1} in the gill uptake and flux experiments respectively. In RN, Cu^{2+} comprised 50 % and 14 % at the maximum tested measured Cu concentrations of 2500 and 900 μg L^{-1} in the gill uptake and flux experiments respectively.

3.2. Copper uptake in gills

Pre-exposure to RN's DOM for up to 6 days did not affect 3-h Cu uptake in gills of *P. axelrodi* exposed to Cu in either IW or RN (Fig. 1). Newly accumulated Cu concentrations in gills of cardinal tetra (*P. axelrodi*) and dwarf cichlid (*A. agassizii*) after a 3-h exposure to waterborne Cu in either IW or RN water, are shown in Fig. 2. Both species accumulated Cu in their gills in a concentration-dependent fashion.

Rio Negro's DOM protected against Cu uptake in both fish species. Indeed, for the same dissolved Cu concentration, Cu uptake was lower (*p* < 0.0001, F-tests) when exposure occurred in RN compared to IW (Fig. 2a, b). This effect was more apparent in tetras than in cichlids. For cichlids, Cu accumulation at the gill in IW and RN waters was somewhat similar in the region where dissolved Cu concentrations overlap (Fig. 2b). In both fish, the overall lower Cu uptake could be largely attributed to a decrease in waterborne Cu bioavailability via the complexation of
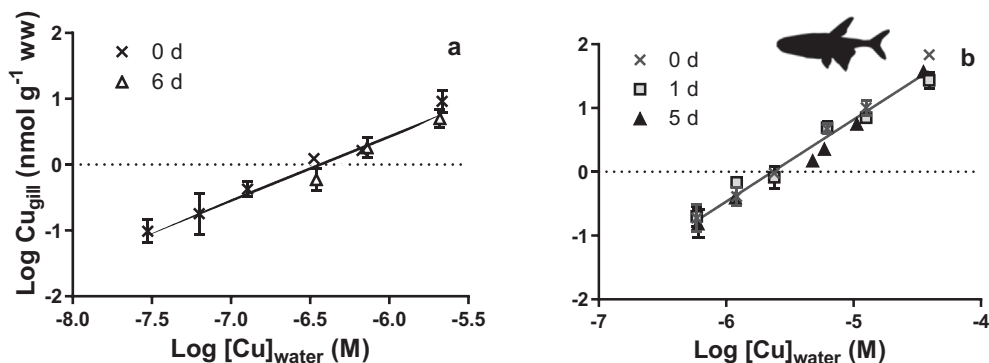
Fig. 1. Log of 3-h Cu uptake in gills of *P. axelrodi*, as a function of log of measured dissolved Cu concentration in a) IW water and b) RN water, after different pre-acclimation durations to RN water. Data are means ± SE, with n = 5. Plain lines are linear models (described in Table SI.1).

Cu^{2+} by DOM. Indeed, Cu uptake was better predicted by the free Cu^{2+} concentration modelled with WHAM7 (Fig. 2c, d). For cardinal tetra (Fig. 2c), there was no water effect (i.e. DOM effect) when uptake data was plotted against $[Cu^{2+}]_{water}$ ($p > 0.05$, F-test). For dwarf cichlid though, a residual water effect was apparent when plotting Cu uptake as a function of $[Cu^{2+}]_{water}$ ($p < 0.001$, F test) (Fig. 2d). In this fish, Cu uptake seemed slightly higher in IW than in RN at $[Cu^{2+}]_{water} < 10^{-8}$ M, but slightly lower in IW than in RN at $[Cu^{2+}]_{water} > 10^{-8}$ M. It is unclear whether these different Cu uptake profiles are the result of species-specific DOM effects on Cu-gill binding. One could also argue that all data for this fish collapse to a single non-linear relationship that may indicate the involvement of various Cu membrane transporters: a high affinity/low capacity transporter at low Cu^{2+} concentrations and a lower affinity/higher capacity transporter at higher Cu^{2+} concentrations. What is evident for this fish is

that the lack of Cu gill data at comparably high $[Cu^{2+}]$ in both waters is preventing a clear comparison of uptake profiles in IW and RN waters. Nevertheless, within the range of Cu concentrations triggering acute effects in *A. agassizii*, Cu uptake remains relatively well predicted on the basis of the free Cu^{2+} concentration (within a factor of ~2).

3.3. Unidirectional sodium fluxes

Baseline Na^+ physiological fluxes were measured in both fish species in Cu-free IW and RN waters (control treatments) (Fig. 3). No significant effect of water type was observed on these Na^+ baseline fluxes in either fish ($p > 0.05$, unpaired *t*-tests). These data showed that Na^+ influx and efflux rates are about 6 times greater in *P. axelrodi* than in *A. agassizii*, yet both species are able to achieve approximate Na^+ balance (i.e. net
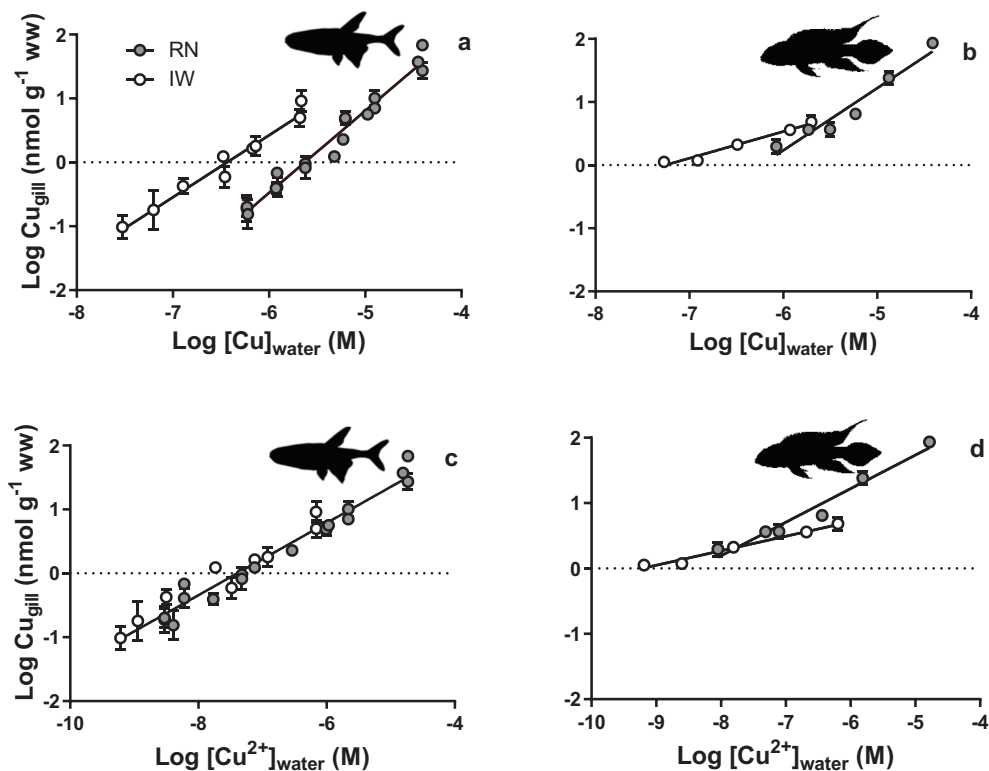

Fig. 2. Log of 3-h Cu uptake in gills of *P. axelrodi* (a and c) and *A. agassizii* (b and d) as a function of the log of the measured dissolved Cu concentration (top panels) and as a function of the log of the modelled Cu^{2+} concentration (bottom panels) in IW water (open circles) and RN water (grey circles). Data are mean ± SE, with n = 5. Plain lines represent linear models (described in Table SI.2 in Supporting information).
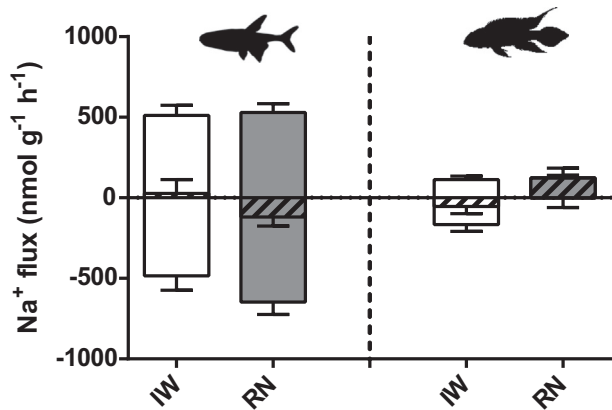
Fig. 3. Unidirectional Na flux rates in *P. axelrodi* (left panel) and *A. agassizii* (right panel) exposed to Cu-free IW and RN waters. Sodium net flux rates are the dashed bars, influx rates are the positive plain bars and the efflux rates are the negative plain bars. Data are mean ± SE, with n = 18 and 12 for *P axelrodi* in RN and IW respectively, and n = 6 and 5 for *A. agassizii* in RN and IW respectively.

flux rates close to zero) in both waters. These observations are in agreement with current knowledge on the different ion regulation strategies employed by Characidae and Cichlidae species to maintain their Na$^+$ balance (Gonzalez et al., 2005; Val and Wood, 2022). Amazonian Characiformes, such as *P. alxelrodi*, have a high affinity and high capacity Na$^+$ transport systems that can sustain high rates of Na$^+$ influx to compensate for the high rates of Na$^+$ diffusive loss in the extremely dilute Rio Negro waters (Gonzalez et al., 2002). This Na$^+$ uptake system also appears to be insensitive to low pH (Gonzalez and Preest, 1999). In contrast, Cichliformes, such as *A. agassizii,* have an unspecialized (acid-sensitive) Na$^+$ transport system (Gonzalez et al., 2002). Yet, they are able to maintain ion balance in acidic and dilute waters because of very low gill permeability that limits Na$^+$ diffusive loss rates (Gonzalez et al., 2002).

As for Cu-gill uptake, pre-exposure to RN water for up to 6 days did not affect Na$^+$ fluxes in *P. axelrodi* later exposed in either IW or RN (Fig. SI.1 in the Supplemental information). Copper exposure resulted in concentration-dependent rates of net Na$^+$ loss to both species (Fig. 4). The levels of net Na$^+$ loss were similar in both fish for a same Cu exposure concentration, but the mechanisms behind these losses were different, as outlined below. This comparable sensitivity is in agreement with a previous study showing
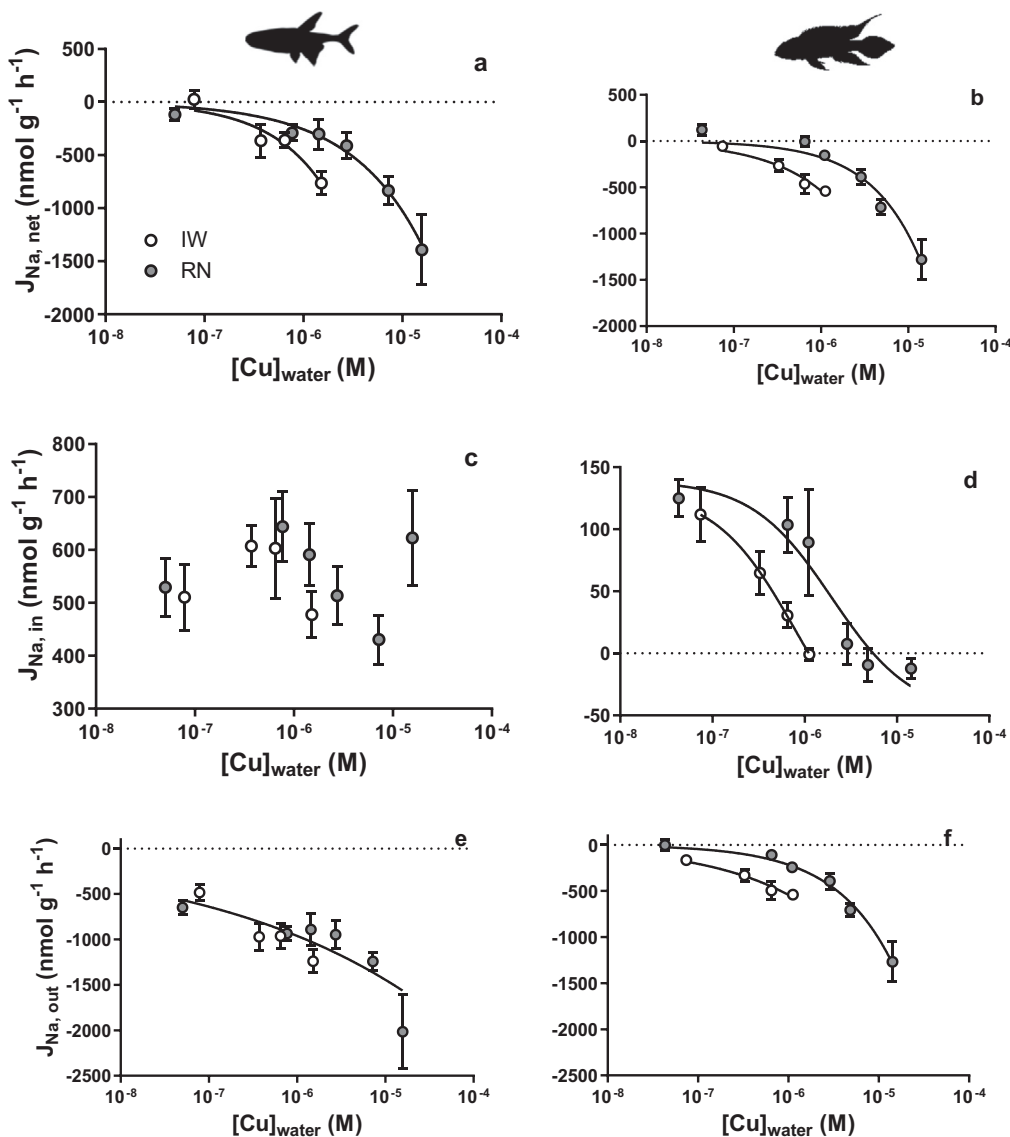

Fig. 4. Sodium net flux (a, b), influx (c, d) and efflux (e, f) rates in *P. axelrodi* (left panels) and in *A. agassizii* (right panels), as a function of measured dissolved Cu concentration in either IW water (open circles) or RN water (grey circles). Data are mean ± SE, with n = 18 and 12 for *P. axelrodi* in RN and IW respectively and with n = 6 and 5 for *A. agassizii* in RN and IW respectively. The solid lines represent fitted models (described in Table SI.3 in Supporting information).

these two species having similar 96-h LC50s for waterborne Cu (Duarte et al., 2009). Failure of Na^+ homeostasis is considered to be the key mechanism of toxicity of acute Cu exposure in freshwater (Grosell et al., 2002). Proposed mechanisms for Cu-induced Na^+ net loss are (i) a decrease in Na^+ gill influx, via direct competition at the apical Na^+ channel, inhibition of branchial carbonic anhydrase (CA), inhibition of the basolateral Na/K-ATPase, and/or inhibition of the apical v-type H^+-ATPase; and (ii) an increase in Na^+ branchial efflux via an increase in the branchial epithelium permeability, induced by the displacement of calcium from the tight junctions (Chowdhury et al., 2016; Grosell et al., 2002; Laurén and McDonald, 1987, 1995; Zimmer et al., 2012). In *P. axelrodi*, the net Na^+ loss (Fig. 4a) was solely attributable to a concentration-dependent promotion of Na^+ efflux (Fig. 4e), as Na^+ influx rates were unaffected during the 3-h Cu exposure (Fig. 4c). On the other hand, in *A. agassizii*, the net Na^+ loss (Fig. 4b) was the result of both concentration-dependent inhibitions of Na^+ influx (Fig. 4d) and stimulation of Na^+ efflux rates (Fig. 4f). More precisely, at

low Cu concentrations ($[Cu^{2+}] < 10^{-8}$ M), Cu effects on both Na^+ influx and efflux rates contributed in similar proportions to the net Na^+ loss in cichlids. At higher Cu concentrations ($[Cu^{2+}] > 10^{-8}$ M), the important net Na^+ loss became mainly attributed with promotion of Na^+ efflux, as Na^+ influx was completely inhibited. While we cannot exclude that inhibition of Na^+ influx might have eventually occurred with longer Cu exposure time in *P. axelrodi* as Cu builds up in the gills, there is a mechanistic basis in support for the lower Cu-sensitivity of $J_{Na, in}$ in cardinal tetras. Indeed, as mentioned earlier, Characiformes have a highly specialized branchial Na^+ uptake system (Gonzalez et al., 2002). This transport system might be particularly resistant to Cu exposure, in a similar way that it has been shown to be resistant to very low pH levels (Gonzalez and Preest, 1999). Consistent with this reasoning, Braz-Mota et al. (2018) showed that 24–96 h exposures of both fish species to waterborne Cu at 50 % of their respective 96-h LC50s led to a marked initial decrease in branchial activities of CA in *A. agassizii,* while no change was observed in *P. axelrodi*. Despite waterborne Cu not significantly affecting Na^+ uptake rates in
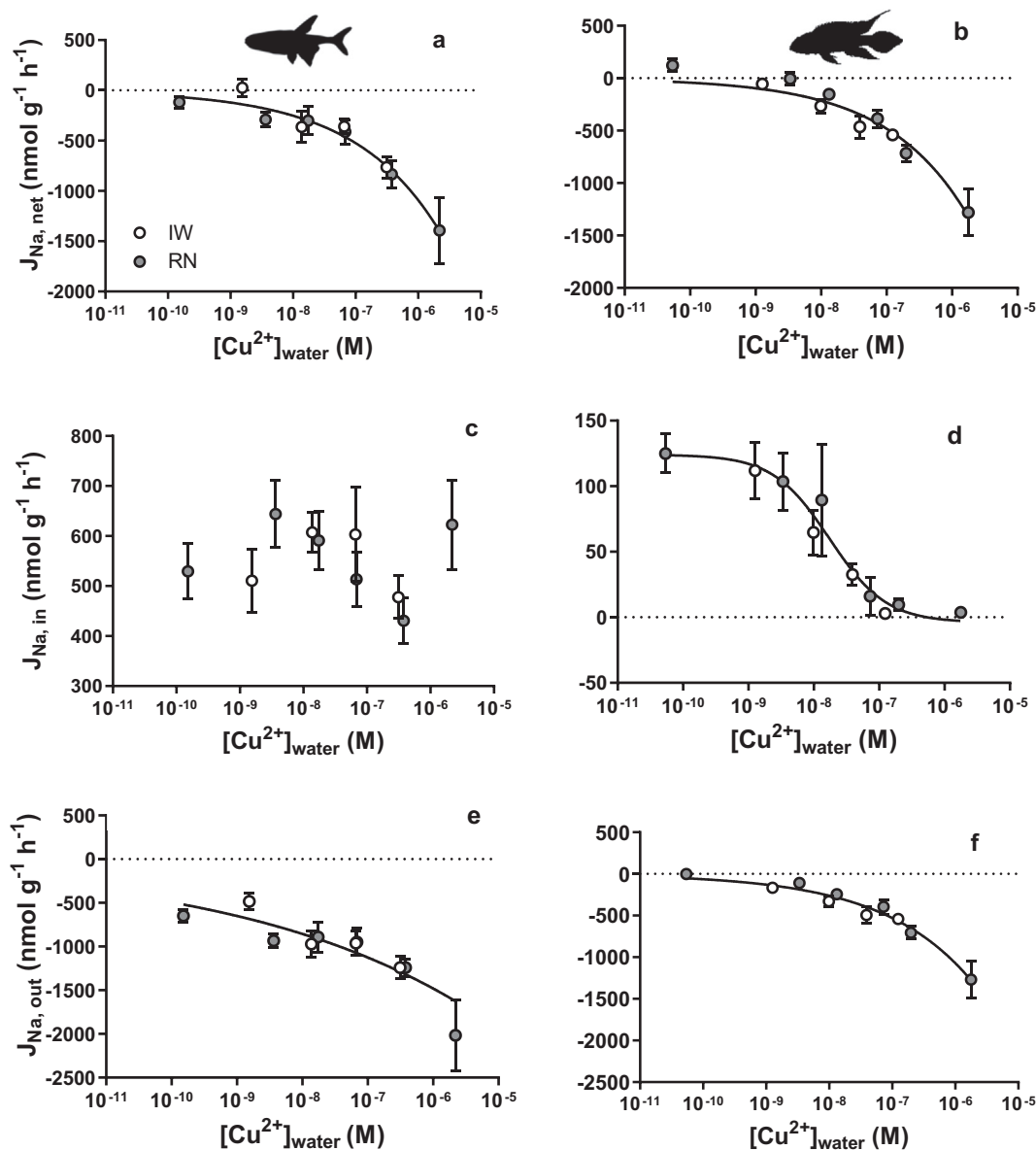

Fig. 5. Sodium net flux (a, b), influx (c, d) and efflux (e, f) rates in *P. axelrodi* (left panels) and *A. agassizii* (right panels) as a function of modelled Cu^{2+} concentration in either IW water (open circles) or RN water (grey circles). Data are mean ± SE, with n = 18 and 12 for *P. axelrodi* in RN and IW respectively and with n = 6 and 5 for *A. agassizii* in RN and IW respectively. The solid lines represent fitted models (described in Table SI.4 in Supporting information).

P. axelrodi in the present study, a large promotion of Na^+ efflux in this fish led to both fish exhibiting similar net Na^+ losses for a same Cu exposure concentration.

Sodium unidirectional fluxes of fish exposed to Cu in RN were globally less affected than fish exposed in IW (Fig. 4), showing that DOM protects against acute Cu toxicity. Graphing these data as a function of modelled free Cu^{2+} concentration led to an overlapping of the IW and RN data (Fig. 5), suggesting a geochemical-based DOM protection, as further discussed below.

3.4. Nature of DOM protection on Cu uptake and toxicity

Pre-acclimation to DOM had no effect on subsequent Cu uptake (Fig. 1) and the decrease in Cu uptake in fish gills exposed to RN water (vs. IW water) followed the modelled decrease free Cu^{2+} concentration (Fig. 2). These data suggest that, if DOM molecules were bound to fish gills under the tested conditions, these molecules did not affect the gill environment to an extent that would affect metal uptake. Thus, this study shows that Rio Negro's DOM protection against Cu uptake in fish is largely associated with its metal complexation properties, as expected from bioavailability-based concepts. These findings are in disagreement with Matsuo et al. (2005), who showed that a 10-day pre-acclimation to DOC led to an 86 % decrease in 3-h Cu uptake in gills of the Amazonian teleost tambaqui (*Colossoma macropomum*). However, while this study was conducted at comparable pH and ionic strength, the tested DOC concentrations (DOC = 0.9 vs 20, 30 and 80 mg L^{-1}) used by Matsuo et al. (2005) were higher than in our study (DOC = 1.4 vs. 10 mg L^{-1}). Furthermore, Matsuo et al. (2005) employed Aldrich humic acid, rather than native DOMs from Amazon waters.

Similar to effects on Cu uptake, DOM did not appear to have physiological effects on baseline Na^+ regulation or on Cu-induced Na^+ regulation impairment in either fish, under the conditions tested in the present study. First, no effect of water type was observed on Na^+ baseline fluxes in either fish (Fig. 3). Second, as for Cu uptake in gills, pre-exposure to RN water for up to 6 days did not affect Na^+ fluxes in *P. axelrodi* later exposed in either IW or RN (Fig. SI.1 in the Supplemental information). Finally, although DOM clearly protected against Na^+ regulation dysfunction caused by Cu (Fig. 4), in both species the modelled Cu^{2+} concentration was an excellent predictor of acute Cu effects on Na^+ flux rates (Fig. 5). This was true despite the different mechanisms of toxicity in the two species (stimulated unidirectional Na^+ efflux alone in *P. axelrodi* versus inhibited unidirectional Na^+ influx and stimulated unidirectional Na^+ efflux *A. agassizii*; Fig. 4). Thus, there was no evidence that this protection had a physiological component. Along with the Cu uptake data, these data support the traditional geochemical mechanism of DOM protection against metal toxicity: a pure result of free metal binding and consequent decrease in metal bioavailability.

Very few studies have explicitly investigated the possible physiological protection from blackwater DOM against metal toxicity (Matsuo et al., 2004, 2005). Instead, most studies have focused on DOM's support of ion regulation under low ion and pH levels (Duarte et al., 2016, 2018; Gonzalez et al., 2002, 2005; Wood et al., 2003). In the present study, the apparent absence of DOM physiological effects on fish baseline ionoregulation, Cu uptake and acute toxicity, may be in part due to test water chemistry. Binding of DOM to gills is favored at low pH (Campbell et al., 1997), so test waters with lower pH might have favored the occurrence of DOM physiological effects. This pH-dependency on DOM effects was notably observed by Wood et al. (2003) with elasmobranch *Potamotrygon* sp. exposed to either Rio Negro water or DOM-free INPA well water. In this latter study, while DOM helped fish ionoregulation at pH 4, blackwater DOM caused only very modest shifts in Na^+ influx and efflux rates at pH ~ 6. DOC concentrations in the Rio Negro can be as high as 30 mg L^{-1}, while its pH can be as low as 4 (Val and Almeida-Val, 1995; Val and Wood, 2022). Using these latter DOC and pH levels would represent extreme yet still environmentally realistic test conditions to unequivocally evaluate the relevance of this potential DOM physiological protection

against metal toxicity. Related to this, positive physiological effects of DOM have been observed using higher DOM concentrations and lower pH levels (Matsuo et al., 2005; Matsuo and Val, 2007).

4. Conclusions

In the present study, there was clear evidence that DOM from the Rio Negro protected cardinal tetras and dwarf cichlids against waterborne acute Cu toxicity. However, there was no indication that this protection had a significant physiological basis. Indeed, pre-acclimation to DOM had very little effect on subsequent measurements of Cu uptake and toxicity, exposure to DOM had no effect on Na^+ baseline fluxes in the fish, and DOM effects on Cu uptake and toxicity could be explained on the basis of bioavailability of the free Cu^{2+} ion alone ($[Cu^{2+}]_{water}$). The conventional geochemical mechanism of protection by DOM held true in the present study: DOM reduced Cu bioavailability by metal-complexation, which decreased the free Cu^{2+} concentration in the water, leading to a reduction in Cu uptake in gills and a subsequent reduction in Cu effects on branchial Na^+ regulation. The prevalence of this geochemical-type protection over a physiological-type protection agrees with the BLM conceptual framework, supporting its potential use to assess the risk of Cu in these Amazonian waters under the conditions tested in this study. Further studies should nevertheless be conducted at test conditions that optimize DOM-gill binding: lower pH and higher DOM concentrations.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.157032.

References

Bourgeault, A., Ciffroy, P., Garnier, C., Cossu-Leguille, C., Masfaraud, J.-F., Charlatchka, R., Garnier, J.-M., 2013. Speciation and bioavailability of dissolved copper in different freshwaters: comparison of modelling, biological and chemical responses in aquatic mosses and gammarids. Sci. Total Environ. 452–453, 68–77. https://doi.org/10.1016/j.scitotenv.2013.01.097.

Braz-Mota, S., Campos, D.F., MacCormack, T.J., Duarte, R.M., Val, A.L., Almeida-Val, V.M.F., 2018. Mechanisms of toxic action of copper and copper nanoparticles in two Amazon fish species: dwarf cichlid (Apistogramma agassizii) and cardinal tetra (Paracheirodon axelrodi). Sci. Total Environ. 630, 1168–1180. https://doi.org/10.1016/j.scitotenv.2018.02.216.

Bryan, S.E., Tipping, E., Hamilton-Taylor, J., 2002. Comparison of measured and modelled copper binding by natural organic matter in freshwaters. Comp. Biochem. Physiol. C: Toxicol. Pharmacol. 133, 37–49. https://doi.org/10.1016/S1532-0456(02)00083-2.

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

Campbell, P.G.C., 1995. Interactions between trace metals and aquatic organisms: a critique of the free-ion activity model. In: Campbell, P.G.C. (Ed.), Metal Speciation and Bioavailability in Aquatic Systems. John Wiley and Sons, New York, NY, pp. 45–102.

Campbell, P.G.C., Twiss, M.R., Wilkinson, K.J., 1997. Accumulation of natural organic matter on the surfaces of living cells: implications for the interaction of toxic solutes with aquatic biota. Can. J. Fish. Aquat. Sci. 54, 2543–2554.

Campbell, P.G.C., Chapman, P.M., Hale, B.A., 2006. Risk assessment of metals in the environment. Chemicals in the Environment: Assessing and Managing Risk. 21, pp. 102–131.

Chowdhury, M.J., Girgis, M., Wood, C.M., 2016. Revisiting the mechanisms of copper toxicity to rainbow trout: time course, influence of calcium, unidirectional Na(+) fluxes, and branchial Na(+), K(+) ATPase and V-type H(+) ATPase activities. Aquat. Toxicol. 177, 51–62. https://doi.org/10.1016/j.aquatox.2016.05.009.

Crémazy, A., Wood, C.M., Smith, D.S., Ferreira, M.S., Johannsson, O.E., Giacomin, M., Val, A.L., 2016. Investigating copper toxicity in the tropical fish cardinal tetra (Paracheirodon axelrodi) in natural amazonian waters: measurements, modeling, and reality. Aquat. Toxicol. 180, 353–363. https://doi.org/10.1016/j.aquatox.2016.10.011.

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. Environ.Toxicol. Chem. 38, 2708–2718. https://doi.org/10.1002/etc.4586.

Croteau, M.-N., Luoma, S.N., Topping, B.R., Lopez, C.B., 2004. Stable metal isotopes reveal copper accumulation and loss dynamics in the freshwater bivalve corbicula. Environ. Sci. Technol. 38, 5002–5009. https://doi.org/10.1021/es049432q.

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. Environ. Toxicol. Chem. 20, 2383–2396. https://doi.org/10.1002/etc.5620201034.

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. J. Comp. Physiol. B. 188, 793–807. https://doi.org/10.1007/s00360-018-1169-y.

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. https://doi.org/10.1016/j.ecoenv.2008.10.003.

Duarte, R.M., Smith, D.S., Val, A., 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.

Gonzalez, R.J., Preest, M.R., 1999. Ionoregulatory specializations for exceptional tolerance of ion-poor, acidic waters in the neon tetra (paracheirodon innesi). Physiol. Biochem. Zool. 72, 156–163. https://doi.org/10.1086/316651.

Gonzalez, R.J., Wilson, R.W., Wood, C.M., Patrick, M.L., Val, A.L., 2002. Diverse strategies for ion regulation in fish collected from the ion-poor, acidic Rio Negro. Physiol. Biochem. Zool. 75, 37–47. https://doi.org/10.1086/339216.

Gonzalez, R.J., Wilson, R.W., Wood, C.M., 2005. Ionoregulation in tropical fishes from ion-poor, acidic blackwaters. Fish Physiology, The Physiology of Tropical Fishes. Academic Press, pp. 397–442. https://doi.org/10.1016/S1546-5098(05)21009-9.

Grosell, M., Nielsen, C., Bianchini, A., 2002. Sodium turnover rate determines sensitivity to acute copper and silver exposure in freshwater animals. Comp. Biochem. Physiol. C: Toxicol. Pharmacol. 133, 287–303. https://doi.org/10.1016/S1532-0456(02)00085-6.

Laurén, D.J., McDonald, D.G., 1987. Acclimation to copper by rainbow trout, Salmo gairdneri: physiology. Can. J. Fish. Aquat. Sci. 44, 99–104. https://doi.org/10.1139/f87-012.

Laurén, D.J., McDonald, D.G., 1995. Effects of copper on branchial ionoregulation in the rainbow trout, Saimo gairdneri Richardson. J. Comp. Physiol. B. 155, 635–644.

Matsuo, A.Y.O., Val, A.L., 2007. Acclimation to humic substances prevents whole body sodium loss and stimulates branchial calcium uptake capacity in cardinal tetras Paracheirodon axelrodi (Schultz) subjected to extremely low pH. J. Fish Biol. 70, 989–1000. https://doi.org/10.1111/j.1095-8649.2007.01358.x.

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 softwater. Aquat. Toxicol. 70, 63–81. https://doi.org/10.1016/j.aquatox.2004.07.005.

Matsuo, A.Y.O., Wood, C.M., Val, A.L., 2005. Effects of copper and cadmium on ion transport and gill metal binding in the amazonian teleost tambaqui (Colossoma macropomum) in extremely soft water. Aquat. Toxicol. 74, 351–364. https://doi.org/10.1016/j.aquatox.2005.06.008.

Mattigod, S., Sposito, G., 1979. Chemical modeling of trace metal equilibria in contaminated soil solutions using the computer program geochem. Chemical Modeling in Aqueous Systems, ACS Symposium Series. American Chemical Society, pp. 837–856 https://doi.org/10.1021/bk-1979-0093.ch037.

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: Ionoregulation, acid–base regulation, ammonia excretion, and metal toxicity. J. Exp. Zool. A Ecol. Genet. Physiol. 335, 843–863. https://doi.org/10.1002/jez.2468.

Niyogi, S., Wood, C.M., 2004. Biotic ligand model, a flexible tool for developing site-specific water quality guidelines for metals. Environ. Sci. Technol. 38, 6177–6192. https://doi.org/10.1021/es0496524.

Paquin, P.R., Gorsuch, J.W., Apte, S., Batley, G.E., Bowles, K.C., Campbell, P.G.C., Delos, C.G., Di Toro, D.M., Dwyer, R.L., Galvez, F., Gensemer, R.W., Goss, G.G., Hogstrand, C., Janssen, C.R., McGeer, J.C., Naddy, R.B., Playle, R.C., Santore, R.C., Schneider, U., Stubblefield, W.A., Wood, C.M., Wu, K.B., 2002. The biotic ligand model: a historical overview. Comp. Biochem. Physiol. C: Toxicol. Pharmacol. 133, 3–35. https://doi.org/10.1016/S1532-0456(02)00112-6.

Playle, R.C., Dixon, D.G., Burnison, K., 1993. Copper and cadmium binding to fish gills: modification by dissolved organic carbon and synthetic ligands. Can. J. Fish. Aquat. Sci. 50, 2667–2677. https://doi.org/10.1139/f93-290.

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 Cu2+-OH-, cl-, CO32-, SO42-, and PO43- systems (IUPAC technical Report). Pure Appl. Chem. 79, 895–950. https://doi.org/10.1351/pac200779050895.

Sioli, H., 1968. Hydrochemistry and Geology in the Brazilian Amazon Region - Harald Sioli - Google Livres.

Tipping, E., 2002. Cation Binding by Humic Substances. Cambridge University Press, Cambridge.

Tipping, E., Lofts, S., Sonke, J.E., 2011. Humic ion-binding model VII: a revised parameterisation of cation-binding by humic substances. Environ. Chem. 8, 225–235.

USEPA, 2007. Aquatic Life Ambient Freshwater Quality Criteria - Copper (2007 Revision) (No. EPA-822-R-07-001). Office of Water, Washington, DC.

Val, A.L., Almeida-Val, V.M.F.de, 1995. Fishes of the Amazon and Their Environment: Physiological and Biochemical Aspects, Zoophysiology. Springer-Verlag, Berlin Heidelberg.

Val, A.L., Wood, C.M., 2022. Global change and physiological challenges for fish of the Amazon today and in the near future. J. Exp. Biol. 225, jeb216440. https://doi.org/10.1242/jeb.216440.

Vigneault, B., Percot, A., Lafleur, M., Campbell, P.G.C., 2000. Permeability changes in model and phytoplankton membranes in the presence of aquatic humic substances. Environ. Sci. Technol. 34, 3907–3913. https://doi.org/10.1021/es001087r.

Wood, C.M., Matsuo, A.Y.O., Wilson, R.W., Gonzalez, R.J., Patrick, M.L., Playle, R.C., Luis Val, A., 2003. Protection by natural Blackwater against disturbances in ion fluxes caused by low pH exposure in freshwater stingrays endemic to the Rio Negro. Physiol. Biochem. Zool. 76, 12–27. https://doi.org/10.1086/367946.

Wood, C.M., Al-Reasi, H.A., Smith, D.S., 2011. The two faces of DOC. Aquat. Toxicol. 105, 3–8. https://doi.org/10.1016/j.aquatox.2011.03.007.

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. Aquat. Toxicol. 122–123, 172–180. https://doi.org/10.1016/j.aquatox.2012.06.010.