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Investigating copper toxicity in the tropical fish cardinal tetra (*Paracheirodon axelrodi*) in natural Amazonian waters: Measurements, modeling, and reality

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ABSTRACT

Copper at high concentrations is an ionoregulatory toxicant in fish and its toxicity is known to be strongly modulated by the water chemistry. The toxicity of Cu to the tropical fish cardinal tetra (*Paracheirodon axelrodi*) was investigated in waters from two major rivers of the Amazon watershed: the Rio Negro (filtered <0.45 μm, pH 5.6, DOC = 8.4 mg L^{-1}, Na = 33 μM, Ca = 8 μM) and the Rio Solimões (filtered <0.45 μm, pH 6.7, DOC = 2.8 mg L^{-1}, Na = 185 μM, Ca = 340 μM), as well as in a natural "reference water" (groundwater) which was almost DOC-free (pH 6.0, DOC = 0.34 mg L^{-1}, Na = 53 μM, Ca = 5 μM). Acute 96-h mortality, Cu bioaccumulation and net flux rates of Na^+, Cl^-, K^+ and total ammonia were determined in *P. axelrodi* exposed in each water. Copper speciation in each water was determined by two thermodynamic models and by potentiometry, and its toxicity was predicted based on the biotic ligand model (BLM) framework. Our results indicate that high Na^+ loss is the main mode of toxic action of Cu in *P. axelrodi*, in accordance with general theory. Cardinal tetra showed a particularly high ability to tolerate Cu and to maintain Na^+ balance, similar to the ability of this and other endemic Rio Negro species to tolerate low pH and ion-poor conditions. Cu toxicity was lower in Rio Negro than in the other two waters tested, and the free [Cu^{2+}] at the LC50, as determined by any of the three speciation methods tested, was approximately 10-fold higher. This variation could not be captured by a realistic set of BLM parameters. At least in part, this observation may be due to gill physiological alterations induced by the abundant dissolved organic matter of the Rio Negro. The implication of this observation is that, for metals risk assessment in tropical waters, similar to the Rio Negro, care must be used in applying BLM models developed using temperate DOC and temperate species.

1. Introduction

Despite being an essential nutrient, Cu at high levels can disturb fish gill ionoregulatory functions, leading to detrimental disturbance of sodium (Na^+), chloride (Cl^-) and sometimes also ammonia homeostasis (Laurén and McDonald, 1985; Grosell et al., 2002; Grosell, 2012). As for many other hydrophilic trace metals, Cu bioaccumulation and toxicity (i.e. bioavailability) can be mathematically described and hence predicted by the biotic ligand model (BLM) in most fresh waters. The BLM stipulates that metal bioavailability is proportional to the amount of metal bound to sensitive sites at the biological surface (e.g. ion transporters at the fish gill) referred to as "biotic ligands". This amount is in turn proportional to (i) the concentration of free metal in solution (e.g. Cu^{2+}), which is modulated by the aqueous concentration of metal-complexing ligands (e.g. HCO_3^-, dissolved organic matter (DOM)) and to (ii) the aqueous concentration of cations competing with the free metal for binding to the biotic ligands (e.g. H^+, Ca^{2+}, Na^+: protective cations). Thus, for a given organism, metal toxicity is modulated by the water chemistry. While this model remains a simplistic view of metal ecotoxicity (e.g. Na^+ protection may be not only of competitive but also of physiological nature, as discussed in Section 4.1), the overall success of the BLM in decreasing uncertainties in predicting tox-

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icity at different water chemistries represents a major progress. Computerized versions of the BLM (e.g. Windward BLM, formerly HDR-Hydroqual BLM) have been built to evaluate site-specific risks in temperate freshwaters, where the BLM was originally developed (Di Toro et al., 2001; Santore et al., 2001; Paquin et al., 2002a; Niyogi and Wood, 2004; Campbell et al., 2006). However, its applicability to the usually softer and less buffered tropical freshwaters remains much less studied (Markich and Camilleri, 1997; Franklin et al., 2000; Franco-de-Sá and Val, 2014). Yet, tropical waters, as in the Amazon basin, are not only known for their extremely rich biodiversity, they can also display remarkably diverse physico-chemical characteristics (pH, ionic strength, DOM concentrations, etc.), in relation to the contrasting properties of the soil and vegetation cover of their respective catchment areas (Konhauser et al., 1994; Val and Almeida-Val, 1995). The main objective of this study was to evaluate the applicability of the BLM to predict Cu toxicity to a tropical freshwater fish, in two waters of the Amazon basin that differ greatly in their geochemistry: the Rio Negro and the Rio Solimões, as well as in a low DOM "reference laboratory water".

The "black waters" of the Rio Negro and the "white waters" of the Rio Solimões represent two of the major types of Amazon waters (Val and Almeida-Val, 1995). Rio Negro (noted "RN" in this manuscript) is one of the two major source rivers of the Amazon. It has an acidic pH (typically from 4 to 6, but can be $\leq$3.5 in some areas; Walker and Henderson, 1996) and is extremely ion-poor (major cations $\leq$40 μmol L^{-1}), which led it to be considered as "slightly contaminated distilled water" by Sioli (1968). It is however rich in optically dark DOM (8–15 mg L^{-1} of C) which gives it a dark tea colour (Duarte et al., 2016). Rio Solimões (noted "RS" in this manuscript) is the name commonly given to the Amazon River above its confluence with RN at the city of Manaus (capital of Amazonas State, Brazil). It is rich in suspended silts carried from the Andes foothills, giving it a light milky brown colour. It has a much higher dissolved minerals content than RN, although it is still considered a soft water according to international standards (hardness <100 mg L^{-1} as $CaCO_3$). Other contrasting properties from RN are its circumneutral pH and lower DOM concentrations typically around 3 mg C L^{-1} (Leenheer, 1980; Ertel et al., 1986). Background Cu concentrations are typically low in both waters (in the order of low μg L^{-1}, this study; Konhauser et al., 1994). However, the increasing human activities in the Amazon region, including mining, industrial and urban development, could pose a significant threat to the freshwater ecosystem. Notably, many urban effluents from the city of Manaus have been shown to present Cu concentrations that often exceed Brazilian water quality criteria of <0.14 μM or 9 μg L^{-1} Cu (Silva et al., 1999; Santana and Barroncas, 2007). For example, Sampaio (2000) measured up to 16 μM (1000 μg L^{-1}) of Cu in effluents from the industrial sector of Manaus. The third water tested in this study, as a "reference laboratory water", was the groundwater available at the National Institute of Amazon Research (INPA, Manaus, Brazil), noted "IW" in this manuscript. This water presents a very poor ionic content very similar to that in RN, but a slightly higher pH and a very low DOM concentration.

The tropical fish selected for this study was the cardinal tetra *Paracheirodon axelrodi*. The cardinal tetra is a small teleost fish endemic to the Rio Negro basin. It is both ecologically and economically important, as it is the most abundant fish species (21%) in the mid Rio Negro basin and the most requested Amazonian ornamental fish in the world market, representing 80% of all fish exported annually from the Amazonas State (Chao et al., 2001). Its Na^+ uptake mechanisms have been studied extensively (Gonzalez et al., 1998; Gonzalez and Wilson, 2001; Matsuo and Val, 2007; Wood et al., 2014).

In this study, Cu bioaccumulation and acute toxicity (96-h mortality) were determined with cardinal tetras exposed in both filtered (<0.45 μm) RN and RS and in unfiltered IW. The BLM was used to predict Cu toxicity in each water, using measured water chemistry and free Cu^{2+} concentrations obtained from three methods: two thermodynamic software models (the Windward BLM in speciation mode and the Windermere Humic Aqueous Model = WHAM) and a direct analytical method (Cu^{2+} Ion Selective Electrode, ISE). Additionally, we investigated the mechanisms of acute Cu toxicity in cardinal tetra by assessing the disturbance of Na^+, Cl^-, K^+ and "total ammonia" (i.e. NH_3 and NH_4^+, noted ammonia in this manuscript) balance in the fish exposed in each water. These latter tests measured net flux rates during three exposure periods: 0–6 h, 6–12 h and 12–24 h.

2. Materials and methods

2.1. Experimental animals

Experimental work was approved by the Ethics Committee on Animal Experiments of INPA under registration number 047/2012, and conformed to national animal care regulations. Cardinal tetras (*Paracheirodon axelrodi*, 70 ± 6 mg wet weight), which had been collected from black waters in the wild, were purchased from a commercial dealer in Manaus and transported to the Laboratory of Ecophysiology and Molecular Evolution at INPA (LEEM-INPA). They were kept in aerated "reference water" (IW, INPA groundwater) at 28 ± 1 °C for at least one week before testing (composition given in Table 1) and were fed daily with commercial dry food pellets. Food was withheld for 24 h prior to experiments, and all experiments were conducted at 28 ± 1 °C.

2.2. Exposure solutions

Waters from RN and RS were collected upstream of Manaus in mid-November 2015, respectively in the areas of Praia de Paricatuba (S 3°5′41.5", W 60°21′19.6") and of the Caldeirão Embrapa research station (S 3°15′20.1", W 60°14′52.0"), while IW was collected directly from the tap at LEEM-INPA. Collected RN and RS waters were filtered through a 0.45 μm polyethersulfone (PES) membrane (Membrane Solution, Dallas, TX, USA), in order to remove the effect of particles in this study. Collected IW was used unfiltered due to its lack of suspended solids. Waters were kept at 4 °C in the dark until used in experiments.

A Cu stock solution was prepared from $CuSO_4{\cdot}5H_2O$ (99% pure, Sigma Aldrich) and spiked into the exposure waters 24 h before each test, to allow equilibration.

2.3. Acute toxicity tests

Fish were acclimated for 24 h in the appropriate Cu-free exposure solutions prior to each test. They were then exposed for 96 h to control conditions (no added Cu) and to nominal Cu concentrations ranging from 0.049 to 32 μM in RN (8 treatments), from 0.016 to 24 μM in RS (6 treatments) and from 0.39 to 2.4 μM in IW (4 treatments). For each treatment, 10 fish were exposed together in 1.2-L plastic containers filled with 1 L of aerated exposure solution. Fish were not fed over the course of the test, including during the acclimation period. Every day, mortality was recorded, dead fish were removed, solutions were renewed (90% of their volume), pH was measured and water samples were collected and filtered (<0.45 μm, cellulose nitrate membrane, Corning, NY, USA) for subsequent water chemistry analyses (e.g. Cu and major cations concentrations, see Section 2.5). These water samples consisted of both 24 h-old experimental and new renewal solutions. Since very little variation in composition was observed between old and new waters, only half of the samples were analyzed for water chemistry: two new and two old water samples per 96 h toxicity test (i.e. n = 4, averaged together in Table 1). At the end of the test, surviving

Table 1
Physical chemistry of the experimental filtered RS, filtered RN and IW. Mean ± 1 SD (n = 4 except for [Al] and [Fe] where n = 1).

	Filtered RS	Filtered RN	IW
Temperature (°C)	28 ± 0.5	28 ± 0.5	28 ± 0.5
pH	6.7 ± 0.1	5.6 ± 0.1	6.0 ± 0.1
DOC (mg C L^{-1})	2.79 ± 0.10	8.44 ± 0.10	0.34 ± 0.02
Alkalinity (mEquiv L^{-1})	0.66 ± 0.02	0.12 ± 0.01	0.14 ± 0.04
Na (μM)	185 ± 12	33 ± 6	53 ± 2
K (μM)	32 ± 4	11 ± 3	15 ± 1
Ca (μM)	340 ± 20	8 ± 2	5 ± 2
Mg (μM)	51 ± 5	4.6 ± 0.3	4.1 ± 2.4
Cl (μM)	109 ± 8	29 ± 5	25 ± 4
Al (μM)	1.3	6.2	0.57
Fe (μM)	4.6	3.0	0.059
Cu (μM and μg L^{-1})	0.016 ± 0.002 μM (1.0 ± 0.1 μg L^{-1})	0.049 ± 0.011 μM (3.1 ± 0.7 μg L^{-1})	0.11 ± 0.03 μM (7.2 ± 1.6 μg L^{-1})

fish were rinsed for 5 min in 1 mM of ethylenediamine tetraacetic acid (EDTA, ACS grade) in the appropriate water to remove surface-bound Cu, then euthanized with 250 mg L^{-1} of MS-222. They were dissected, by separating the head (including the gills) from the rest of the body and both sections were weighed. Due to the very small size of these fish, it was not practical to dissect the gills, and the head was used as a surrogate (see Results). The heads were digested in 2 N HNO_3 (Trace Metal grade) for one week at 30 °C to determine 96-h head Cu contents. In RN and IW tests, the 96-h LC50 values (concentration of dissolved Cu generating 50% fish mortality) and their associated 95% confidence levels were obtained based on mortality observed at each measured dissolved Cu concentration, with the Toxicity Relationship Analysis Program (TRAP) version 1.22 (USEPA, 2002) using the Tolerance Distribution analysis type and the Gaussian Distribution model. In the RS test where only one partial kill was observed, the Spearman-Kärber method was used to estimate the 96-h LC50 value and its 95% confidence levels (Spearman, 1908; Kärber, 1931).

2.4. Flux rate measurements

In addition to controls (no added Cu), tested Cu concentrations (nominal values) ranged from 0.4 to 16 μM in RN (4 treatments), from 0.4 to 8 μM in RS (3 treatments) and from 0.4 to 0.8 μM in IW (2 treatments). The upper concentrations were selected to correspond approximately to the 96-h LC50 determined in the acute toxicity tests. For each treatment, 6 fish were exposed in individual opaque 22-mL plastic vials. Prior to the test, fish were acclimated for 12 h to the vials filled with 20 mL of aerated Cu-free exposure solution. At t = 0 h, this solution was replaced by the exposure medium. For Cu, Na^+, Cl^-, K^+ and ammonia analyses, 5-mL water aliquots were collected at the beginning and at the end of each flux period (0–6 h, 6–12 h, 12–24 h), with the exposure media being completely renewed for the 12–24 h flux period. Fish were not fed over the course of the test, including during the acclimation period. At the end of the test, fish were rinsed with 1 mM EDTA, euthanized, dissected, weighed and acid-digested (to obtain the 24-h head Cu content) following the same procedure as for the toxicity tests. Net flux rates (in nmol g^{-1} h^{-1}) were calculated with the following equation (Wood, 1992) for each of the 3 flux periods:

$$J_{x,\,net} = \frac{([x]_i - [x]_f) \cdot V}{m \cdot \Delta t} \quad (1)$$

with $[x]_i$ and $[x]_f$ representing the aqueous concentrations of compound x respectively at the beginning and at the end of the flux period (in nmol L^{-1}), V the volume of exposure medium (in L), m the wet weight of the fish (in g) and Δt the duration of the flux period (in h).

Note that some fish died in some experiments at high [Cu]. The flux rates of these fish were only calculated for the flux period in which they were still alive.

2.5. Analytical techniques

Copper concentrations in water and in digested fish tissues were measured by atomic absorption spectrometry (AAS) using a graphite furnace technique (Perkin-Elmer AAnalyst 800 AA spectrophotometer, Norwalk CT, USA). A reference material (dogfish liver DOLT-2, National Research Council Canada) and method spikes were analyzed, and provided Cu recoveries between 90 and 110%. Concentrations of total aqueous Ca and Mg were measured by AAS (Varian AA240FS, Santa Clara, CA, USA), while K and Na were measured using a flame photometer (a 910 Digital Flame Photometer, Instrumentação Analítica São Paulo, SP, Brazil). Dissolved Al and Fe concentrations were obtained by inductively coupled plasma mass spectrometry (Agilent 7700×, Santa Clara, CA, USA). Aqueous ammonia and Cl^- concentrations were measured with the colorimetric assays developed by Verdouw et al. (1978) and Zall et al. (1956) respectively. Dissolved organic carbon (DOC) concentrations were measured with a TOC analyzer (Shimadzu TOC-VCSH, Canby, OR, USA). Titratable alkalinity was determined by titration of 10-mL water samples, continuously aerated, with standardized 0.02 N HCl to pH 4.00, using microburettes (Gilmont, Great Neck, NY, USA), as described by McDonald and Wood (1981).

2.6. BLM prediction of LC50 values

The BLM is an equilibrium model composed of two main components: (i) a metal speciation component and (ii) a component describing reactions at the biotic ligands. For component (i), we tested three methods to determine Cu speciation: two thermodynamic methods and an analytical method, which are described in Section 2.6.1. Section 2.6.2 provides the mathematical expressions for reactions at the biotic ligand and the methodology used to calculate the LC50 values in each tested water.

2.6.1. Copper speciation calculation

Firstly, [Cu^{2+}] concentrations ("free Cu^{2+}") were modelled with the speciation mode of the Windward BLM software (W-BLM) Research version 3.1.2.37 (http://www.windwardenv.com/biotic-ligand-model/), using measured physico-chemistry as model inputs. Note that this speciation component of W-BLM uses a set of WHAM version 5 thermodynamic constants. For metal binding 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).

Secondly, $[Cu^{2+}]$ concentrations were modelled using the speciation software WHAM version 7 (Tipping et al., 2011), with the same DOM assumptions as above. Contrary to W-BLM software, WHAM7 allows Al and Fe concentrations as inputs. To account for possible transport of small Al and Fe(III) colloids through the 0.45 μm membrane used for water filtration, $Al(OH)_3$ and $Fe(OH)_3$ were allowed to precipitate in WHAM7 (note that this decision did not affect Cu speciation in the end). After critical review of the editable WHAM7 thermodynamic database, we replaced the default log K_{CuHCO3} of 14.62 (Mattigod and Esposito, 1979) by 12.13, as recommended by the IUPAC (Powell et al., 2007). The precision of each $[Cu^{2+}]$ estimate (given as 95% confidence intervals) was computed based on uncertainties in physico-chemistry measurements, using a Monte-Carlo routine included in the WHAM7 model.

Lastly, $[Cu^{2+}]$ concentrations were also measured in filtered RN and RS using a Cu^{2+} Ion Selective Electrode (ISE, Orion Model 94-29, Boston, MA, USA). No measurement was performed in IW, because such low Cu buffering makes free ion measurements impossible at the levels of Cu tested here. Because cupric ISE analyses require sufficient conductivity in solution to work properly, the ionic strength of all samples was adjusted to 0.01 M by addition of K_2SO_4 (99%, Sigma Aldrich). The electrode was calibrated (using 3 standards in the range 10^{-6}–$10^{-3.5}$ M) directly into concentration units at the same ionic strength. Electrode response was consistently within 5% of a Nernstian slope. Each sample was titrated 3 to 4 times and all data pooled for analysis. The waters titrated using ISE were collected at the same time and site as the waters used for the fish toxicity and flux tests. For each water, a linear regression between the log of added [Cu] and the log of measured $[Cu^{2+}]$ was obtained with the SigmaPlot® software, along with the 95% prediction bands. This equation and prediction bands were then used to estimate $[Cu^{2+}]$ and the 95% confidence on this prediction at untested [Cu] concentrations (e.g. at the observed LC50).

2.6.2. Copper LC50 calculation

The BLM is an equilibrium-based model which assumes that metal toxicity is directly proportional to the fraction of biological targets (called biotic ligands, noted "BL") bound by the metal. For Cu, this fraction f_{CuBL} is expressed by the following equation:

$$f_{CuBL} = \frac{\{\Sigma CuBL\}}{\{BL\}} = \frac{K_{CuBL}\cdot\left[Cu^{2+}\right] + K_{CuOHBL}\cdot K_{CuOH}\cdot\left[Cu^{2+}\right]\cdot\left[OH^{-}\right]}{1 + K_{CuBL}\cdot\left[Cu^{2+}\right] + K_{CuOHBL}\cdot K_{CuOH}\cdot\left[Cu^{2+}\right]\cdot\left[OH^{-}\right] + K_{CaBL}\cdot\left[Ca^{2+}\right] + K_{MgBL}\cdot\left[Mg^{2+}\right] + K_{NaBL}\cdot[Na^{+}] + K_{HBL}\cdot\left[H^{+}\right]} \quad (2)$$

Where {} are densities on the gills (in nmol g^{-1} wet wt), [] are concentrations in the water (in mol L^{-1}), and K_{CBL} is the formation constant between BL and cation C (C: Cu^{2+}, $CuOH^+$, Mg^{2+}, Ca^{2+}, Na^+ or H^+). Note that {ΣCuBL} is the sum of Cu^{2+} and $CuOH^+$ bound to BL, as the toxicity of $CuOH^+$ is generally thought to contribute to the overall toxicity in acute Cu BLMs (De Schamphelaere et al., 2002; De Schamphelaere and Janssen, 2002). Also, note that it is the ion activities and not ion concentrations that are considered in the BLM. However, in the soft waters tested in this study, the difference between these two quantities was negligible. From Eq. (2), the free Cu^{2+} concentration at 50% mortality can be derived:

$$\left[Cu^{2+}\right]50 = \frac{f^{50}_{CuBL}}{1 - f^{50}_{CuBL}} \cdot \frac{1 + K_{CaBL}\cdot\left[Ca^{2+}\right] + K_{MgBL}\cdot\left[Mg^{2+}\right] + K_{NaBL}\cdot[Na^{+}] + K_{HBL}\cdot\left[H^{+}\right]}{K_{CuBL} + K_{CuOHBL}\cdot K_{CuOH}\cdot\left[OH^{-}\right]} \quad (3)$$

For Cu acute toxicity in fish, the following log K values were derived from experiments with fathead minnows and rainbow trout (Playle et al., 1993; Santore et al., 2001): log K_{CuBL} = 7.4, log K_{CuOHBL} = 6.2, log K_{HBL} = 5.4, log K_{CaBL} = 3.6, log K_{MgBL} = 3.6 and log K_{NaBL} = 3.0. It is assumed that log K values can be extrapolated within taxonomically similar groups (e.g. fish) and that only the intrinsic sensitivity (modulated by f^{50}_{CuBL}) varies among species (Di Toro et al., 2001; Santore et al., 2001; De Schamphelaere et al., 2002; De Schamphelaere and Janssen, 2002; Deleebeeck et al., 2007; Van Sprang et al., 2009; Schlekat et al., 2010). Thus, as an initial approach, we calculated the Cu LC50 values to cardinal tetra exposed in each water with these log K values, by adjusting only f^{50}_{CuBL}. The following steps were performed for each speciation method (W-BLM, WHAM7 and ISE):

(i) For the LC50 measured in each water ($LC50_{obs}$), the corresponding $[Cu^{2+}]50_{obs}$ was estimated with the speciation method.
(ii) The value of f^{50}_{CuBL} was adjusted by iteration to obtain a geometric mean of 1 for the ratio of $[Cu^{2+}]50_{calc}$ (obtained with Eq. (3)) and $[Cu^{2+}]50_{obs}$ among the three waters, using the Solver analysis tool in Excel®.
(iii) From the resulting $[Cu^{2+}]50_{calc}$ in each water, the corresponding $LC50_{calc}$ was estimated with the speciation method (inverse of step 1). Note that in the case of the W-BLM, this step consists in running the software in toxicity mode with the adjusted f^{50}_{CuBL}.

Based on the results of these exercises, in a subsequent approach, we tested adjusting the log K values to improve the modeling. By convention, the $LC50_{calc}$ values are considered acceptable when they are within a factor of 2 from $LC50_{obs}$ values. Additionally, the obtained f^{50}_{CuBL} should not be close to 1 (i.e. the BL should not be saturated with Cu at 50% mortality) as the model then loses sensitivity. This latter situation occurs when $K_{CuBL}\cdot[Cu^{2+}] + K_{CuOHBL}\cdot K_{CuOH}\cdot[Cu^{2+}]\cdot[OH^{-}]$ » $1 + K_{CaBL}\cdot[Ca^{2+}] + K_{MgBL}\cdot[Mg^{2+}] + K_{NaBL}\cdot[Na^{+}] + K_{HBL}\cdot[H^{+}]$ in Eq. (3). None of these two conditions were met with the above toxicity modeling using the existing log K values of the acute Cu W-BLM for fish. Thus, we tested adjusting the log K values to obtain (i) a decrease in f^{50}_{CuBL} absolute values ($\leq$0.5 as an arbitrary condition) and (ii) the minimum relative variation among f^{50}_{CuBL} values in each water, using the Solver analysis tool in Excel®. Indeed, according to the BLM theoretical framework, this value should be the same in these three different waters (Di Toro et al., 2001). Adjustment of log K_{HBL} generated the highest changes in the model performance, because of its higher default value coupled with the high pH variations observed between the three waters. Note that on the other hand, adjustment of log K_{CuBL} has basically no impact on the model performance, but this value can be varied to bring its coupled parameter f^{50}_{CuBL} to a credible value $\leq$0.5.

2.7. Statistical analyses

Most data are reported as means ± SEM or as means with their 95% confidence intervals. Simple comparisons were performed using a Student's *t*-test and multiple comparisons using a *t*-test with a False Discovery Rate (FDR) correction. The effect of increasing dissolved Cu concentration on each measured net flux rate was assessed by a single factor ANOVA followed by a post-hoc Tukey's HSD test. Significant difference was accepted at the $p < 0.05$ level.

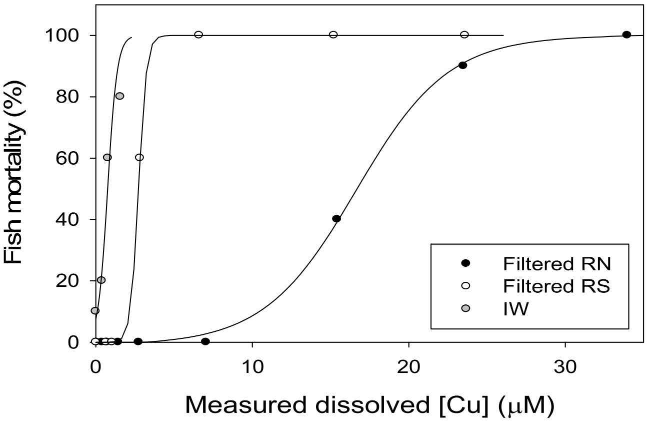

Fig. 1. Percentage mortality of *P. axelrodi* exposed for 96 h in filtered Rio Negro (black circles), filtered Rio Solimões (open circles) and INPA water (grey circles), as a function of measured dissolved Cu concentration. Regressions with Gaussian (normal) distribution in TRAP 1.22.

3. Results

3.1. Water physico-chemistry

The compositions of tested RS, RN and IW are given in Table 1. Rio Negro and IW were close in ionic composition but DOC concentration was much lower in IW. As expected, RS presented much higher ionic concentrations than IW and RN, with a higher pH and an intermediate DOC concentration. Copper background concentrations were relatively low, with however a significantly greater level in the "reference water" (IW) which was groundwater.

3.2. Cu effects: 96 h acute toxicity

The acute toxicity of Cu to cardinal tetras exposed in RN, RS and IW is shown in Fig. 1 and the corresponding 96-h LC50 values are presented in Table 2 ($LC50_{obs}$). The $LC50_{obs}$ measured in IW ($56 \pm 18\ \mu g\,L^{-1}$) in this study was very similar to the one measured by Duarte et al. (2009) for the same fish species in the same water ($46 \pm 7\ \mu g\,L^{-1}$) – i.e. ~0.8 μM. All LC50 values were significantly different from each other, with Cu toxicity to *P. axelrodi* decreasing in the order IW > RS > RN. Copper in IW was ~3 times and ~19 times more toxic than in RS and in RN respectively, while Cu in RS was ~6 times more toxic than in RN. The great majority of mortality occurred within the first 24 h of exposure.

3.3. Cu effects: sodium, chloride, potassium and ammonia net flux rates

In this section, we first present the net flux rates of Na^+, Cl^-, K^+ and ammonia from *P. axelrodi* exposed in the "reference water" (IW) over the three flux periods (Fig. 2). Based on these results, we then show flux rates in RS and RN for the first flux period only in the main text (Fig. 3), with flux rates in the subsequent periods shown in Supporting information (Figs. S1 and S2). Contrary to toxicity data, net flux rates are presented as a function of nominal rather than measured dissolved [Cu], for convenience in graph plotting. The bias from this approximation was however not important, as percentages of Cu recovery (measured vs. nominal) were 95 ± 11% in IW, 96 ± 9% in RN and 89 ± 10% in RS. Negative flux rates indicate internal loss from the fish to the external medium, while positive flux rates indicate internal gain by the fish via net uptake from the water.

Fig. 2 presents the 0–6 h, 6–12 h and 12–24 h net flux rates of Na^+, Cl^-, K^+ and ammonia from cardinal tetras exposed to 0, 0.4 and 0.8 (~$LC50_{obs}$ value) μM of Cu in IW. In the absence of Cu, approximate net balance was observed for Na^+ and Cl^- over the entire 24 h exposure. Potassium was slightly lost, probably because this element is acquired mostly from the food (Wood and Bucking, 2011), which was withheld in the experiments. In agreement with previous findings on this species (Wood et al., 2014) ammonia was excreted at a high rate (see right-hand axis) from the fish over the entire course of the test, this compound being the major metabolic waste product in teleost fish (Wilkie, 2002). As Cu concentration increased, Na^+, K^+ and Cl^- loss rates tended to increase, while total ammonia excretion rate remained unaffected. This preliminary test in IW indicated that Cu effects on *P. axelrodi* ion regulation are already visible in the first flux period. In fact, Cu effects were generally more marked between 0 and 6 h, as *P. axelrodi* exhibited less negative flux rates in the subsequent periods. This probably reflected a decline in internal concentrations, and therefore in the gradients for loss, rather than recovery, as fish started to die in these periods. In consequence, we only present 0–6 h net flux rates for the RN and RS comparison (Fig. 3), with the two other flux periods available in the Supporting information (Figs. S1 and S2).

Fig. 3 presents the 0–6 h net flux rates of Na^+, Cl^-, K^+ and ammonia from cardinal tetras exposed to 0, 0.4, 3.2 (~$LC50_{obs}$ in RS), 0.8 and 16 (~$LC50_{obs}$ in RN) μM of Cu. No mortality was observed within this time period. The same Cu effects as in IW were observed in these two waters, that is a stimulation of Na^+ and Cl^- loss at a similar magnitude, a less substantial increase in K^+ loss and no impact on ammonia excretion. The levels of Na^+ loss were higher in RS than in RN for the same [Cu] (Fig. 2), which is consistent with the acute toxicity data (cf. Fig. 1 and Table 2). The comparison with IW was limited by the much lower $LC50_{obs}$ value in this water: the only shared exposure [Cu] for these three waters was 0.4 μM, where no significant difference in Na^+ loss rate was observed. However, the very high Na^+ loss rate at 0.8 μM Cu in IW (Fig. 2) indicates that, as for mortality, ionoregulatory disturbance effects are stronger in this very low DOC water than in filtered RN and RS.

3.4. Copper bioaccumulation in P. axelrodi

The 24-h (Fig. 4a) and 96-h (Fig. 4b) Cu head contents, measured in the surviving fish from the flux and toxicity tests respectively, were compared in RN and RS (no data for IW). In BLM applications, 24-h Cu accumulations are normally preferred over the 96-h Cu accumulations. However, the 24-h Cu head concentrations were very variable and no apparent trend was observed between Cu content in the gill and dissolved Cu concentration in the water ($p > 0.05$, ANOVA). Hence, we also provide the less variable 96-h data, which show that bioaccumulation of Cu was similar in the heads of cardinal tetra exposed in both waters for the same [Cu] exposure. In the BLM, gill metal content is normally the concentration of interest, especially the 24-h gill accumulation at the LC50, classically referred to as the LA50 for accumulation predictive of 50% lethality at 96 h. However, due to their very small size, tetras' gills could not be easily dissected and therefore we decided to estimate gill Cu contents from head contents. Tetra gills represent 17 ± 1% of the head weight (mean ± SEM, n = 6). Assuming that Cu in the head is mostly bound to the gills because of their high surface area (Evans et al., 2005), gill Cu content was estimated, by dividing the head accumulation (after background subtraction) by 0.17. A linear relationship was observed between gill Cu content and dissolved Cu concentration at 96 h ($R^2 = 0.87$ in RN and $R^2 = 0.86$ in RS, SigmaPlot®), which allowed estimating the 96-h LA50 and its 95% prediction intervals at $x = LC50_{obs}$. These 96-h LA50 values are given in Table 2 and were significantly 7-fold higher in RN than in RS ($p < 0.05$, *t*-test). These different 96-h LA50 values constitute a clear deviation from the premises of the BLM, as discussed in Section 4.3.

Table 2
Observed dissolved Cu concentration ($LC50_{obs}$), corresponding estimates of Cu^{2+} concentrations ($[Cu^{2+}]50_{obs}$), with each speciation method) and of Gill-Cu accumulation at 50% fish mortality (LA50) in filtered RS, filtered RN and IW. Estimates with 95% confidence intervals (unavailable for W-BLM $[Cu^{2+}]50$ modeling). Estimates not sharing the same lower-case or upper-case letter are significantly different among waters, as evaluated by a *t*-test (with correction for false discovery rate for multiple comparisons).

	$LC50_{obs}$ (μM and $\mu g\,L^{-1}$)	$[Cu^{2+}]50_{obs}$ (μM)	LA50 ($nmol\,g^{-1}$ wet wt)
Filtered RS	3.05 [2.21–3.90] μM[a] 194 [140–248] $\mu g\,L^{-1}$	W-BLM: 0.11 WHAM7: 0.42 [0.076 – 1.5][a] ISE: 0.80 [0.20 – 3.3] [A]	24 h: p>0.05, ANOVA 96 h: 20 [1–39][a]
Filtered RN	17.2 [14.0–20.3] μM [b] 1090 [887–1290] $\mu g\,L^{-1}$	W-BLM: 1.3 WHAM7: 4.2 [1.2–13][b] ISE: 8.1 [2.9 – 17] [B]	24 h: $p > 0.05$, ANOVA 96 h: 130 [70–200][b]
IW	0.886 [0.314–1.46] μM [c] 56.3 [19.9 – 92.6] $\mu g\,L^{-1}$	W-BLM: 0.22 WHAM7: 0.78 [0.27–2.2][a] ISE: not measured	Not measured

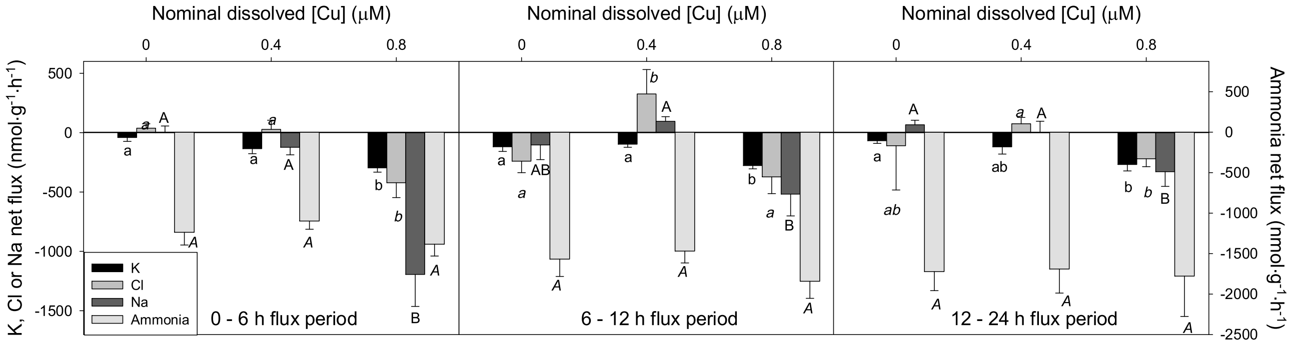

Fig. 2. The 0–6 h, 6–12 h and 12–24 h net flux rates of K^+, Cl^-, Na^+ (left axis) and ammonia (right axis) in *P. axelrodi* exposed to Cu in INPA groundwater. For the net flux rates of a specific moiety in the same time period, means not sharing the same lower-case or upper-case letter are significantly different across different Cu concentrations (single factor ANOVA with post-hoc Tukey's HSD test). Mean ± 1 SEM (n = 6 for 0–6 h and 6–12 h and n = 4 for 12–24 h due to fish mortality).

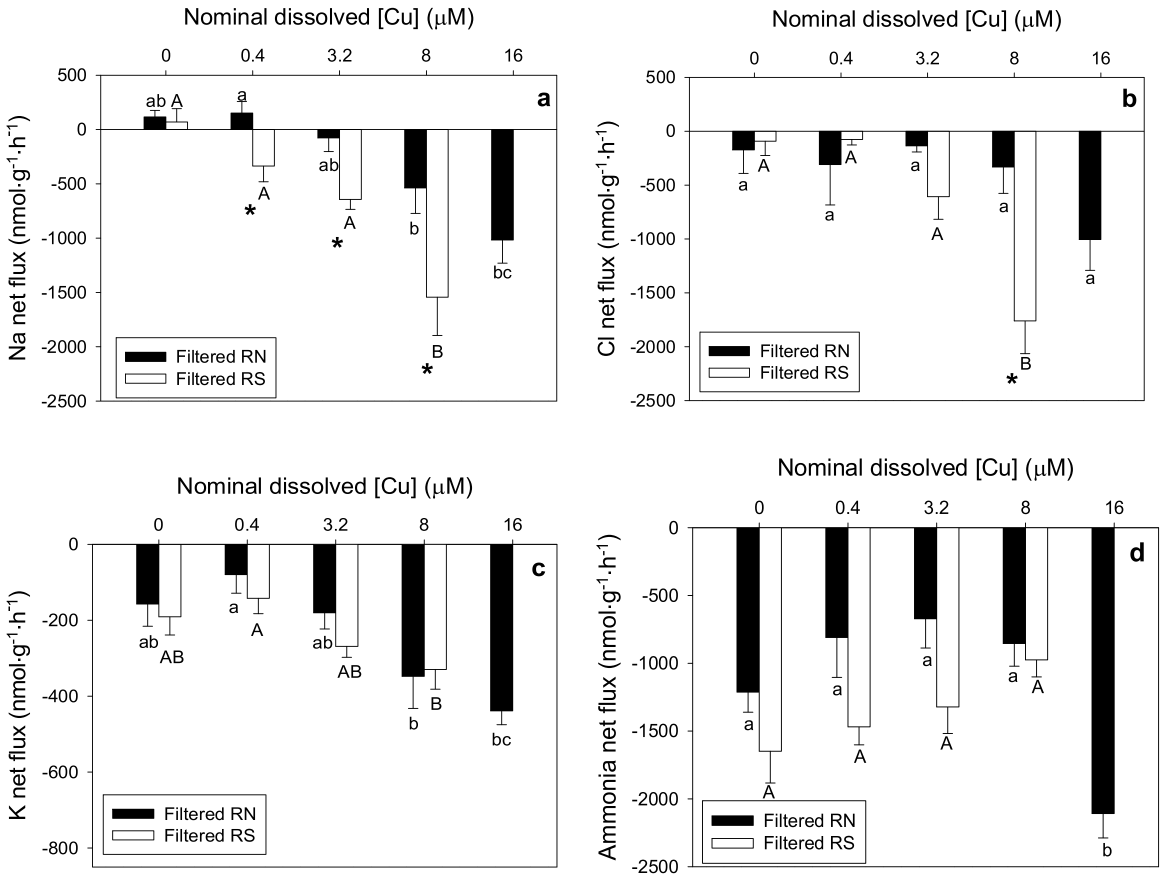

Fig. 3. The 0–6 h net flux rates of (a) Na^+, (b) Cl^-, (c) K^+ and (d) ammonia in *P. axelrodi* exposed to Cu in filtered Rio Negro (black bars) and filtered Rio Solimões (white bars). Different lower-case or upper-case letters and asterisks indicate significant differences across different Cu concentrations (single factor ANOVA with post-hoc Tukey's HSD test) and water sources (*t*-test) respectively. Mean ± 1 SEM (n = 6).

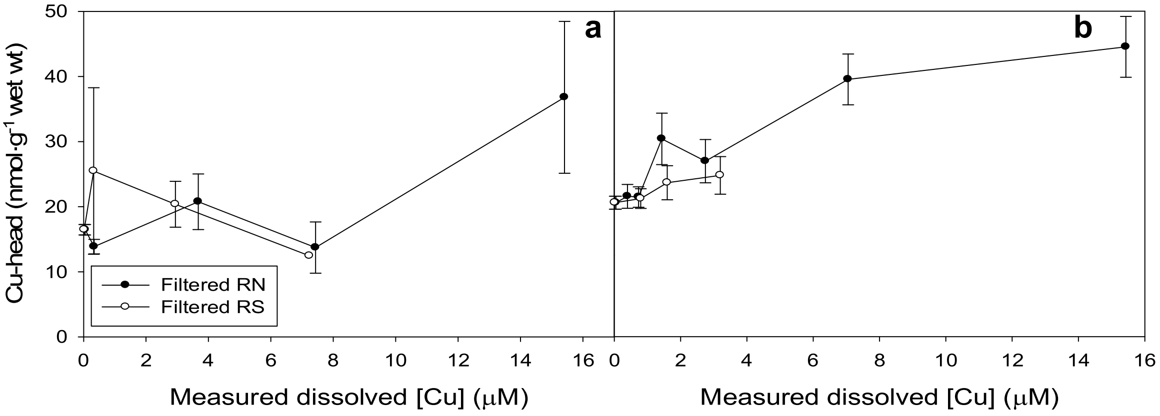

Fig. 4. Copper bioaccumulation at a) 24 h and b) 96 h in heads of *P. axelrodi* exposed in filtered Rio Negro (black circles) and in filtered Rio Solimões (open circles), as a function of the dissolved Cu concentration. Mean ± 1 SEM (n = 4–12).

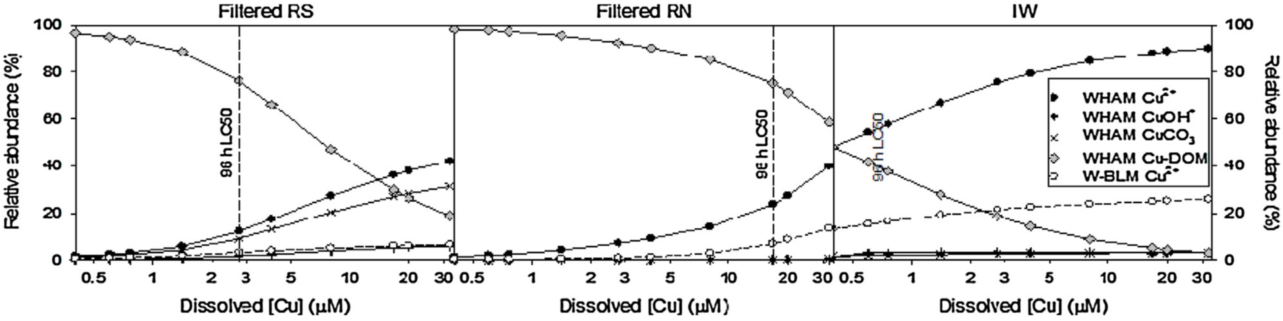

Fig. 5. Mean relative abundance of important chemical species of Cu [Cu^{2+}, $CuOH^+$, $CuCO_3$, Cu-DOM] (i.e. with abundance >5%) as a function of dissolved Cu concentration in filtered RS, filtered RN and IW. Speciation was computed with WHAM7 and W-BLM (%Cu^{2+} only). The x-axis is in log scale.

3.5. Aqueous copper speciation

The WHAM7-calculated Cu speciation is given for each water in Fig. 5, for the ranges of dissolved [Cu] used in each acute toxicity test. The dominant Cu species was Cu^{2+} in IW and Cu-DOM in both filtered RN and RS. In each water, increasing dissolved [Cu] resulted in an increase in % Cu^{2+} and % $CuCO_3$ as % Cu-DOM decreased, indicating a model-predicted gradual saturation of Cu binding sites on DOM. The % Cu^{2+} was the highest in IW, while it was not significantly different in RS and RN, despite the higher DOM concentration in RN. This similar % Cu^{2+} was the result of the higher displacement of Cu-DOM by Al and Fe and the lower Cu-CO_3 binding in RN than in RS, as predicted by the thermodynamic calculations.

The % Cu^{2+} obtained with W-BLM are also given in Fig. 5 (note: most metal complexes concentrations are not available in W-BLM). These proportions were lower than WHAM7 proportions (on average by a factor 5 in RN and by a factor 4 in RS and IW). These discrepancies are mostly explained by the log K_{CuHCO3} difference, which was 14.62 in W-BLM, but updated to 12.13 in the editable WHAM7 thermodynamic database. However for RN, the absence of the relatively high [Al] (and [Fe]) as an input variable in W-BLM also explains a significant portion of this discrepancy (about half of it at the LC50). Thus, WHAM7 modeling with the updated log K_{CuHCO3} should provide better Cu^{2+} predictions than W-BLM. This was confirmed by the closer [Cu^{2+}] between WHAM7 calculations and direct measurements by ISE (Fig. 6), as described below.

The Cu-ISE measurements are given in Fig. 6. They show the same increase in % Cu^{2+} with the increase of [Cu] in RN and RS as the WHAM7 and W-BLM predictions. In both waters, the relationship log [Cu^{2+}] = f(log [Cu]) was linear (slope = 1.19 and intercept = 0.47, $R^2 = 0.92$, n = 10 for RS; slope = 1.35 and intercept = 1.34, n = 33, $R^2 = 0.95$ for RN). In the [Cu] range of the toxicity tests, the $[Cu^{2+}]_{ISE}$ predictions were around 2-fold higher than the $[Cu^{2+}]_{WHAM7}$ predictions. Considering the substantial uncertainties which often surround metal speciation measurements and modeling in natural waters, a difference of a factor 2 could be considered as quite satisfactory. Furthermore, when using either the ISE linear regressions or WHAM7 to predict [Cu^{2+}] at a given [Cu] (see e.g. [Cu^{2+}] at $LC50_{obs}$ in Table 2), no significant differences could be found between the two estimations due to relatively large prediction uncertainties. We however can hypothesize that the differences could come from uncertainties in the constants characterizing Cu-DOM complexation in WHAM7, as DOM binding properties can vary greatly with the DOM source (Luider et al., 2004). However, this discrepancy could also partially come from bias in the ISE measurement. Indeed, WHAM7 predicts that the addition of K_2SO_4 (3.33 mM to reach I = 0.01 M) in the ISE analyses leads to displacement of Cu bound to DOM by K^+ and to complexation of Cu^{2+} by the $SO_4{}^{2-}$. The former effect being greater than the latter, K_2SO_4 addition resulted in a net $[Cu^{2+}]_{ISE}$ overestimation that was however relatively small: ×1.4 in RN and only ×1.1 in RS at the LC50 values of [Cu].

In Table 2, we provide the concentrations of free ionic Cu^{2+} eliciting 50% of mortality in *P. axelrodi* in the three waters. These values were calculated with each of the three methods from the different $LC50_{obs}$ values observed in each water. Whatever the method, the resulting $[Cu^{2+}]50_{obs}$ were similar in RS and IW and an order of magnitude higher in RN.

3.6. Observed versus predicted LC50 values

In Table 3, BLM predictions are given for each water using the three speciation methods (W-BLM, WHAM7 and ISE), with the existing log K values for Cu in the acute fish W-BLM, then with

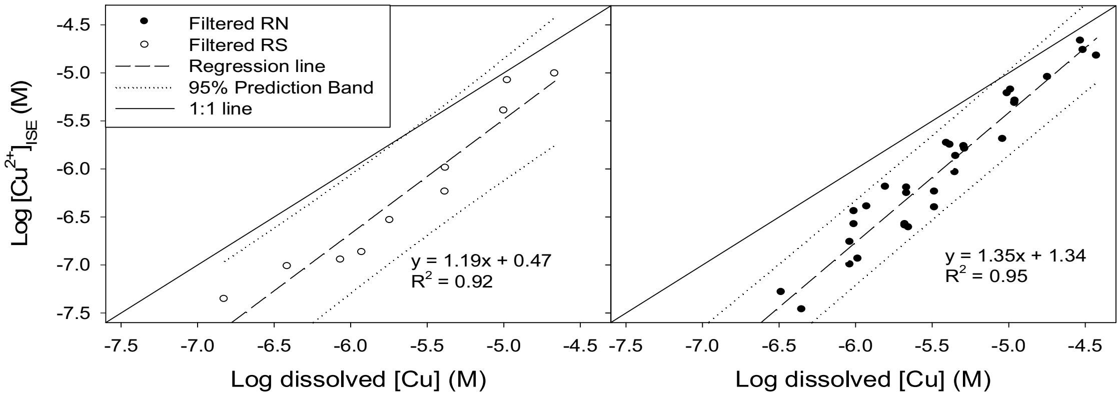

Fig. 6. Log of the free Cu^{2+} concentration measured with an Ion Selective Electrode in RS (left) (n = 10) and filtered RN (right) (n = 33) (T = 25 °C, I = 0.01 M), as a function of the log of total added dissolved Cu concentration.

Table 3
Modelled fraction of Cu bound to BL (f^{50}_{CuBL}), free Cu^{2+} concentration ($[Cu^{2+}]50_{calc}$) and dissolved Cu concentration ($LC50_{calc}$) at 50% mortality and ratio of $LC50_{calc}$:$LC50_{obs}$. These BLM calculations were performed in filtered RS and RN and in IW, based on the three speciation methods (W-BLM, WHAM7 and Cu-ISE) and with default and adjusted BLM log K values.

Speciation	Water	Default BLM log K values[a]				Adjusted BLM log K values[b]			
		f^{50}_{CuBL}	$[Cu^{2+}]50_{calc}$ (μM)	$LC50_{calc}$ (μM)	$\frac{LC50_{calc}}{LC50_{obs}}$	f^{50}_{CuBL}	$[Cu^{2+}]50_{calc}$ (μM)	$LC50_{calc}$ (μM)	$\frac{LC50_{calc}}{LC50_{obs}}$
	Filtered RS		0.472	9.90	3.2		0.124	3.83	1.3
W-BLM	Filtered RN	0.81	0.290	9.89	0.57	0.37	0.778	15.2	0.89
	IW		0.230	1.02	1.2		0.326	1.43	1.6
	Filtered RS		1.66	7.38	2.4		0.333	2.95	0.97
WHAM7	Filtered RN	0.94	1.02	7.12	0.41	0.43	3.20	14.1	0.82
	IW		0.809	0.966	1.1		1.29	1.52	1.7
	Filtered RS		3.24	9.89	3.2		0.769	2.95	0.97
Cu-ISE	Filtered RN	0.97	2.00	6.15	0.36	0.49	8.42	17.8	1.0
	IW		n.a.	n.a.	n.a.		n.a.	n.a.	n.a.

[a] Default values: log K_{CuBL} = 7.4, log K_{CuOHBL} = 6.2, log K_{HBL} = 5.4, log K_{CaBL} = 3.6, log K_{MgBL} = 3.6 and log K_{NaBL} = 3.0.
[b] Default values with adjusted log K_{HBL} = 7.1, 7.6 and 7.9 for W-BLM, WHAM7 and ISE speciation respectively.

adjusted log K values. Firstly, using the default log K values, the f^{50}_{CuBL} values providing the best fit of our data were 0.81, 0.94 and 0.97 with W-BLM, WHAM7 and ISE respectively. These values were very close to 1, indicative of a BL almost saturated with Cu at 50% mortality, an unlikely situation. Whatever the prediction method used, the $LC50_{calc}$ was under-estimated in RN (factor >2, except with W-BLM), very slightly overestimated in IW (factor <2) and over-estimated in RS (factor >2). As a result, the range of $LC50_{calc}$: $LC50_{obs}$ varied from 6 to 9 fold for the different methods, which represents an important deviation from the BLM, as the observed toxicity variation among the three waters cannot be captured by the model when using its default log K values and a single adjusted f^{50}_{CuBL}.

Since $[Cu^{2+}]50_{obs}$ was the highest in RN, the highest cation protection was expected in this water. Only H^+ was present at the highest concentration in RN (2.5× and 13× higher than in IW and RS respectively) (cf. Table 1). Thus, protection by H^+ had to be increased to a point where cation protection in RN became greater than in RS where $[Ca^{2+}]$, $[Mg^{2+}]$ and $[Na^+]$ were respectively 68×, 11× and 7.2× higher. Not surprisingly, the adjusted log K_{HBL} values providing the best toxicity prediction (and satisfying the condition f^{50}_{CuBL}<0.5) were very high: 7.1, 7.6 and 7.9 for W-BLM, WHAM7 and ISE modeling respectively. These values seem unrealistically high (see Discussion). When RN was removed from the modeling, Cu toxicity in IW and RS could be satisfactorily predicted with the default BLM parameters, where only log K_{CuBL} was decreased to 6.7 for the sole purpose of bringing f^{50}_{CuBL} <0.5.

4. Discussion

4.1. Mechanisms of copper toxicity in the cardinal tetra

Copper effects on branchial ionoregulation have been relatively well studied (see Grosell, 2012 for a review). Freshwater fish naturally lose ions by passive diffusion from the gill to the more dilute external media. To maintain ion homeostasis, fish need to actively (i.e. at the cost of energy) uptake these elements from the water. Failure of Na^+ homeostasis is believed to be the key toxicity target of Cu in fish. Proposed mechanisms for Cu-induced Na^+ loss are (i) a decrease in Na^+ branchial 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 Ca from the tight junctions (Laurén and McDonald, 1985, 1987a,b; Vitale et al., 1999; Grosell et al., 2002; Chowdhury et al., 2016). It has been proposed that mortality occurs when the fish loses approximately 30% of its exchangeable plasma Na^+ pool (Grosell et al., 2002), which is typically in the range of 40–50 μmol g^{-1} wet wt (Paquin et al., 2002b). The mortality of cardinal tetra observed in our toxicity tests is consistent with the rate of Na^+ loss measured in the flux tests, with the same trend observed between the three waters (IW > RS > RN). For example, at 8 μM of Cu in filtered RS, 100% and 83% fish died within 24 h respectively in the toxicity and the flux test (33% within 6–12 h, then 83% within 12–24 h, Supporting information Figs. S1 and S2). The mean net

0–12 h Na^+ flux rate was around $-1\ \mu mol\ g^{-1}\ h^{-1}$. Assuming a typical exchangeable Na^+ pool of $45\ \mu mol\ g^{-1}$ in *P. axelrodi*, then a 30% Na^+ plasma loss was reached after 13.5 h of exposure. Thus, we believe there is little doubt that Cu acute toxicity observed in cardinal tetras in this study is mainly due to Na^+ homeostasis disturbance.

In our study, Cu also stimulated Cl^- and K^+ loss in *P. axelrodi*. These Cu effects are commonly reported, but they are usually considered as secondary mechanisms of Cu toxicity (Laurén and McDonald, 1985; Grosell et al., 2002; Grosell, 2012). Finally, note that impairment of fish ammonia excretion has been proposed as an important mechanism of Cu toxicity in fish, with inhibition of Na/K-ATPase, CA and blockade of Rhesus glycoprotein as suggested causes (Lim et al., 2015). However, our data suggest that it is not an important mechanism for Cu acute toxicity in *P. axelrodi,* in accordance with recent findings that ammonia excretion does not depend on Na^+ uptake in this species (Wood et al., 2014).

4.2. Mechanisms of copper tolerance in the cardinal tetra

When using log K values from the existing Cu acute fish BLM, f^{50}_{CuBL} adjusted values were close to 1, illustrating the very low sensitivity of cardinal tetra to Cu compared to the temperate fish for which the BLM was calibrated (the fathead minnow and the rainbow trout). Our estimations of gill LA50s $\left(= f^{50}_{CuBL} \cdot \{BL\}\right)$ from the measured Cu-head contents (cf. Table 2) were accordingly high compared to the LA50s of a few $nmol\ g^{-1}$ typically reported for temperate fish such as the rainbow trout and the fathead minnow (Playle et al., 1993; Di Toro et al., 2001; Santore et al., 2001; Niyogi and Wood, 2004). Indeed, these $LA50_{obs}$ in RN were even above the default maximum biotic ligand capacity ($\{BL\}$) of $30\ nmol\ g^{-1}$ in the W-BLM. We should however note that our estimations of LA50 s are based on the assumption that most metal accumulated in the head of cardinal tetras is bound to the gills, due to their extremely high surface area (Evans et al., 2005). The possible resulting overestimation of LA50s may partially, but probably not fully explain their uncommonly high values.

A high tolerance of cardinal tetras to Cu is not a surprise, since this species, and more generally fish endemic to the Rio Negro, are known to exhibit a remarkably high tolerance against ionoregulatory disturbances. Fish in the Rio Negro basin thrive in a very ion-poor and acidic environment, where both ion deficiency and low pH both would exert tremendous ionoregulatory stress to most temperate fish, via inhibition of Na^+ influx and stimulation of Na^+ efflux (Kwong et al., 2014). However, Rio Negro fish have adapted to this very challenging habitat, by notably acquiring impressive net Na^+ uptake abilities (generally very high Na^+ affinities, high Na^+ uptake rates, low permeabilities, and resistance to disruption by H^+), which appear to be supported through unique and novel mechanisms (Gonzalez et al., 1998, 2002; Gonzalez and Preest, 1999; Gonzalez and Wilson, 2001; Matsuo and Val, 2007; Wood et al., 2014). This high Na^+ branchial uptake ability may explain the intrinsic high tolerance of Rio Negro fish to H^+- or Cu-induced imbalance of Na^+ homeostasis (Matsuo et al., 2005; Duarte et al., 2009).

4.3. Possible explanations for the low Cu toxicity in Rio Negro water

Because of the high LC50 observed in the extremely soft and acidic Rio Negro water, only a very large increase in the log K_{HBL} up to ~7.6 could lead to acceptable LC50 predictions for all three waters. Such a high value seems unrealistic. First, this strong protection by H^+, overshadowing protection by Ca^{2+}, Mg^{2+} and Na^+ in RS, is in disagreement with the strong hardness protective effect observed against Cu acute toxicity in *P. axelrodi* by Duarte et al. (2009). Secondly, the optimized log K_{HBL} is two log-units higher than the commonly reported pKa for biological surfaces (e.g. cell wall, fish gills), ranging from 4 to 5.4 (Cusimano et al., 1986; Playle et al., 1993).

Deviations from BLM predictions in tests at variable pH have sometimes been attributed to the bioavailability of metal complexes, such as $CuOH^+$ in fish (Allen and Hansen, 1996; De Schamphelaere et al., 2002; De Schamphelaere and Janssen, 2002). In our waters though, $CuOH^+$ had a very low abundance relative to Cu^{2+} (one to two orders of magnitude lower), making it unlikely that $CuOH^+$ significantly contributed to the toxicity. The bioavailability of a metal-carbonate complex such as $CuCO_3$ has been hypothesized in a few studies for Pb and Cu in unicellular algae and in *Daphnia magna* (De Schamphelaere et al., 2002; Slaveykova and Wilkinson, 2003; Bayen et al., 2009). However, except in the study from Bayen et al. (2009) on a green algae, no unequivocal evidence could be demonstrated, as co-variation of parameters with potentially confounding effects occurred (Sánchez-Marín et al., 2013). In fish, to our knowledge, the bioavailability of metal-carbonate complexes has not been proposed. Furthermore, although the proportion of $CuCO_3$ was higher in RS than in RN, it was too low in IW to account for the 20 times lower LC50 observed in IW compared to RN.

The 96-h LA50 estimated values (derived from fish Cu-head contents) were higher in RN than in RS, by approximately a factor of 7. This difference could indicate a discrepancy between the real-world situation and the current theoretical framework of the BLM. Failures of the BLM occur when one or more assumptions underlying the model are not respected (Campbell, 1995). One of these assumptions is that the physiology of the organism remains unchanged in the tests. In that context, our data could indicate that fish in RN are physiologically better protected against Cu-induced Na^+ homeostatic disturbance than in the other waters. Based on relatively recent studies, we hypothesize that this physiological protection in Rio Negro could be provided by its DOM. Until recently, the ameliorative effect of DOM on metal toxicity was solely attributed to its metal binding properties, which are integrated in the BLM. However, binding of DOM to biological surfaces, a fast process that has been notably demonstrated for fish gills (Campbell et al., 1997), may rapidly alter organism physiology in a way that protects against metal toxicity (Wood et al., 2011). Indeed, recent evidence show that DOM has effects on Na^+ transport, diffusive permeability and electrical properties of fish gills in a manner that can promote Na^+ homeostasis (see Steinberg et al., 2006 and Wood et al., 2011 for reviews). These DOM beneficial effects have been proposed to contribute to Rio Negro's fish tolerance to low pH and ion poor conditions. Indeed, in fish exposed to low pH, DOM has been shown to support Na^+ influx (increase of transport capacity, sometimes accompanied by a change in affinity) and, usually more importantly to prevent Na^+ (and Cl^-) efflux (Gonzalez et al., 1998, 2002; Gonzalez and Wilson, 2002; Wood et al., 2003; Matsuo and Val, 2007; Duarte et al., 2016). The mechanisms involved remain largely speculative. Dissolved organic matter binding to the gill could alter the net charge on tight paracellular junctions and stabilize them the same way Ca^{2+} does, resulting in a decrease in gill permeability and hence in lower diffusive ion losses (Wood et al., 2011). Hypotheses for stimulated Na^+ uptake rates include higher Na^+ concentrations at the gill boundary layer when DOM is present, a favourable alteration in -trans-epithelial potential, an augmented Na/K-ATPase activity and changes in fluidity of the lipoprotein bilayers in the transcellular pathway (Wood et al., 2011). The extent of these physiological effects appears to increase with decreasing pH as DOM binding to biological surface increases, suggesting a hydrogen bonding or a hydrophobic bonding sorption mechanism (Campbell et al., 1997). Furthermore, the DOM source may also greatly influence the magnitude of these effects, with optically darker, more aromatic, and larger DOM molecules (such as the very allochthonous ones in Rio Negro) being generally more effective (Matsuo et al., 2004; Wood et al., 2011; Duarte et al., 2016). Indeed, the same DOM characteristics that cause physiological effects at the gills also seem to protect against metal toxicity (Al-Reasi et al., 2011, 2013). Studies indicating that DOM-induced physiological effects help to protect against metal toxicity include Matsuo et al. (2004), Matsuo et al. (2005), Glover and Wood (2004), and Giacomin et al. (2013). In the present study, the Rio Negro environment may have presented ideal conditions for detecting this protective role of DOM, with low pH and allochthonous-rich DOM present at high concentrations. Further studies are needed to fully appreciate the environmental relevance and potential regulatory implications of this direct protection from DOM against metals targeting ionoregulatory balance in aquatic organisms.

4.4. Recommendations on Cu speciation determination

For a fish with a given sensitivity, the accuracy of BLM LC50 predictions is strongly dependent on the accuracy of the determination of the metal aqueous speciation, mostly the free ion concentration. In the present study, we tested three methods to determine the free Cu^{2+} concentration in the three waters at 50% fish mortality: two modeling methods (W-BLM and WHAM7) and an analytical method (Cu-ISE). Each method provided different estimates of the free Cu^{2+} concentration. Speciation modeling obviously depends on the availability and the choice of the thermodynamic constants, as well as on the concentrations of binding sites. This study reinforces the fact that, prior to modeling, the model's thermodynamic database should be critically reviewed and edited when necessary and possible. Additionally, our analyses emphasize the potentially important influence of Al and Fe in metal speciation, as these metals can significantly reduce DOM metal-complexing capacity. High dissolved Al and Fe concentrations are common in tropical waters containing high amounts of DOM, such as the Rio Negro (Benedetti et al., 1997). Direct speciation measurements are always preferable over modeling estimates, on condition that handling of the samples does not affect Cu speciation. For ISE measurements in soft tropical waters, the necessary leveling of ionic strength (by addition of a large amount of electrolyte) may potentially introduce significant bias in the free metal concentration via competition (K^+ displacement of Cu^{2+} from DOM) and complexation (Cu^{2+} with $SO_4{}^{2-}$) reactions in the water (Rachou et al., 2007). These are all important factors for consideration in future BLM work, especially in tropical waters.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aquatox.2016.10.011.

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