Environmental Pollution 341 (2024) 122988

Contents lists available at ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol

The biotic ligand model as a promising tool to predict Cu toxicity in amazon blackwaters☆

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ARTICLE INFO

Keywords:
LC_{50}
Biotic ligand model (BLM)
Rio negro
Copper speciation
Characiformes
Siluriformes
Cichliformes

ABSTRACT

The Rio Negro basin of Amazonia (Brazil) is a hotspot of fish biodiversity that is under threat from copper (Cu) pollution. The very ion-poor blackwaters have a high dissolved organic carbon (DOC) concentration. We investigated the Cu sensitivity of nine Amazonian fish species in their natural blackwaters (Rio Negro). The acute lethal concentration of Cu (96 h LC_{50}) was determined at different dilutions of Rio Negro water (RNW) in ion-poor well water (IPW), ranging from 0 to 100%. The IPW was similar to RNW in pH and ionic composition but deficient in DOC, allowing this parameter to vary 20-fold from 0.4 to 8.3 mg/L in tests. The Biotic Ligand Model (BLM; Windward version 3.41.2.45) was used to model Cu speciation and toxicity over the range of tested water compositions, and to estimate lethal Cu accumulations on the gills (LA_{50}). The modeling predicted a high relative abundance of Cu complexes with DOC in test waters. As these complexes became more abundant with increasing RNW content, a concomitant decrease in free Cu^{2+} was observed. In agreement with this modeling, acute Cu toxicity decreased (i.e. 96 h LC_{50} values increase) with increasing RNW content. The three most sensitive species (*Hemigrammus rhodostomus, Carnegiella strigatta* and *Hyphessobrycon socolofi*) were Characiformes, whereas *Corydoras schwartzi* (Siluriformes) and *Apistogramma agassizii* (Cichliformes) were the most tolerant. These sensitivity differences were reflected in the BLM-predicted lethal gill copper accumulation (LA_{50}), which were generally lower in Characiformes than in Cichliformes. Using these newly estimated LA_{50} values in the BLM allowed for accurate prediction of acute Cu toxicity in the nine Amazonian fish. Our data emphasize that the BLM approach is a promising tool for assessing Cu risk to Amazonian fish species in blackwater conditions characterized by very low concentrations of major ions but high concentrations of DOC.

1. Introduction

The Rio Negro basin of Amazonia (Brazil) is a hotspot of biodiversity, and sustains 8% of the world's freshwater ichthyofauna (Val and Almeida-Val, 1995), but it is under increasing threat from anthropogenic activities (Val and Wood, 2022). One of these is increasing copper (Cu) pollution. Copper is an essential microelement involved in multiple physiological and biochemical reactions, and is naturally found in freshwater environments at concentrations typically ranging from 0.2 to 30 μg/L [0.003–0.47 μM] (USEPA, 2007). However, high Cu concentrations of anthropogenic origin are widely recognized to cause ionoregulatory and respiratory disturbances that can be lethal to fish (reviewed by Grosell, 2012). Due to expanding mining, agricultural and industrial activities, elevated Cu concentrations have been reported in Amazon waters, at levels often exceeding the environmental limits for freshwater protection allowed by the Brazilian Environmental National Council (9 μg/L) (Conselho Nacional do Meio Ambiente, 2005; Lima et al., 2015; Duarte and Val, 2020). In the Rio Negro basin, industrial activities are the most significant sources of Cu contamination, especially during the dry season in areas surrounding major cities like

☆ This paper has been recommended for acceptance by Wen-Xiong Wang.

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https://doi.org/10.1016/j.envpol.2023.122988
Received 31 July 2023; Received in revised form 11 November 2023; Accepted 16 November 2023
Available online 20 November 2023

Manaus, where Cu enrichment in water and sediment has been reported reaching values so high as > 2100 μg/L and 40 mg/kg, respectively (Geissler, 1999; Pinto et al., 2009; Santana, 2016; Jacaúna et al., 2020). The latter Cu water levels are two orders of magnitude above the CONAMA guideline, and over acute toxicity thresholds measured in local fish species (Matsuo et al., 2005; Duarte et al., 2009; Crémazy et al., 2022, 2016). Regulatory enforcement in Brazil currently focuses on protection against acute toxicity, and there is an urgent need for a tool that will predict acute Cu toxicity across a range of native species.

The increase in human pressure and associated risks of metal pollution to Amazonian freshwater ecosystems are increasing the need for the development of water quality criteria (WQC) that adequately protect aquatic communities. For Cu, bioavailability-based approaches that account for local water chemistry, such as the mechanistic Biotic Ligand Model (BLM) or the empirical Multi-Linear Regression (MLR) models derived from it, are considered as best regulatory practices (Santore et al., 2001; Paquin et al., 2002; Niyogi and Wood, 2004; Mebane et al., 2020). Unfortunately, a recent review (Souza et al., 2023) notes that bioavailability-based approaches such as the BLM have been largely overlooked to date in Brazil. The BLM is a chemical equilibrium-based theoretical framework that stipulates that metal toxicity is directly related to the concentration of metal bound to biological sites of toxicity (e.g. gills) called biotic ligands (BLs). This metal-BL association is highly modulated by water quality parameters through competition between free metal ion (e.g. Cu^{2+}) and protective aqueous cations (e.g. Ca^{2+}, Na^{+} and H^{+}) for binding to BLs, and by complexation of metal ions with aqueous inorganic (e.g. HCO_3^- and OH^-) and organic (dissolved organic carbon (DOC)) ligands (Niyogi and Wood, 2004; Mebane et al., 2020). In particular, DOC is known to be very protective against Cu toxicity because of its strong ability to bind free Cu^{2+}, thereby reducing its bioavailability (e.g., McGeer et al., 2002; Crémazy et al., 2017). Various BLMs have been developed and validated over the years for a variety of temperate freshwater species and metals (Santore et al., 2001; Paquin et al., 2002; Niyogi and Wood, 2004; Mebane et al., 2020; Garman et al., 2020), and BLMs are used in several countries around the world to derive site-specific WQCs (Niyogi and Wood, 2004; Zhang et al., 2017; Ryan et al., 2018). These latter BLMs have been calibrated in a wide variety of freshwater conditions, notably pH (4.9–9.2), concentrations of DOC (0.05–29.65 mg/L), Ca^{2+} (0.2–120.2 mg/L), Mg^{2+} (0.02–51.9 mg/L), and Na^{+} (0.16–236.9 mg/L) (HydroQual, Inc., 2007; McConaghie & Matzke, 2016). However, BLMs have yet to be calibrated and evaluated for fishes of the Amazon basin under their local water quality conditions (extremely soft water, pH (3.6–5.5), DOC (2.3–71 mg/L)) (Ríos-Villamizar et al., 2020). Instead, Cu is currently regulated with a single water quality guideline (WQG) for all Brazilian freshwaters (Conselho Nacional do Meio Ambiente, 2005). To test and calibrate BLMs, it is necessary to conduct toxicity tests at varying water chemistry representative of natural conditions, in order to i) determine fish sensitivity to metals, and to ii) evaluate the effects of water chemistry on metal toxicity.

Rather than using a single WQG for all water bodies, a bioavailability-based regulatory approach appears to be suitable for the Amazon basin, which comprises markedly different waters typically divided into three main types whose composition also fluctuates with annual seasons: the "black waters" from the Negro River drainage area (acidic pH, very low content of major cations and anions and high DOC concentration); the "white waters" from the Solimões/Amazonas River (near neutral pH, relatively higher amount of suspended particles and nutrients, and lower DOC content); and "clear waters" from the Tapajós River (neutral pH and low DOC and ionic levels) (Furch, 1984; Sioli, 1984).Despite the high natural acidity of blackwaters, the pH of Rio Negro water has recently been reported to reach 6.42–6.98 in some areas (Del Rio Calvo & Oliveira, 2020). As expected, recent studies have shown that the sensitivity of one well-studied Amazonian fish species (*Paracheirodon axelrodi*) to metals (copper and nickel) was strongly modulated by the differences in water composition among the different types of water (Crémazy et al., 2016, 2019; 2022; Holland et al., 2017). Yet, when assessing the applicability of the BLM to predict acute Cu toxicity in tropical conditions, the BLM appeared to overestimate Cu toxicity to *P. axelrodi* in Rio Negro water (Crémazy et al., 2016). These authors hypothesized that blackwater DOC can induce physiological adjustments in gills that exerts a protective role against Cu toxicity, which the BLM was not built to take into account. In contrast, a subsequent study (Crémazy et al., 2022) did not support this interpretation. Alternatively, this toxicity over-estimation by the BLM might be associated with a relatively greater ameliorating effect of hardness in extremely soft waters. Indeed, in Ni toxicity studies with invertebrates, increased protectiveness from hardness cations (Ca^{2+} and Mg^{2+}) has been observed when tests were conducted in very soft (and very-low DOC) waters (Deleebeeck et al., 2007; Kozlova et al., 2009; Peters et al., 2018).

The present study aimed to determine acute Cu toxicity to nine Amazonian fish species representing the three main fish Orders found in blackwaters (i.e. Characiformes, Cichliformes and Siluriformes) under a range of conditions using different dilutions of Rio Negro water, and to evaluate the accuracy of the BLM at predicting the observed Cu toxicity. Many of these species are economically important for the ornamental fish trade. Here, the BLM was used to model the influence of blackwater conditions on Cu speciation, to estimate lethal Cu accumulations on the gills (LA_{50}) in the selected fish species, and to predict acute Cu toxicity across a range of Amazon blackwater conditions. A particular focus was on the role of blackwater DOC in ameliorating Cu toxicity. The current study was therefore intended to further evaluate the applicability of the BLM for predictions of Cu toxicity to Amazonian fish, and to refine it as required for this purpose. The ultimate goal is to contribute to the development of site-specific acute WQC for tropical blackwaters.

2. Material and methods

2.1. Fish acquisition and acclimation

We selected nine teleost fish species from three main Orders of the Amazon: the Characiformes – *Carnegiella strigatta* (0.28 ± 0.01 g), *Paracheirodon axelrodi* (0.24 ± 0.01 g), *Hemigrammus rodosthomus* (0.34 ± 0.02 g) and *Hyphessobrycon socolofi* (0.87 ± 0.03 g); the Cichliformes – *Dicrossus maculatus* (0.68 ± 0.01 g), *Apistogramma hippolytae* (1.21 ± 0.83 g) and *Apistogramma agassizii* (1.27 ± 0.15 g); and the Siluriformes – *Corydoras schwartzi* (0.94 ± 0.02 g) and *Otocinclus hasemani* (0.39 ± 0.02 g). The animals were acquired from an ornamental fish dealer (Turky's Aquariums; Manaus, AM) and transported to the Laboratory of Ecophysiology and Molecular Evolution (LEEM), at the Brazilian National Institute for Research of the Amazon (INPA) located in Manaus (AM, Brazil). All fish species had been collected from their natural environment near Barcelos city (~500 km upstream from Manaus, 0° 57'.596″S, 62° 55'.368″W) from small blackwater streams of the Rio Negro basin. This region is historically recognized as a pristine area with no known sources of metal contamination. Animals were acclimated for at least two weeks to laboratory conditions, in aerated tanks (150 L) supplied with flow-through of very soft water from INPA wells (average composition: pH = 6.88; [Na^{+}] = 57.4; [K^{+}] = 30.4; [Ca^{2+}] = 16.2; [Mg^{2+}] = 3.7; [Cl^{-}] = 48.0 μM; temperature = 28 °C, alkalinity = 5.33 mg $CaCO_3$/L; DOC = 0.41 mg C/L), hereafter referred to as ion-poor water (IPW). Fish were fed once daily to satiation with commercial dry food pellets. Feeding was suspended 48 h before and during the experiments. All experiments were performed in accordance with current Brazilian National guidelines for care and use of laboratory animals, and with the guidelines of the National Research Council (USA) Guide for the Care and Use of Laboratory Animals. For all species, the fish were of mixed sex, though sex was not determined in the individual assays.

2.2. Toxicity assays

To verify the influence of Rio Negro water DOC water on the acute toxicity of copper, we determined the 96 h Cu toxicity for all fish species across a range of dilutions of Rio Negro water (RNW) with IPW. This latter water has a chemical composition that resembles the ion-poor water of the Rio Negro, but without the high DOC levels (Wood et al., 2003). Six test solutions were prepared with the following % of RNW: 0% (i.e. 100% IPW), 20%, 40%, 60%, 80% and 100% (i.e. undiluted RNW). All toxicity tests were conducted within one year. Throughout this year, fresh RNW was collected weekly to avoid important changes in DOC properties by long-term water storage. Water from the Rio Negro was directly pumped (submersible pump JENECA HM-6081) to a 1000-L fibreglass tank and transported to the INPA laboratory.

For each fish species/water type combination, we conducted a toxicity test with 10 Cu concentrations ranging from 10 to 2000 μg Cu/L and a control (Cu-free water). Prior to initiating the toxicity assays, fish were acclimated for at least two weeks in holding tanks (150 L) containing aerated water at each of the 6 water compositions tested (i.e., 0, 20, 40, 60, 80 and 100% RNW). The results of the 0% RNW tests (i.e. 100% IPW) have been reported previously (Duarte et al., 2009), and these tests were performed at the same time as the other tests. During the acclimation period the mortality levels in holding tanks were always lower than 10% to all fish species.

To start the tests, a group of 10 individuals of each fish species was transferred to each of 11 plastic test tanks (10 tanks for Cu exposure and one control group) and allowed to settle for at least 24 h prior to the experimental set-up. A ratio of 0.8 g of fish/L of experimental solution was maintained in each test tank. The test tanks were connected to a header tank (150 L) to which an appropriate aliquot of 1 M $CuCl_2.2H_2O$ (Sigma-Aldrich, ACS reagent >99.0%) stock solution was added. The test solutions were prepared 24 h before the test to allow chemical equilibration. To initiate the 96 h toxicity tests, water in the test tanks was carefully replaced with the appropriate experimental solution with copper, and a gradual flow-through from the header tank was initiated, so that 80% of the water volume was replaced every 24 h. No mortality of fish in control group was recorded in any of the toxicity trials with different dilutions of RNW. The water in tanks was continuously aerated during the experiments, and tests were run under a 12:12-h dark:light cycle. Fish mortality, pH and the concentration of major ions (Na^+, K^+, Ca^{2+}, Mg^{2+} and Cl^-) in water of each test tank was recorded every 12 h. Water samples (~5 ml) were every 12 h from all the test tanks and the header tank to determine the total copper concentration, and samples were acidified with 100 μl of 1N HNO_3 and stored at 4 °C for later copper analysis. The concentrations of DOC, sulphate and alkalinity in test solutions were determined once during the preparation of the different dilutions of RNW at each toxicity trial.

2.3. Analytical methods

Copper concentrations were measured by atomic absorption spectrophotometry with a graphite furnace technique (PerkinElmer Analyst 800 AA spectrophotometer). The AA spectrophotometer was calibrated using a multielement standard solution (PerkinElmer) to obtain a five-point standard curve for copper, which included four standards and one blank (Milli Q water – Millipore). The concentrations of the major cations (i.e., Na^+, K^+, Ca^{2+} and Mg^{2+}) were also determined by AA spectrophotometry with a flame technique (PerkinElmer Analyst 800 AA spectrophotometer). Water concentrations of Cl^- were measured using the colorimetric assay described by Zall et al. (1956). DOC concentration in water samples (except 100% IPW) was determined through the combustion and oxidation of organic carbon in samples, with subsequent generation of CO_2 that was measured by a non-dispersive infra-red (NDIR) detector using a Total Organic Carbon (TOC) Analyzer (Apollo 900, Teledine Tekmar. A commercial standard (Phoenix TOC Validation set, Teledyne Tekmar) was used to obtain a four points standard curve, which includes three standards and one blank (Milli Q water – Millipore). Water temperature and pH were measured using a multi-parameter probe (YSI), and dissolved oxygen was kept always above 80% saturation. Alkalinity was measured by the standard titration method (APHA, 1998).

2.4. Data analysis and BLM modelling

For all fish species, the copper LC_{50} values for 96 h exposure (96 h LC_{50}) and their upper and lower 95% confidence limits were obtained from the plots of the percentage of fish mortality versus the total copper concentrations in the experimental solution, using the trimmed Spearman-Karber method (Hamilton et al., 1977).

The Biotic Ligand Model software from Windward Environmental (version 3.41.2.45; available at: https://www.windwardenv.com/biotic-ligand-model/) was used. This BLM is the basis of the US Environmental Protection Agency's recommended freshwater ambient water quality criteria (AWQC) for copper. The BLM was first employed to obtain Cu aqueous speciation and the lethal Cu accumulation (LA_{50} nmol/g wet weight) at the biotic ligand for each fish species (i.e., the Cu accumulation on BL that leads to 50% mortality). This modeling was performed at the observed 96 h LC_{50} values, using water quality parameters measured for each toxicity test and assuming humic acid percentages (HA) of 10% (default value in the Windward BLM), 50% and 90% for the DOC, since the exact composition was unknown. For each fish species, we used a linear regression analysis to determine if LA_{50} values (i.e. estimated fish sensitivity to Cu) are affected by the alterations of water chemical composition in the toxicity tests. Secondly, mean LA_{50} values (geometric mean of LA_{50} values calculated under the tested range of RNW dilutions) at each tested % of HA were compared among fish species within the Orders of Characiformes and Cichliformes, as well as among the Orders themselves, by a one-way ANOVA followed by Tukey's *a posteriori* test. Differences in mean LA_{50} between the two Siluriformes fish species were evaluated by a paired *t*-test.

Finally, toxicity predictions were performed using the acute Cu BLM available in the Windward BLM software, with the same DOC quality assumptions mentioned above. The predictions were conducted using the newly derived LA_{50} values estimated for each Amazonian fish species separately, and also using the existing rainbow trout LA_{50} value (3.7 nmol/g wet weight) in the Windward BLM. In both these scenarios, the BLM-predicted 96h LC_{50} values were plotted against the experimentally observed 96h LC_{50} values, for all fish species and the various %HA values mentioned above. On these graphs, the 1:1 line shows perfect agreement, and boundary lines show 2-fold deviation from perfect agreement (i.e., ratios of 0.5 and 2 between observed and predicted 96h LC_{50} values).

3. Results

3.1. Toxicity test data

3.1.1. Water quality

For each toxicity test, the mean water quality parameters are presented in Table 1 and Supplementary Fig. S1. Note that the 100% RNW treatment for *D. maculatus* was missing due to limited fish availability. We observed some variability in certain water quality parameters (e.g. pH, Na and Ca concentrations) of IPW and RNW used to make up the exposure solutions (Table 1 and Supplementary Fig. S1). These variations were expected and reflect natural seasonal water quality oscillation in a dynamic system, as fresh IPW and RNW were repeatedly collected over a full year for this study. Overall, in the toxicity tests, water pH ranged from 5.5 to 7.4, [DOC] varied from 0.41 to 8.27 mg/L, [Ca^{2+}] varied from 5.0 to 42.9 μM, [Mg^{2+}] varied from 2.5 to 9.8 μM, [Na^+] varied from 21.3 to 79.6 μM, [K^+] varied from 4.3 to 56.5 μM, [Cl^-] varied from 14.4 to 117 μM, and alkalinity varied from 2.00 to 6.66 mg/L $CaCO_3$ (Table 1 and Supplementary Fig. S1). In general, for

Table 1
Water quality measured in the test solutions, and the experimentally observed 96 h LC_{50} values (with 95% confidence intervals) for the nine species of fish.

Fish species	Toxicity test	96h-LC_{50} (μgCu/L)	Temp	pH	DOC	HA	Ca^{2+}	Mg^{2+}	Na^{+}	K^{+}	SO_4^{2-}	Cl^{-}	Alkalinity
			°C		(mg C/L)	(%)	(μmol/L)						($mgCaCO_3$/L)
C. strigatta	INPA water[a]	26.3 [21.0 –32.8]	28	6.73	0.41	10	5.0	2.5	36.1	18.4	10.4	50.5	5.08
	20% RN	54.7 [39.5 –69.9]	28	5.98	2.97	10	41.4	5.1	79.6	9.0	8.7	86.3	3.06
	40% RN	79.4 [61.7 –97.1]	28	6.64	3.61	10	33.4	5.1	27.0	11.5	6.4	31.6	5.33
	60% RN	148 [135 –161]	28	6.54	4.59	10	7.0	5.1	62.7	7.4	5.2	52.8	3.66
	80% RN	172 [155 –190]	28	6.43	5.69	10	12.0	5.1	44.8	8.4	3.5	52.7	3.00
	100% RN	307 [272 –344]	28	6.69	7.45	10	25.5	8.1	34.4	11.5	1.8	24.3	4.00
P. axelrodi	INPA water[a]	45.9 [39.1 –53.7]	28	6.46	0.41	10	17.2	3.0	63.5	35.3	10.4	51.3	5.12
	20% RN	172 [149 –197]	28	5.75	2.24	10	11.2	3.0	21.3	12.3	8.7	31.3	3.15
	40% RN	297 [258 –340]	28	5.69	3.26	10	14.0	5.5	23.5	14.3	6.4	29.3	3.33
	60% RN	347 [299 –381]	28	6.49	4.19	10	16.7	7.6	22.2	12.8	5.2	29.9	4.30
	80% RN	402 [348 –464]	28	6.53	4.95	10	22.2	7.6	25.2	15.3	3.5	53.0	4.31
	100% RN	672 [597 –756]	28	6.65	7.48	10	42.9	9.8	25.7	9.2	1.8	111.7	6.66
H. rhodostomus	INPA water[a]	12.8 [10.4 –15.7]	28	7.05	0.41	10	9.7	3.8	52.2	36.3	10.4	46.8	5.60
	20% RN	81.2 [71.7 –92.1]	28	6.78	2.24	10	10.0	3.4	30.5	21.2	8.7	21.2	3.00
	40% RN	158 [131 –189]	28	6.4	3.26	10	14.2	5.5	32.6	18.9	6.4	40.9	4.30
	60% RN	205 [229 –273]	28	5.99	4.2	10	16.7	6.8	31.3	19.8	5.2	14.4	3.15
	80% RN	313 [286 –340]	28	6.44	5.02	10	23.5	8.9	52.2	29.7	3.5	66.3	5.00
	100% RN	443 [408 –468]	28	6.5	7.48	10	42.9	9.8	25.7	9.2	1.8	117.1	6.66
H. socolofi	INPA water[a]	26.9 [24.1 –30.0]	28	6.84	0.41	10	9.2	3.0	60.5	28.1	10.4	50.2	5.08
	20% RN	38.1 [31.0 –45.2]	28	7.19	1.52	10	8.7	2.5	60.1	4.9	8.7	39.5	4.00
	40% RN	113 [94.0 –133]	28	6.2	2.16	10	11.5	3.4	68.3	4.3	6.4	36.4	2.33
	60% RN	267 [237 –296]	28	6.91	4.59	10	40.7	8.1	63.5	7.7	5.2	74.2	3.50
	80% RN	358 [324 –392]	28	6.8	6.49	10	17.0	8.5	30.9	9.7	3.5	103.0	3.00
	100% RN	414 [376 –452]	28	6.81	7.45	10	25.2	8.9	33.9	13.8	1.8	37.2	3.25
D. maculatus	INPA water[a]	39.3 [29.6 –52.3]	28	6.65	0.41	10	32.9	6.4	70.1	24.6	10.4	60.6	5.40
	20% RN	121 [85.6 –155]	28	6.39	2.46	10	25.7	3.8	38.7	12.3	8.7	73.3	2.50
	40% RN	213 [163 –262]	28	6.36	3.52	10	32.7	4.7	74.4	11.0	6.4	67.7	2.20
	60% RN	236 [203 –269]	28	5.54	4.4	10	38.7	5.5	67.4	10.2	5.2	67.1	3.33
	80% RN	333 [274 –391]	28	6.8	6.15	10	37.4	8.1	22.6	9.5	3.5	64.0	3.10
A hippolytae	INPA water[a]	33.0 [22.6 –47.9]	28	7.12	0.41	10	19.2	4.7	66.1	56.5	10.4	39.2	5.60
	20% RN	271 [220 –322]	28	6.98	1.96	10	26.0	3.8	73.5	9.2	8.7	61.5	6.33
	40% RN	304 [217 –391]	28	6.75	3.25	10	23.7	5.5	53.1	9.7	6.4	69.1	2.00
	60% RN	603 [553 –653]	28	6.12	4.4	10	16.5	6.4	71.8	7.9	5.2	45.7	5.67
	80% RN	831 [753 –908]	28	6.39	5.69	10	10.5	7.6	55.3	9.7	3.5	80.4	3.00
A. agassizii	INPA water[a]	40.2 [25.3 –63.7]	28	6.89	0.41	10	32.9	6.4	70.1	24.6	10.4	41.5	5.50
	20% RN	248 [192 –319]	28	5.86	2.24	10	11.5	3.8	67.0	20.7	8.7	66.3	3.15
	40% RN	430 [338 –548]	28	6.54	3.26	10	13.7	5.5	40.0	18.9	6.4	48.5	3.00
	60% RN	600 [531 –799]	28	6.61	4.2	10	18.5	6.8	55.3	26.1	5.2	49.4	3.00
	80% RN	771 [670 –886]	28	7.05	4.95	10	24.7	7.6	60.5	24.0	3.5	77.9	5.33
	100% RN	1090 [981 –1190]	28	6.47	7.48	10	34.4	8.9	34.8	14.3	1.8	99.6	6.66
C. schwartzi	INPA water[a]	53.0 [38.1 –73.9]	28	7.38	0.41	10	12.0	3.4	54.4	29.2	10.4	54.7	4.98
	20% RN	260 [227 –293]	28	6.15	2.46	10	25.7	3.8	38.7	12.3	8.7	73.3	2.30
	40% RN	394 [332 –456]	28	6.13	3.52	10	32.7	4.7	74.4	11.0	6.4	67.7	2.10
	60% RN	442 [393 –490]	28	6.1	3.6	10	28.5	7.6	61.4	10.7	5.2	42.9	3.33
	80% RN	452 [388 –515]	28	5.92	4.4	10	38.7	5.5	67.4	10.2	3.5	67.1	2.67
	100% RN	915 [737 –1090]	28	6.26	8.27	10	37.4	8.5	22.6	9.5	1.8	64.0	3.00
O. hasemani	INPA water[a]	18.0 [14.8 –22.2]	28	6.81	0.41	10	8.5	3.0	44.8	20.2	10.4	47.7	5.60
	20% RN	66.6 [42.8 –90.4]	28	7.16	1.96	10	26.0	3.8	73.5	9.2	8.7	61.5	6.33
	40% RN	212 [189 –254]	28	6.78	3.25	10	23.7	5.5	53.1	9.7	6.4	69.1	2.00
	60% RN	325 [288 –363]	28	6.87	4.59	10	40.7	8.1	63.5	7.7	5.2	74.2	3.10
	80% RN	440 [385 –495]	28	6.48	6.49	10	17.0	8.5	30.9	9.7	3.5	103.0	3.20
	100% RN	454 [391 –516]	28	6.23	7.61	10	24.7	7.6	26.1	9.5	1.8	72.5	3.33

[a] Data from Duarte et al. (2009).

the different species toxicity tests, as the % RNW increased in the test solution, there were significant increases in the DOC ($p < 0.0001$), Mg^{2+} ($p < 0.0001$), Ca^{2+} ($p = 0.0044$), and Cl^{-} ($p = 0.0065$) concentrations, and significant decreases in SO_4^{2-} ($p < 0.0001$), K^{+} ($p = 0.0004$) and Na^{+} ($p = 0.002$) concentrations (F-tests) (Supplementary Fig. S1). On the other hand, pH and alkalinity appeared to be unaffected by RNW content ($p > 0.1$) (F-tests) (Supplementary Fig. S1).

3.1.2. Copper toxicity

The experimentally determined 96 h LC50 values are shown in Table 1 for all nine species of fish in the various test waters. These values generally increased with % RNW. More precisely, increasing the RNW content from 0 to 100% resulted in substantial reductions in copper toxicity (seen as increases in LC_{50} values) to *D. maculatus* (8.5- fold: 39.3–333 μg Cu/L), *C. strigatta* (11.7-fold: 26.3–307 μg Cu/L), *P. axelrodi* (14.7-fold, 45.9–672 μg Cu/L), *H. socolofi* (15.4-fold, 26.9–414 μg Cu/L), *C. schwartzi* (, 53.0–915 μg Cu/L 17.3-fold), *O. hasemani* (25.2-fold, 18.0–454 μg Cu/L), *A. hippolytae* (25.2-fold, 33.0–831 μg Cu/L), *A. agassizzi* (27.1-fold, 40.2–1.087 μg Cu/L) and *H. rhodostomus* (34.6-fold, 12.8–443 μg Cu/L). Overall, these trends represent average reduction in copper toxicity of 19.1 times for Characiformes, 20.2 times for Cichliformes and 21.2 times for Siluriformes.

3.2. Model predictions

3.2.1. Copper aqueous speciation

The predicted Cu speciation is presented in Table 2 for a humic acid (HA) content of 10%. In the test waters, the dominant Cu species were predicted to be Cu-DOC complexes, with average relative abundances ranging from 84.2% (in IPW) to 96.5% (in 100% RNW) (Table 2). The most dominant inorganic copper species was $CuHCO_3^{-}$, with an average relative abundance of 9.2% (ranging from 1.5% to 18.74%) in IPW and

Table 2
The BLM-calculated relative abundances (%) of main copper species in test solutions, and lethal copper accumulation at 50% toxicity (LA_{50}). In this analysis, all BLM calculations were performed using 10% of humic acid (HA).

Fish species	Toxicity test	Relative abundance of main Cu species in test water (%)					LA_{50} (nmol/g wet wt)	
		Cu^{2+}	Cu-DOC	$CuOH^+$	$CuHCO_3^+$	$CuCO_3$	Individual tests	Geometric mean
C. strigatta	INPA water[a]	2.56	91.48	0.49	5.23	0.37	1.87	2.03
	20% RN	0.94	97.95	<0.1	1.18	<0.1	2.97	
	40% RN	0.16	99.48	<0.1	0.37	<0.1	0.97	
	60% RN	0.12	99.71	<0.1	0.19	<0.1	1.34	
	80% RN	0.12	99.76	<0.1	0.16	<0.1	1.47	
	100% RN	0.11	99.72	<0.1	0.20	<0.1	2.35	
P. axelrodi	INPA water[a]	9.17	70.72	0.94	18.74	0.72	16.32	16.7
	20% RN	3.98	91.51	<0.1	5.23	<0.1	17.88	
	40% RN	4.40	90.52	<0.1	6.11	<0.1	21.64	
	60% RN	1.00	97.33	0.11	1.85	<0.1	14.28	
	80% RN	0.87	97.65	0.11	1.61	<0.1	14.14	
	100% RN	0.85	96.85	0.14	2.38	0.14	17.20	
H. rhodostomus	INPA water[a]	0.66	97.44	0.26	1.46	0.22	0.76	4.16
	20% RN	0.22	99.48	<0.1	0.29	<0.1	1.51	
	40% RN	0.54	98.56	<0.1	0.98	<0.1	5.50	
	60% RN	0.94	98.06	<0.1	1.25	<0.1	9.10	
	80% RN	0.65	98.09	<0.1	1.36	<0.1	9.91	
	100% RN	0.43	98.46	<0.1	1.20	<0.1	9.20	
H. socolofi	INPA water[a]	2.81	90.42	0.70	5.68	0.52	5.60	3.27
	20% RN	<0.1	99.72	<0.1	0.14	<0.1	0.29	
	40% RN	1.23	97.68	<0.1	1.20	<0.1	7.89	
	60% RN	0.39	98.96	0.11	0.57	<0.1	6.10	
	80% RN	0.15	99.67	<0.1	0.19	<0.1	3.61	
	100% RN	0.16	99.63	<0.1	0.23	<0.1	4.26	
D. maculatus	INPA water[a]	7.87	73.33	1.25	16.75	0.99	13.61	9.83
	20% RN	0.98	98.04	<0.1	1.01	<0.1	7.02	
	40% RN	1.15	97.86	<0.1	1.04	<0.1	11.03	
	60% RN	3.26	92.84	<0.1	4.45	<0.1	16.55	
	80% RN	0.26	99.40	<0.1	0.35	<0.1	5.25	
A hippolytae	INPA water[a]	3.47	86.01	1.63	7.64	1.34	7.49	17.9
	20% RN	3.92	84.13	1.33	9.97	1.26	22.19	
	40% RN	1.59	96.93	0.32	1.34	0.10	16.77	
	60% RN	4.19	86.88	0.20	9.85	0.17	25.57	
	80% RN	3.10	93.46	0.27	3.97	0.13	25.88	
A. agassizii	INPA water[a]	6.62	75.95	1.83	14.30	1.47	12.58	22.2
	20% RN	6.39	86.00	0.17	8.27	<0.1	23.34	
	40% RN	3.56	92.09	0.44	4.51	0.21	24.08	
	60% RN	3.74	91.62	0.55	4.74	0.26	25.52	
	80% RN	2.67	90.29	1.07	5.94	0.89	25.18	
	100% RN	2.76	90.09	0.29	7.68	0.30	26.21	
C. schwartzi	INPA water[a]	4.97	77.90	4.27	9.77	3.11	13.47	21.8
	20% RN	5.02	90.53	0.25	4.76	<0.1	22.83	
	40% RN	4.88	91.27	0.24	4.22	<0.1	24.37	
	60% RN	5.28	88.11	0.24	7.27	0.12	25.20	
	80% RN	4.44	91.28	0.13	4.88	<0.1	24.07	
	100% RN	1.80	96.33	0.12	2.32	<0.1	23.55	
O. hasemani	INPA water[a]	1.53	94.48	0.35	3.42	0.29	2.36	4.90
	20% RN	0.16	99.28	<0.1	0.42	<0.1	0.87	
	40% RN	0.66	98.71	0.14	0.56	<0.1	8.11	
	60% RN	0.66	98.38	0.17	0.85	<0.1	10.27	
	80% RN	0.38	99.17	<0.1	0.53	<0.1	8.83	
	100% RN	0.42	99.11	<0.1	0.60	<0.1	9.16	

[a] Data from Duarte et al. (2009).

of 2.1% (ranging from 0.20% to 7.7%) in RNW (Table 2). The next most abundant species were the ones recognized as bioavailable in the Windward BLM: Cu^{2+} with an average relative abundance of 4.4% (ranging from 0.7% to 9.2%) in IPW and of 1.1% (ranging from 0.1% to 3.1%) in RNW, and $CuOH^+$ with an average abundance of 1.3% (ranging from 0.3% to 4.3%) in IPW and of 0.1% (ranging from <0.1% to 0.3%) in RNW (Table 2). Overall, the increment of RNW content tended to reduce the relative abundance of the main inorganic copper species ($CuHCO_3^+$, Cu^{2+}, $CuOH^-$ and $CuCO_3$ respectively) in favour of increasing the % Cu-DOC complexes in the test solutions. When increasing the % HA content in the model from 10% to 50 and 90%, the relative abundance of Cu-DOC complexes increased (>90% in both water types), while the abundance of $CuHCO_3^-$, Cu^{2+} and $CuOH^+$ decreased (Supplementary Table S1).

3.2.2. Fish sensitivity to copper

The BLM-derived LA_{50} values indicated interspecific variations in sensitivity to Cu (Table 2, Fig. 1A). Using 10% HA, the Characiformes fish exhibited the lowest estimated LA_{50} values (i.e. highest sensitivity) compared to Cichliformes and Siluriformes fish (Fig. 1B). The same pattern was seen using 50% HA, but at 90% HA the LA_{50} values for Characiformes were significantly lowered only compared to Cichliformes (Supplementary Fig. S2). The LA_{50} comparison among the species within main fish Orders also revealed that the Characiformes species *C. strigatta* (2.0, 0.4 and 0.1 nmol/g), *H. rhodostomus* (4.2, 1.0 and 0.3 nmol/g and *H. socolofi* (3.3, 0.7 and 0.2 nmol/g) had significantly lower LA_{50} values compared to *P. axelrodi* (16.7, 8.9 and 3.5 nmol/g) at all the % HA values tested (i.e., 10, 50 and 90% HA, respectively) (Fig. 1A; Supplementary Fig. S2). Similarly, within the Siluriformes (catfishes), *O. hasemani* (one of the most sensitive species overall) displayed

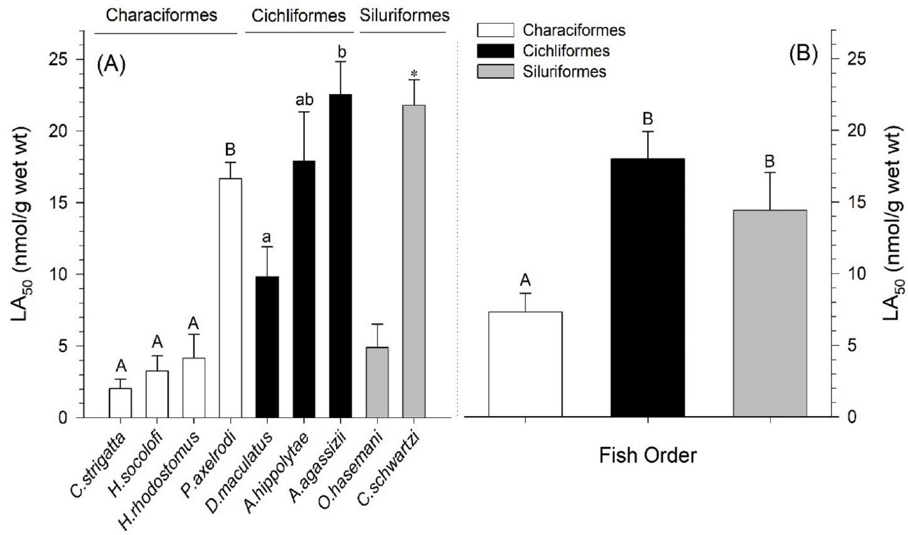

Fig. 1. Mean lethal Cu accumulation (LA_{50}) estimated from the different toxicity tests by the Windward acute Cu BLM (with 10% humic acid) for **A)** individual Amazonian fish species and **B)** fish orders. Data are mean ± SEM (n = 5–6 in left panel and n = 12–19 in the right panel). In the left panel (A), different capital and lower-case letters represent significant differences among Characiformes fish and Cichliformes fish species respectivey, whereas asterisk (*) represents significant differences between Siluriformes species. In the right panel (B), different capital letters represent significant differences among the fish Orders.

significantly lower values of LA_{50} (4.9, 1.2 and 0.3 nmol/g) compared to the highly tolerant species *C. schwartzi* (21.8, 16.0 and 8.2 nmol/gw) (Fig. 1A; Supplementary Fig. S2). Within the Cichliformes, *D. maculatus* showed LA_{50} values that were significantly reduced (9.8 and 3.5 nmol/g) compared to *A. agassizii* (22.6 and 17.6 nmol/g) using 10 and 50%HA in the BLM, respectively (Fig. 1A; Supplementary Fig. S2), but at 90% HA the estimated values of LA_{50} were not different between *D. maculatus* (1.2 nmol/g), *A. hippolytae* (4.9 nmol/g) and *A. agassizii* (10.4 nmol/g) (Supplementary Fig. S2).

BLM-estimated LA_{50} values were plotted across the dilutions of RNW in Fig. 2. There was moderate evidence of a decrease in apparent sensitivity to copper as RNW conditions increased for *H. rhodostomus*, *O. hasemani* and *A. agassizii*, as suggested by the significant positive correlation between the LA50 value and the %RNW content for these species. More precisely, the LA_{50} value increased by 12.2-fold for *H. rhodostomus* ($R^2 = 0.87$, $p = 0.01$) (Fig. 2A), by 2.1-fold for *A. agassizii* ($R^2 = 0.72$, $p = 0.03$) (Fig. 2B) and by 3.9-fold for *O. hasemani* ($R^2 = 0.66$, $p = 0.05$) (Fig. 2C) with increasing RNW content. For the remaining fish species, changes in the LA_{50} were not significantly explained by the content of RNW in test solution ($p > 0.05$) (Fig. 2).

3.2.3. Copper toxicity predictions

Overall, using the newly derived LA50 values (geometric mean of all LA_{50} values for that species, Table 2) at 10% HA, the BLM predicted almost all LC50 values within a factor of two (Fig. 3). When using the sensitivity parameter of the temperate fish *Oncorhynchus mykiss* (LA50 = 3.7 nmol/g), the BLM overestimated Cu toxicity to the most tolerant fish species (e.g., *C. schwartzi*, *A. agassizzi*, *A. hippolytae* and *P. axelrodi*) (Supplementary Fig. S3A). When the % HA was changed from 10% (Fig. S3A) to 50% (Fig. S3B) and 90% (Fig. S3C) in the latter modelling, then the discrepancy between observed and predicted LC_{50} values became worse.

4. Discussion

4.1. Amazonian fish sensitivity to copper

In the present study, three of the four Characiformes species (*P. axelrodi* was a marked exception) and the Cichliformes species *D. maculatus*, were the most sensitive to copper, while the other two Cichliformes species (*A. agassizzi* and *A. hippolytae*) and the Siluriformes species *C. schwartzi* were the most tolerant. These sensitivity differences might reflect marked inter-species differences in branchial Na^+ uptake mechanisms. Copper is broadly recognized as an ionoregulatory stressor to freshwater fishes, directly acting on Na^+ (and Cl^-) homeostasis (reviewed by Grosell, 2012), with detrimental effects on gill physiology quite similar to those observed in fish exposed to low water pH (Gonzalez et al., 2006; Kwong et al., 2014). The depletion of internal Na^+ pools following acute Cu exposure is associated with **i)** stimulation of Na^+ effluxes due to increased branchial epithelium permeability (as Cu^{2+} displaces Ca^{2+} ions from cellular tight junctions) and to **ii)** decreased Na^+ branchial uptake via direct Cu^{2+} and Na^+ competition at Na^+ channels and via inhibition of enzymes involved with Na^+ uptake (e.g. Na^+/K^+-ATPase, carbonic anhydrase and V-type H^+-ATPase) (Grosell et al., 2002; Matsuo et al., 2005; Grosell, 2012; Crémazy et al., 2016). It follows that the sensitivity to copper exposure might be predicted and explained by Na^+ turnover rates, where species with lower control of branchial ionic permeability and high affinity Na^+ uptake mechanisms tend to be more sensitive to copper (Grosell et al., 2002; Morris et al., 2021). Teleost fish from blackwaters of the Rio Negro present remarkable adaptations in their branchial ionoregulatory mechanisms, especially to maintain Na^+ homeostasis, to thrive in ion-poor acidic blackwaters (Gonzalez et al., 2002, 2006; Wood et al., 2014; Morris et al., 2021). Below, we provide possible explanations for the copper sensitivity differences among our tested fish species, based on species-specific adaptations in Na^+ homeostasis.

We found that three Characiformes fish species are among the four most sensitive species to copper. This finding suggests that this group of species could serve as potential models for the development of risk assessment procedures environmentally and ecologically relevant for the protection of Amazonian blackwaters against metal contamination. High sodium turnover rates have been reported in Characiformes species such as *Hemigrammus* sp. and *C. strigatta*, which display low control of branchial ionic permeability (high Na^+ efflux rates) during exposure to environmental acidity in ion-poor water, but show a low-pH insensitive Na^+ uptake system, characterized by high influx rates (J_{max}) and a high affinity for Na^+ uptake (low K_m) (Gonzalez et al., 2002, 2006; Val and Wood, 2022). This efficient Na^+ transport system may increase Cu uptake (via ion mimicry) and gill Cu accumulation, resulting in apparent low Cu tolerance observed in three out of four Characiformes fish species

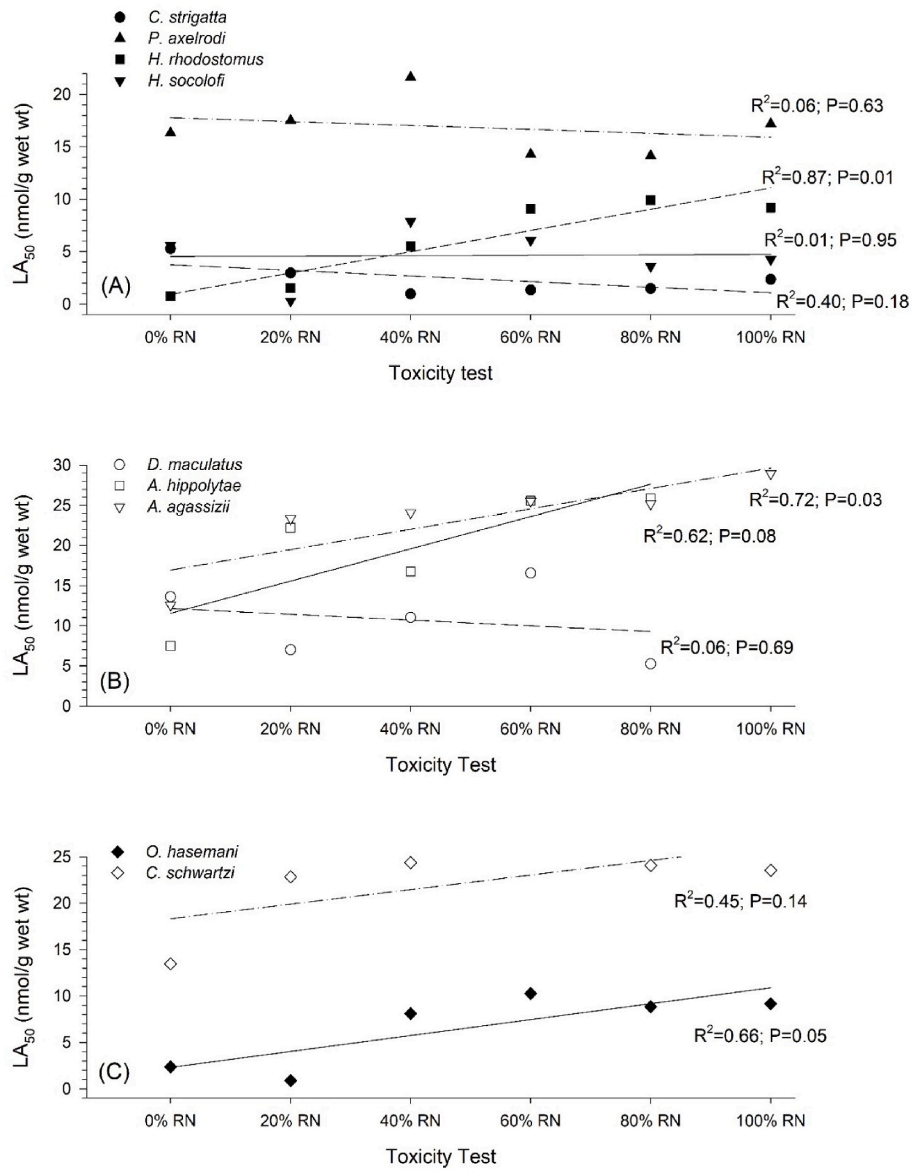

Fig. 2. Effect of increasing content of Rio Negro Water (RNW) in the test solutions on the estimated lethal accumulation (LA_{50}) by the BLM (with 10% humic acid) to nine Amazonian fish species. (A) Characiformes, (B) Cichliformes and (C) Siluriformes fish species. The relationship was calculated through a Linear Regression analysis for each fish species.

in our study. An exception was *P.* axelrodi, which appeared tolerant to Cu in our study and Crémazy et al. (2016). Perhaps the effects of DOC on gill physiology of this species might explain this contrasting response (see Discussion below).

In contrast to Characiformes, a less specialized ion uptake system with lower Na^+ uptake rates, lower affinity for Na^+ (high Km) and a lower intrinsic permeability of gills with a high control of efflux rates, even in ion-poor acidic waters, has been seen in cichliform fishes such as *Apistogramma* sp and *Pterophyllum scalare* (Gonzalez et al., 2002, 2006; Duarte et al., 2013; Val and Wood, 2022). Thus, the higher Cu tolerance displayed by both *Apistogramma* species studied likely reflects a stronger resistance to increased ionic diffusive losses in the gills induced by Cu^{2+}. Meanwhile, the higher Cu sensitivity of the cichliform *D. maculatus* might be explained by the two-fold lower weight compared to both *Apistogramma* species, since smaller fishes are thought to be less tolerant to metals than larger fishes (Grosell et al., 2002). Few studies have evaluated both the Na^+ transport system and diffusive loss rates in Siluriformes in blackwater conditions. In an earlier investigation, Gonzalez et al. (2002) revealed that *Corydoras julii* employs a mixed mechanism for Na^+ homeostasis, displaying relatively low Na^+ uptake affinity but high uptake rates, and a relatively poor control of diffusive efflux rates at low pH. Thereby, the specializations in Na^+ uptake system in this group might help fish to avoid severe imbalances in Na^+ homeostasis and contribute to the high Cu tolerance seen in *C. schwartzi*. Therefore, the emerging picture suggests that species-specific specializations of branchial unidirectional Na^+ fluxes are behind the differences in magnitude of the relative sensitivity/tolerance to copper in Amazonian species in blackwaters, and among the main fish Orders analysed.

4.2. Effects of water quality on copper toxicity

The present study is the first to report Cu 96-h LC50 values for the following eight Amazonian fishes in their natural river waters: *C. strigatta*, *H. rodosthomus*, and *H. socolofi*, *D. maculatus*, *A. hippolytae*, *A. agassizii*, *C. schwartzi* and *O. hasemani*. Only the cardinal tetra (*P. axelrodi)* has been previously assessed in Rio Negro water, with a reported 96-h LC50 value of 1090 [887–1290] μg L^{-1} (mean and 95% confidence interval) by Crémazy et al. (2016), relatively similar to the

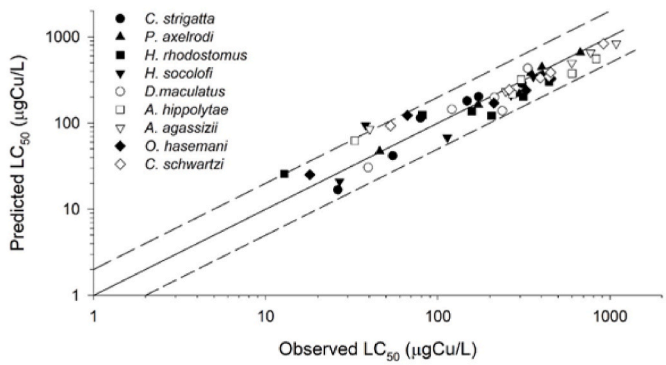

Fig. 3. Relationship between experimentally observed and BLM-predicted 96 h LC_{50} for nine Amazonian fish species in bioassays with different dilutions of natural Rio Negro water. Predicted 96 h LC_{50} values were calculated by the Windward acute Cu BLM, using water quality parameters in Table 1 and the species-specific LA_{50} in Table 2 (geometric mean values). The plain line the perfect agreement line (1:1 line) and the dashed lines represent a 2-fold deviation between observed and predicted 96h LC_{50} values (2:1 and 1:2 lines).

value of 672 [597 −756] μg L^{-1} in our study.

The role of water quality parameters on Cu toxicity to Amazonian fish species has been recently evaluated (Matsuo et al., 2005; Duarte et al., 2009; Crémazy et al., 2016; Dal Pont et al., 2017; Crémazy et al., 2019; Crémazy et al., 2022), while these effects have been widely described to several temperate freshwater fish species (reviewed by Grosell, 2012). In our study the substantial decrease in Cu toxicity (i.e. increases in 96-h LC50 values) with the increase in test water RNW content was likely mostly due to the ~20-fold increase in DOC concentration, and to a lesser extent to the concomitant ~2-fold increases in Ca^{2+} and Mg^{2+} concentrations from 0 to 100% RNW (cf. Table 1 and Supplementary Fig. S1). The protection by Ca^{2+} and Mg^{2+} concentrations is attributed to the decrease in Cu binding to the BL as these major cations compete with Cu^{2+} for binding to the BL. The protection by DOC is attributed to the strong decrease in aqueous Cu^{2+} concentration due to high complexation of copper by DOC molecules, as %RNW increases (cf. Table 2) (see Al-Reasi et al., 2011, 2013 for more details).

Previous studies have also proposed that a proportion of the protective effect of DOC against metal toxicity could be due to physiological effects of DOC on branchial epithelium permeability and ion uptake of fishes (Glover and Wood, 2004; Wood et al., 2011; Crémazy et al., 2016; Holland et al., 2017; Morris et al., 2021). This hypothesis was notably raised by Crémazy et al. (2016) who found that BLM overestimated copper toxicity to *P. axelrodi* in RNW. This hypothesis is based on studies that have shown that DOC stimulates Na^{+} (and Cl^{-} and Ca^{2+}) uptake in fish gills (Gonzalez et al., 1998, 2002; Wood et al., 2003; Matsuo and Val, 2007; Duarte et al., 2016; Duarte et al., 2018) and reduces diffusive ion losses via increased gill permeability, under acidic conditions (Araújo et al., 2017; Wood et al., 2003; Wood et al., 2011; Duarte et al., 2016; Duarte et al., 2018). These effects appear to be linked with the aromaticity of the DOC, where allochthonous DOC molecules with high molecular weight and darker optical properties, as seen in Rio Negro black waters (Duarte et al., 2016; Johannsson et al., 2020), provide the highest protection against ionoregulatory disturbances (Glover and Wood, 2004; Wood et al., 2011; Crémazy et al., 2016; Holland et al., 2017; Morris et al., 2021). In our study though, there was no strong evidence of a physiological protection from DOC against Cu toxicity, in agreement with a recent study with *P. axelrodi* and *A agassizi* (Crémazy et al., 2022). Indeed, only three out of the nine tested fish species (*H. rhodostomus, O. hasemani and A. agassizii*) showed moderate evidence of a LA50 increase with increase RNW content. Further, when using the geometric mean of the LA50 values determined for all tested species, Cu toxicity predictions by the BLM were within a factor 2 error. The relatively high pH of 6.5 of the RNW used in our study might explain the absence of these effects, which are limited compared to Cu-DOC complexation effects (Crémazy et al., 2022). Indeed, these physiological effects appear to be more important at low pH, possibly due to an increased binding of DOC to gills under acidic conditions (Morris et al., 2021). The discrepancies between the findings of no physiological protective action of DOC against copper toxicity in *P. axelrodi* (present study; Crémazy et al., 2022) and *A agassizi* (Crémazy et al., 2022) versus non-parallel opposite findings in *P. axelrodi* (Crémazy et al., 2016) and *A. agassizi* (present study) indicate the need for further research on this issue across a range of pHs.

4.3. Implications for environmental regulation

Over the last decades, many environmental regulatory agencies worldwide have recommended the establishment of water quality criteria (WQC) for the risk assessment to metal contamination, where the BLM mechanistic approach can be used to predict metal toxicity and provide site-specific regulatory limits for protecting aquatic life (Niyogi and Wood, 2004; Zhang et al., 2017; Ryan et al., 2018; Mebane et al., 2020; Garman et al., 2020). Our data suggest that the Windward BLM can be used for accurate prediction of acute copper toxicity to Amazonian fish, using our newly derived LA50 values. While the Windward BLM tested in this study appears to be a suitable tool for implementing metal bioavailability ERA in the Amazon, similar performance will likely be offered by other available bioavailability-based tools, such as other full-BLM software (e.g. WHAM FTOX, Visual Minteq), or more user-friendly simplified BLM tools (e.g. M-BAT, Bio-met) (Merrington et al., 2016) and empirical MLRs (Schlekat et al., 2020). To further evaluate the applicability of bioavailability-based models in Amazonian waters, we recommend future studies to investigate the role of other water quality parameters, particularly pH. Indeed, this latter parameter varies across a far wider range in the Amazon basin (Val and Almeida-Val, 1995) than the range encountered in the present study. We also encourage research to address the important data and knowledge gap regarding chronic Cu toxicity to Amazonian fish. Indeed, chronic sublethal metal exposures represent more realistic environmental scenarios that acute lethal exposures. While current regulatory practices in Brazil focus on protection against acute toxicity, most jurisdictions favour the use of chronic data over acute data in their ERA frameworks. Until such data become available, a simplifying Acute-to-Chronic Ratio (ACR) approach, notably used by the USEPA (2007), may be considered to assess chronic effects based on acute toxicity. Finally, while the fish used in this study represent an ecologically, economically, and socially important group of Amazonian organisms, they are likely not the most Cu-sensitive taxonomic group in this region. Indeed, small invertebrates (e.g. daphnids) tend to be the most sensitive to chronic Cu exposure (Mebane et al., 2020). Thus, we recommend future studies to characterize the chronic toxicity of Cu in other Amazon-relevant taxonomic groups.

5. Conclusions

In the present study, there was a marked reduction in copper toxicity for all nine Amazonian fish species with the increment in Rio Negro water content in test solutions. The relative sensitive to copper was specie-specific where the three most sensitive species (*Hemigrammus rhodostomus*, *Carnegiella strigatta* and *Hyphessobrycon socolofi*) were Characiformes, whereas the most tolerant were from Siluriformes (*Corydoras schwartzi*) and Cichliformes (*Apistogramma agassizii*) Orders. These sensitivity differences among the fish species were reflected in the BLM-predicted lethal gill copper accumulation (LA_{50}), which were generally lower in Characiformes than in Cichliformes. In the present study, there was clear evidence for geochemical protection by DOC on copper toxicity where Cu-DOC complexes were the most dominant Cu species in all test solutions. Indeed, the DOC concentration was 20-fold higher with the increase in RNW content, leading to a substantial decrease in free Cu^{2+} concentration in water. However, the increment in Ca^{2+} and Mg^{2+} in test solutions with higher RNW content also contributed to reduced copper toxicity in fish, by the increase in competition of major cation with Cu^{2+} for binding sites in gills. The use of newly estimated LA_{50} values improved the accuracy of Windward BLM in predicting acute Cu toxicity to all nine fish species, emphasizing the BLM applicability for the establishment of water quality criteria for the risk assessment to copper contamination in Amazonian blackwater conditions.

Author's statement

I, Rafael M. Duarte, will serve as the corresponding author. The paper is not under consideration for publication elsewhere. All authors participated in the experimental work, the analysis of the data, and/or the writing of the manuscript, and all authors have approved the submission.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Adalberto Luis Val reports financial support was provided by Foundation for Research Support of Amazonas State. Christopher Michael Wood reports financial support was provided by Canada Research Chairs program, and a visiting fellowship from the Science Without Borders Program (CNPq-Brazil). Work in Canada was supported by NSERC (Canada) Discovery grants (RGPIN-2017-03843 to CMW and RGPIN-2019-04400 to AC). Adalberto Luis Val reports financial support was provided by Brazilian National Council for the State Funding Agencies. Adalberto Luis Val reports financial support was provided by Coordination of Higher Education Personnel Improvement.

Data availability

Data will be made available on request.

Acknowledgements

Work in Brazil was supported by FAPEAM (062.01187/2017), CNPq (465540/2014–7) and CAPES (Financial Code 001) through the INCT-ADAPTA grant to ALV. ALV is the recipient of a research fellowship from Brazilian CNPq. CMW was supported by the Canada Research Chairs program, and a visiting fellowship from the Science Without Borders Program (CNPq-Brazil). Work in Canada was supported by NSERC (Canada) Discovery grants (RGPIN-2017-03843, 2023–03714 to CMW and RGPIN-2019-04400 to AC).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2023.122988.

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