

The Effects of Natural Suspended Solids on Copper Toxicity to the Cardinal Tetra in Amazonian River Waters

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Abstract: The aim of the present study was to characterize the effects of particles on metal aquatic toxicity in a tropical system. To this end, we investigated the effects of natural suspended solids on copper (Cu) geochemistry and acute toxicity to the cardinal tetra (*Paracheirodon axelrodi*), in 2 Amazonian rivers with different total suspended solids (TSS) levels: the Rio Negro (low TSS ~ 8 mg L⁻¹) and the Rio Solimões (high TSS ~ 70 mg L⁻¹). The effect of particles on Cu aqueous geochemistry was assessed by measuring total, dissolved, and free ionic Cu concentrations in filtered (<0.45 μm) and unfiltered waters. Furthermore, acute Cu toxicity to fish was assessed in both filtered and unfiltered waters, by measuring physiological net fluxes of Na⁺, Cl⁻, K⁺, and total ammonia (in both river waters) and 96-h fish mortality (in Rio Solimões only). The particles in the Rio Negro were not abundant enough to play a significant role in our study. On the other hand, the Rio Solimões particles bound approximately 70% of total aqueous Cu in our tests. In agreement with bioavailability-based models, this decrease in dissolved (and free ionic) Cu concentration decreased Cu lethality in the 96-h toxicity tests. In the physiological measurements, the best evidence of particle protection was the total alleviation of Cu-induced Cl⁻ losses. These flux tests also suggested that the particles themselves may negatively affect Na⁺ balance in the fish. Overall, the present study supports the use of bioavailability concepts to account for the role of natural suspended solids on metal biological effects in the Amazon River basin. *Environ Toxicol Chem* 2019;38:2708–2718. © 2019 SETAC

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INTRODUCTION

Water physicochemical parameters, such as pH, major cation concentrations, and the concentration of dissolved organic matter (DOM), are known to modulate metal bioavailability in natural waters (Erickson et al. 1996; Niyogi and Wood 2004). Bioavailability models, such as the Biotic Ligand Model (BLM), stipulate that bioavailability of a hydrophilic metal (e.g., copper [Cu]) is usually related to the free metal ion concentration in the water ([M^{z+}]; Allen and Hansen 1996). Within this framework, adsorption of metals on suspended solids (size >0.45 μm) is recognized as a key process controlling metal biogeochemical fate in aquatic ecosystems (Lu and Allen 2001). Indeed, by decreasing the dissolved metal concentration, and thus the free metal concentration within the water column, this process reduces metal bioavailability and toxicity to aquatic

organisms (Erickson et al. 1996). However, some studies have shown that the role of suspended solids on metal biological fate may not be so simple. Notably, there have been reports in the literature that particulate metal could contribute to the bioavailable metal fraction in fish (Erickson et al. 1996; Tao et al. 1999, 2000; Ma et al. 2002). For example, Tao et al. (1999) showed that the gills of neon tetras accumulated a portion of Cu bound to kaolin suspended solids. Proposed mechanisms for particulate metal uptake are via adherence of particles on the gill surface, followed by either direct phagocytic uptake or by desorption of the metal in the gill microenvironment prior to uptake by epithelial metal transporters (Goldes et al. 1986; Martens and Servizi 1993; Tao et al. 2000). Furthermore, suspended solids may act as an additional biological stressor in metal toxicological studies. In fish, high levels of suspended solids have been shown to directly damage the delicate gill structures (Lloyd 1987; Au et al. 2004), as well as to induce stress that can increase fish susceptibility to contaminants (Kjelland et al. 2015). Hence, the total suspended solids (TSS) concentration is an important water quality parameter in

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environmental jurisdictions. For example, in Brazil, the maximal allowable TSS is 500 mg L^{-1} (CONAMA 2005). Despite these evidences of potential confounding effects of suspended solids on metal uptake and toxicity, studies that have explicitly tackled this issue remain scarce, and even more so in surface waters with natural suspended solids. The present study aims at addressing this data gap, by evaluating the potential effects of natural particles on the toxicity of Cu to a tropical fish in Amazonian river waters.

The Amazon River basin is characterized by the great spatial and temporal variations of its abiotic features (e.g., pH, hardness, TSS, dissolved oxygen, and DOM concentrations; Val and Almeida-Val 1995). This environmental diversity, responsible for the unparalleled biological diversity of this region, is perfectly demonstrated just east (downstream) of the city of Manaus, where the “black waters” of the Rio Negro and the “white waters” of the Rio Solimões, meet in a spectacular fashion to form the Amazon River. The Rio Negro is notably characterized by its abundant optically dark dissolved humic acids and by its low TSS. On the other hand, the Rio Solimões is characterized by a milky-brown color produced by its large loads of suspended silts carried from its source in the Andes foothills. The present study focuses on the effects of TSS in both river waters on acute Cu toxicity to fish, whereas a previous study has already characterized the effects of other water chemistry parameters (e.g., pH, DOM, [Ca], etc.) in filtered river waters (Crémazy et al. 2016). In both cases, we chose the cardinal tetra (*Paracheirodon axelrodi*) as our model fish species. This ornamental fish, native to the mid-Rio Negro basin, is currently emerging as a biological model for ecotoxicological studies, due to its conveniently small size and its great ecological and economic importance. Indeed, several metal toxicological studies have been carried out with *P. axelrodi*, in various waters from the Amazonian basin, including the Rio Negro and Rio Solimões (Duarte et al. 2009; Crémazy et al. 2016; Holland et al. 2017; Braz-Mota et al. 2018). Notably, we previously showed that Cu affects gill ionoregulatory functions in cardinal tetras, with the probable key targets being Na^+ and Cl^- homeostasis (Crémazy et al. 2016), as for temperate freshwater species (Niyogi and Wood, 2004). These effects occur at Cu concentrations that are 1 to 2 orders of magnitude higher than the natural background levels found in the Rio Negro and Rio Solimões (a few $\mu\text{g L}^{-1}$). Yet, anthropogenic pressure is increasing at a fast pace in the Amazon region, notably around the city of Manaus, an important industrial pole, which extends from upstream to the mixing zone of both rivers. Copper concentrations in the city effluents have been shown to often exceed the Brazilian water quality guideline of $9 \mu\text{g L}^{-1}$ of dissolved Cu (CONAMA 2005), with some effluents presenting concentrations as high as $1000 \mu\text{g L}^{-1}$ (Silva et al. 1999; Sampaio 2000; Santana and Barroncas 2007). Furthermore, there is currently a clear intent to intensify the exploitation of the Amazon’s mineral resources, which include vast ore deposits of copper, tin, nickel, bauxite, manganese, iron ore, and gold (Ministério de Minas e Energia 2011). Although the assessment of mining impact in the Amazon is usually limited to evaluation of deforestation, contamination of river waters is another serious threat

that generally lacks attention. Despite the growing environmental risk in this very ecologically important region, the fate and effects of metals in the Amazon basin are understudied.

In the present study, cardinal tetras were exposed to waterborne Cu spiked into unfiltered and filtered ($<0.45 \mu\text{m}$) waters of the Rio Solimões and the Rio Negro. Physiological indicators of acute Cu toxicity were evaluated by measuring net flux rates of Na^+ , Cl^- , K^+ , and total ammonia (NH_3 and NH_4^+) excretion rates in the fish. Furthermore, when particle effects on ion regulation were observed, standardized 96-h mortality tests were also performed. These biological effects were related to Cu geochemistry/bioavailability in the river waters, by measuring and/or modeling total, dissolved, and free ionic Cu concentrations in the filtered and unfiltered waters. Thus, all water chemistry factors (e.g., pH, DOM, ions, etc.) were taken into account.

MATERIALS AND METHODS

Experimental animals

Cardinal tetras (*Paracheirodon axelrodi*, $70 \pm 6 \text{ mg}$ wet wt) were purchased in November 2015 from a local ornamental fish store and transported to the Laboratory of Ecophysiology and Molecular Evolution at INPA in Manaus (Amazonas, Brazil). They were kept in continuously aerated INPA groundwater (pH = 6.0, dissolved organic carbon concentration [DOC] = 0.3 mg L^{-1} , alkalinity = 0.34 mEq L^{-1} , [Ca] = $5 \mu\text{M}$, [Mg] = $4 \mu\text{M}$, [Na] = $50 \mu\text{M}$, [K] = $15 \mu\text{M}$, [Cl] = $25 \mu\text{M}$), for at least 2 wk before the experiments. This water composition closely resembled that of the Rio Negro, except for its much lower DOC concentration. Acclimation and tests were performed at a water temperature of 28°C . Fish were fed every day with commercial dry food pellets, but feeding was withheld 24 h before and throughout the tests. Experimental work was approved by the Ethics Committee on Animal Experiments of INPA (Registration Numbers 047/2012 and 026/2015) and conformed to national animal care regulations in Brazil.

Exposure waters

Detailed water-collection locations and chemical compositions are given in Crémazy et al. (2016). Briefly, waters from the Rio Negro (pH = 5.6, [DOC] = 8.4 mg L^{-1} , Alkalinity = 0.12 mEq L^{-1} , [Ca] = $8 \mu\text{M}$, [Mg] = $5 \mu\text{M}$, [Na] = $33 \mu\text{M}$, [K] = $11 \mu\text{M}$, [Cl] = $29 \mu\text{M}$) and the Rio Solimões (pH = 6.7, [DOC] = 2.8 mg L^{-1} , Alkalinity = 0.66 mEq L^{-1} , [Ca] = $340 \mu\text{M}$, [Mg] = $51 \mu\text{M}$, [Na] = $190 \mu\text{M}$, [K] = $32 \mu\text{M}$, [Cl] = $110 \mu\text{M}$) were collected in mid-November 2015, at approximately 20 km upstream of their confluence east of Manaus. Waters were immediately transported in clean 25-L carboys to the Laboratory of Ecophysiology and Molecular Evolution at INPA, where they were kept in the dark at 4°C until 24 h before the experiments. For tests in particle-free water, large aliquots of the collected waters were filtered with a glass vacuum filtration device connected to an electrical pump, through rapid-flow $0.45 \mu\text{m}$ polyethersulfone membranes (47 mm diameter, Membrane Solutions). This filtration was performed on the same day as water collection. All tests were performed within

3 wk of water collection. Exposure waters were spiked with $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (99%, Sigma Aldrich) 24 h prior to each test, to allow for chemical equilibration.

Na^+ , K^+ , Cl^- , and total ammonia net flux tests

For these tests, cardinal tetras were exposed to Rio Negro and Rio Solimões waters (unfiltered and filtered) spiked with various Cu concentrations (4 treatments, including the control). Our previous study (Crémazy et al. 2016) showed that the Rio Negro water was significantly more protective than Rio Solimões water against Cu toxicity, due to its much higher DOC content. Therefore, different concentration ranges were used, with the upper Cu concentrations corresponding approximately to the 96-h lethal concentration of Cu causing 50% of fish mortality (96-h LC50) values previously observed in each of the filtered waters (Crémazy et al. 2016). Nominal Cu concentrations were 0, 25, 200, and 500 $\mu\text{g L}^{-1}$ in Rio Solimões water and 0, 200, 500, and 1000 $\mu\text{g L}^{-1}$ in Rio Negro water. For each treatment, $n = 6$ fish were exposed in individual 22-mL plastic vials filled with 20 mL of the appropriate water. Continuous aeration ensured that particles remained in suspension. The fish were acclimated to these conditions (in Cu-free water) for 12 h prior to the beginning of the flux test. After replacement of the acclimation water with test waters, flux measurements were carried out as detailed in Crémazy et al. (2016). Briefly, small aliquots of test waters were collected and filtered using syringe-tip filters ($<0.45 \mu\text{m}$ cellulose nitrate membrane, 25 mm diameter, Corning) at the start and end of 3 flux periods (0–6 h, 6–12 h, 12–24 h), to measure dissolved Na^+ , Cl^- , K^+ , and ammonia concentrations. These concentrations allowed calculation of the Na^+ , Cl^- , K^+ , and ammonia net fluxes ($J_{x,\text{net}}$, in $\text{nmol g}^{-1} \text{h}^{-1}$) with the following equation:

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

with $[x]_i$ and $[x]_f$ representing the dissolved aqueous concentrations of x (x : Na^+ , Cl^- , K^+ , or ammonia) at the beginning and at the end of the flux period respectively (in nmol L^{-1}), V the volume of exposure solution (in L), m the wet weight of the fish (in g), and Δt the duration of the flux period (in h). As explained in Wood (1992), this flux approach provides many advantages over methods based on blood or tissue analyses, including a much greater sensitivity. Note that although the flux rates in unfiltered waters are reported in the present study for the first time, the filtered data have been presented in a previous publication (Crémazy et al. 2016).

Water samples were also collected at $t = 0$ h (start of the first flux period) and at $t = 24$ h (end of third flux period) to measure dissolved Cu concentrations. At the end of the test, fish were euthanized with an overdose of tricaine methanesulfonate (MS-222).

96-h mortality tests

For these toxicity tests, only the effect of Rio Solimões particles was evaluated. We did not evaluate toxicity in filtered

versus unfiltered Rio Negro water, because the suspended solids of Rio Negro 1) were not very abundant (low TSS), 2) bound Cu in negligible amounts in the range of tested Cu concentration, and 3) had a negligible effect on Cu-induced ion imbalance in cardinal tetras (see *Results* section). These various observations indicated that effects of Rio Negro's particles on Cu lethality to fish were extremely unlikely.

Cardinal tetras were acclimated to both unfiltered and filtered Rio Solimões water 24 h prior to the beginning of the tests. The fish were then exposed for 96 h to both waters spiked with 0 (control), 50, 100, 200, 500, 1000, and 1500 $\mu\text{g L}^{-1}$ Cu (nominal concentrations). For each treatment, $n = 10$ fish were exposed in bulk in 1.2-L plastic containers filled with 1 L of aerated solution. This aeration ensured that particles remained in suspension. Every day, mortality was recorded (dead fish were removed) and solutions were renewed (90% of their volumes). In addition, pH was measured and filtered and unfiltered water samples were collected for total and dissolved Cu, dissolved major cations, and DOC concentrations analyses in both fresh and 24-h-old solutions. At the end of the test, the surviving fish were euthanized with an overdose of MS-222. Because of limitations in the amount of river water available for the present study (especially filtered water), it was not logistically feasible to repeat these toxicity tests.

Water analyses

General water chemistry analyses. Total suspended solids (in mg L^{-1}) in Rio Negro and Rio Solimões were measured by comparing the weight of 0.45 μm cellulose nitrate membranes (Whatman, GE Healthcare) before and after filtration of 200 mL of water ($n = 3$ replicates). The filters were dried at 80 °C for 12 h prior to each weighing, both before and after filtration. In the water samples collected from the tests, Cu concentrations were measured by atomic absorption spectrometry (AAS) with a graphite furnace (Perkin-Elmer AAnalyst 800 AA spectrophotometer). A Cu standard solution (PE N9300224, Calibration Solution, Perkin Elmer) was used to calibrate the instrument. Blanks were analyzed every 12 samples to correct for instrument signal drift. A limit of detection of 1 $\mu\text{g L}^{-1}$ was measured (as 3 times the standard deviation of 7 repeated blank measurements). This analysis was done after acidification of the water samples with 1% HNO_3 (Trace metal grade, Fisher Scientific). For the unfiltered water samples, this acidification allowed for the desorption of Cu bound onto particles into the solution. The vials were agitated after HNO_3 addition, particles were allowed to sink overnight, and the supernatant was collected for the Cu concentration analysis. For major cation concentrations, AAS with a flame was used for Ca and Mg (Varian AA240FS), and a flame photometer was used for Na and K (910 Digital Flame Photometer, Instrumentação Analítica). Ammonia and Cl concentrations were measured with colorimetric assays developed by Verdouw et al. (1978) and Zall et al. (1956), respectively. Alkalinity was measured by titrating 10-mL water samples to an endpoint of pH 4.0 with certified 0.02 N HCl (Sigma-Aldrich) using a microburette (Gilmont Instruments), while the sample was continually bubbled with CO_2 -free air. A sealed body 20020 electrode

(Quimis) coupled to a PG1800 pH meter (Gehaka) was used. Finally, the water DOC concentrations were measured with a TOC analyzer (Shimadzu TOC-VCSH).

Copper aqueous speciation analyses. Free Cu^{2+} concentration analyses were performed in parallel to the flux and mortality tests, with a subset of the collected and treated waters. As detailed in Crémazy et al. (2016), we used a cupric ion-selective electrode (ISE, Orion Model 94 – 29) to generate titration curves giving $[\text{Cu}^{2+}]$ as a function of the total Cu concentration added to the various waters. The range of total Cu concentration used was approximately $2\text{--}2000\ \mu\text{g L}^{-1}$ (i.e., $10^{-7.5}\text{--}10^{-4.5}$ M). Because sufficient conductivity is needed for precise cupric ISE measurements, the ionic strength of the water was adjusted to 0.01 M by addition of K_2SO_4 (99%, Sigma Aldrich). The electrode was calibrated directly into concentration units, using 3 standards within this range and at the same ionic strength. Copper speciation in the dissolved phase was modeled with the Windermere Humic Aqueous Model (WHAM), Ver. 7 (Tipping et al. 2011), as previously detailed in Crémazy et al. (2016).

Data analyses

In the tests with unfiltered Rio Negro and Rio Solimões waters, the concentrations of Cu in the particulate phase ($[\text{Cu}]_s$, in $\mu\text{g kg}^{-1}$) were obtained from the total Cu concentrations measured in unfiltered water samples ($[\text{Cu}]_T$, in $\mu\text{g L}^{-1}$), the dissolved Cu concentrations measured in filtered water samples ($[\text{Cu}]_D$, in $\mu\text{g L}^{-1}$), and the measured TSS values (in mg L^{-1}), according to the following equation:

$$[\text{Cu}]_s = \frac{[\text{Cu}]_T - [\text{Cu}]_D}{\text{TSS}} \cdot 10^6 \quad (2)$$

The values of $[\text{Cu}]_s$ and $[\text{Cu}]_D$ were then used to calculate the partitioning coefficient of Cu between the solid and the dissolved phase (K_d , in L kg^{-1}):

$$K_d = \frac{[\text{Cu}]_s}{[\text{Cu}]_D} \quad (3)$$

The effect of particles on Cu speciation in the river waters was assessed by comparing the linear regressions of the measured $\log [\text{Cu}^{2+}]$ as a function of $[\text{Cu}]_T$ in the Cu-ISE analyses, using an extra sum of squares F-test. The 96-h LC50 estimates and their associated 95% confidence levels were calculated by the software Toxicity Relationship Analysis Program, Ver. 1.22 (US Environmental Protection Agency 2002) using the Tolerance Distribution analysis type and the Gaussian Distribution model. When only 1 partial kill (i.e., only 1 mortality percentage between 0 and 100%) was observed in the toxicity test, the Spearman-Kärber method was used instead (Spearman 1908; Kärber 1931). The 96-h LC50 values and their confidence intervals were calculated as a function of the measured total Cu concentration and separately as a function of the measured dissolved Cu concentration. The differences

between these estimates were then assessed with a t test. Finally, the effects of both particle and Cu exposures on Na^+ , Cl^- , K^+ , and ammonia net fluxes were assessed by a two-way analysis of variance with a Tukey test for multiple comparison. All the statistical tests were performed in Prism GraphPad Ver. 6.0, with a significance level of 0.05. Results are given either as means \pm standard errors or as means with the 95% confidence intervals.

RESULTS

Copper geochemistry in the river waters

Chemical composition of filtered Rio Negro and Rio Solimões waters is given in the *Exposure waters* section of *Materials and Methods*. No change in these water chemistry parameters was observed over the course of the present study. Titration measurements in unfiltered versus filtered Rio Solimões water showed that the presence or absence of particles did not affect alkalinity (0.656 ± 0.008 vs 0.662 ± 0.030 mEq L^{-1} , $n=3$). We measured TSS values of $73 \pm 3\ \text{mg L}^{-1}$ ($n=3$) in the Rio Solimões and $7.8 \pm 0.2\ \text{mg L}^{-1}$ ($n=3$) in the Rio Negro. In the Rio Solimões, the concentration of Cu bound to particles, $[\text{Cu}]_s$, ranged from 1.63 to $162\ \text{mg kg}^{-1}$ within the range of total Cu concentrations used in the present study (i.e., $[\text{Cu}]_T = 25\text{--}1500\ \mu\text{g L}^{-1}$). Figure 1 shows that, as $[\text{Cu}]_T$ increased from 25 to $1500\ \mu\text{g L}^{-1}$ in the Rio Solimões, the proportion of Cu bound to particles increased from 58 to 77% (average = $68 \pm 3\%$; Figure 1A) and the K_d increased from 19 100 to 46 800 ($10^{4.28}$ to $10^{4.67}$) L kg^{-1} (average = $32\ 900 \pm 13\ 900$ ($10^{4.47 \pm 0.07}$) L kg^{-1} ; Figure 1B). Based on these K_d values and the previously computed dissolved Cu speciation in Crémazy et al. (2016), the relative abundance of the different chemical species of Cu in the Rio Solimões was estimated (Figure 1C). These calculations showed an increase in the percentage of Cu^{2+} from 0.70 to 3.6% with the increase in $[\text{Cu}]_T$, in parallel with a decrease in Cu-DOM complexes from 39 to 13%.

Particulate Cu could not be detected in the Rio Negro, as the measured $[\text{Cu}]_D$ and $[\text{Cu}]_T$ values were not significantly different (percentage particulate Cu = $1.1\% \pm 1.6$, $n=6$). Thus, the Cu partitioning coefficient could not be assessed in this water.

Figure 2 shows the ISE $[\text{Cu}^{2+}]$ measurements in the filtered and unfiltered Rio Solimões (Figure 2A) and Rio Negro (Figure 2B), with linear regressions of $\log [\text{Cu}^{2+}]$ as a function of $\log [\text{Cu}]_T$. In agreement with WHAM predictions, the percentage of Cu^{2+} increased with increasing $[\text{Cu}]_T$. These ISE measurements also confirmed the above findings that approximately 70% of Cu was bound to particles in the Rio Solimões, while particulate Cu was negligible in the Rio Negro. Indeed, the regression lines of the filtered and unfiltered Rio Solimões waters were significantly different ($p=0.0049$, with $F_{2,22} = 6.82$), with $[\text{Cu}^{2+}]$ in unfiltered water only approximately one-third of the $[\text{Cu}^{2+}]$ in filtered water for the same total Cu concentration (Figure 2A). This difference was attributable to significant differences in the intercepts, as the slopes were virtually identical. On the other hand, the regression lines of the

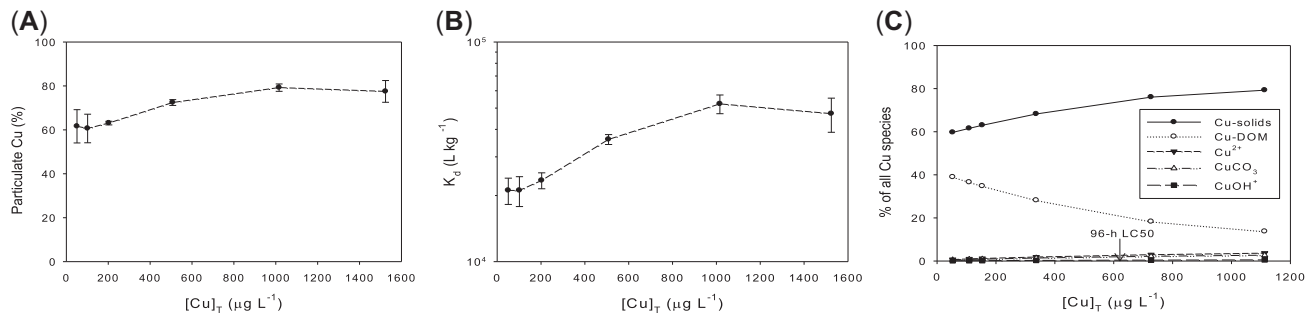


FIGURE 1: Copper (Cu) solid-liquid partitioning and speciation in the Rio Solimões: effects of total Cu concentration on (A) the proportion of Cu in the particulate form, (B) the partitioning coefficient of Cu between the solid and dissolved phase (K_d , given on a logarithmic scale), and (C) the relative abundance of the main forms of Cu in the water. Measurements were made with $n=9$ replicates. In panel C, the 96-h median lethal concentration (96-h LC50) is shown, at which the amount of Cu bound to particles is approximately 3 times the amount of Cu bound to dissolved organic matter (DOM). Copper partitioning in the solids phase was measured in the present study, and Cu speciation in the dissolved phase was modeled with WHAM, Ver. 7 (Tipping et al. 2011), as previously detailed in Crémazy et al. (2016).

filtered and unfiltered Rio Negro waters were not significantly different ($p=0.15$ with $F_{2,19}=2.12$; Figure 2B). The regression results (R^2 and equations) are given in the figure legend boxes.

Na⁺, Cl⁻, K⁺, and ammonia net flux rates

The effects of Cu on Na⁺, Cl⁻, K⁺, and ammonia fluxes in cardinal tetras are depicted in Figures 3 and 4 for tests in Rio Solimões and Rio Negro, respectively. For both unfiltered (dashed bars) and filtered (plain bars) waters, these graphs show the net fluxes of each component, for each flux period (0–6 h, 6–12 h, and 12–24 h), as a function of the total added Cu concentration. Note that a higher range of total Cu concentrations was used in the Rio Negro tests. Nominal rather than measured concentrations are given here for convenience in graph plotting, an acceptable approximation considering the high Cu recovery with the AAS analysis: $88 \pm 11\%$ in Rio Solimões and $94 \pm 10\%$ in Rio Negro.

In the absence of Cu (i.e., control conditions), K⁺ and ammonia were both lost by the cardinal tetras (i.e., $J_{K,net}$ and $J_{ammonia,net} < 0$), and Na⁺ and Cl⁻ were at approximate net balance (i.e., $J_{Na,net}$ and $J_{Cl,net} \sim 0$) throughout the 3 flux periods (Figures 3 and 4, at $0 \mu\text{g L}^{-1}$ Cu). This was generally true for tests with both filtered and unfiltered waters, with the exception that Na⁺ loss rates under control conditions were more negative in the unfiltered than in filtered Rio Solimões water in the 12–24 h flux period (Figure 3A', at $0 \mu\text{g L}^{-1}$ Cu). Addition of Cu mostly altered Na⁺ and Cl⁻ fluxes, whereas changes to K⁺ and ammonia fluxes were rarely and irregularly observed. Indeed, during the 0–6 h period, Cu promoted the net loss of Na⁺ and Cl⁻ in both filtered Rio Solimões (Figures 3A and 3B) and Rio Negro waters (Figures 4A and 4B), as $J_{Na,net}$ and $J_{Cl,net}$ became increasingly negative. These Cu effects were much lower during the 6–12 h (Figures 3A', 3B', 4A', and 4B') and 12–24 h periods (Figures 3A'', 3B'', 4A'', and 4B''). When particles were present in the Rio Solimões, Cu-induced Cl⁻ losses were completely alleviated (Figure 3B), whereas Na⁺ loss rates were unaffected

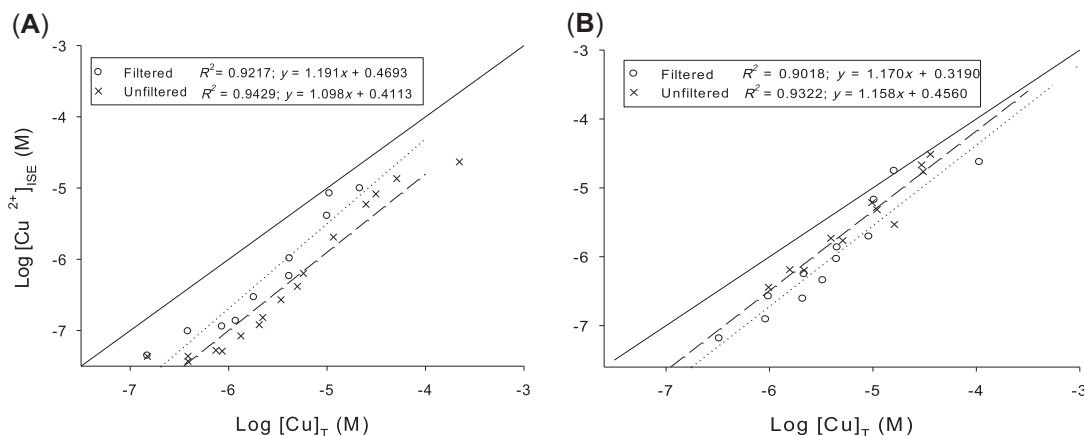


FIGURE 2: Log of the free Cu²⁺ concentration measured with an ion-selective electrode in filtered (circle symbols) and unfiltered (cross symbols) waters of (A) the Rio Solimões and (B) the Rio Negro ($T = 25 \text{ }^\circ\text{C}$, $I = 0.01 \text{ M}$), as a function of the log of total added Cu concentration. Each data point represents 1 measurement (total number is (A) 10 and 16 and (B) 12 and 11 for filtered and unfiltered waters, respectively). The plain lines are the 1:1 line, and the dotted and dashed lines are the regression lines for the filtered and unfiltered treatments respectively (regression R^2 and equations are given in the figure boxes). The regression lines differed significantly between unfiltered and filtered waters for the Rio Solimões ($p = 0.0049$), but not for the Rio Negro.

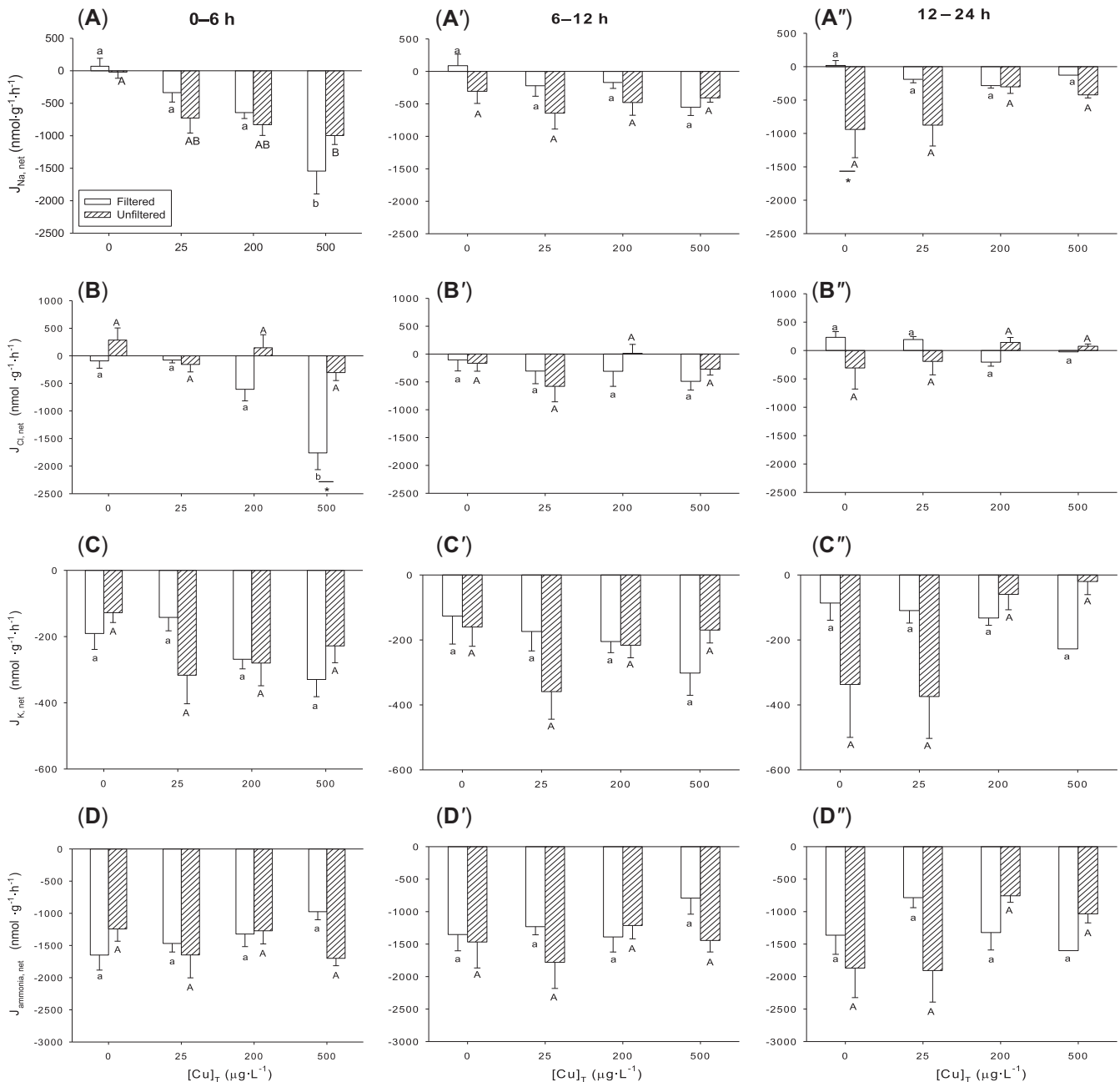


FIGURE 3: The 0–6 h, 6–12 h, and 12–24 h net flux rates of Na^+ (A, A' and A''), Cl^- (B, B', and B''), K^+ (C, C', and C''), and ammonia (D, D', and D'') in *Paracheirodon axelrodi* exposed to varying nominal Cu concentrations in filtered (plain bars) and unfiltered (dashed bars) water of the Rio Solimões. Different lowercase or uppercase letters indicate significant differences across different Cu concentrations for filtered and unfiltered water, respectively, and asterisks indicate significant differences between filtered and unfiltered waters for a given Cu concentration (two-way analysis of variance with post-hoc Tukey's honest significant difference test). Mean \pm standard error ($n = 6$, except at $500 \mu\text{g L}^{-1}$ Cu in filtered water, where $n = 4$ for 6–12 h and $n = 1$ for 12–24 h, due to fish mortality). When comparing Figures 3 and 4, note that the range of Cu concentrations tested in Rio Solimões water (Figure 3) is lower than that tested in Rio Negro water (Figure 4). Note also that the filtered data have been presented in a previous publication (Crémazy et al. 2016).

(Figure 3A). On the other hand, in the Rio Negro, particle effects were only observed on K^+ and ammonia fluxes (Figures 4C'', 4D, and 4D''). Indeed, decreased K^+ loss was observed in the presence of particles, but only during the 12- to 24-h period at 500 and $1000 \mu\text{g L}^{-1}$ of added Cu (Figure 4C''). Increased ammonia loss was observed in the presence of particles, but only at $200 \mu\text{g L}^{-1}$ (0–6 h and 12–24 h) and $500 \mu\text{g L}^{-1}$ (0–6 h) of added Cu (Figures 4D and 4D'').

Copper also affected fish survival during these flux tests, with a higher mortality rate observed in filtered than in unfiltered water for the same added total Cu concentration. Indeed, at the highest $[\text{Cu}]_T$ in the filtered Rio Solimões, 2 fish died during the 6- to 12-h period and an additional 2 during the 12- to 24-h period. On the other hand, no fish died during exposure to the unfiltered Rio Solimões, at any $[\text{Cu}]_T$. During the 12- to 24-h exposure period in the Rio Negro, 1 fish died at $500 \mu\text{g L}^{-1}$ Cu in

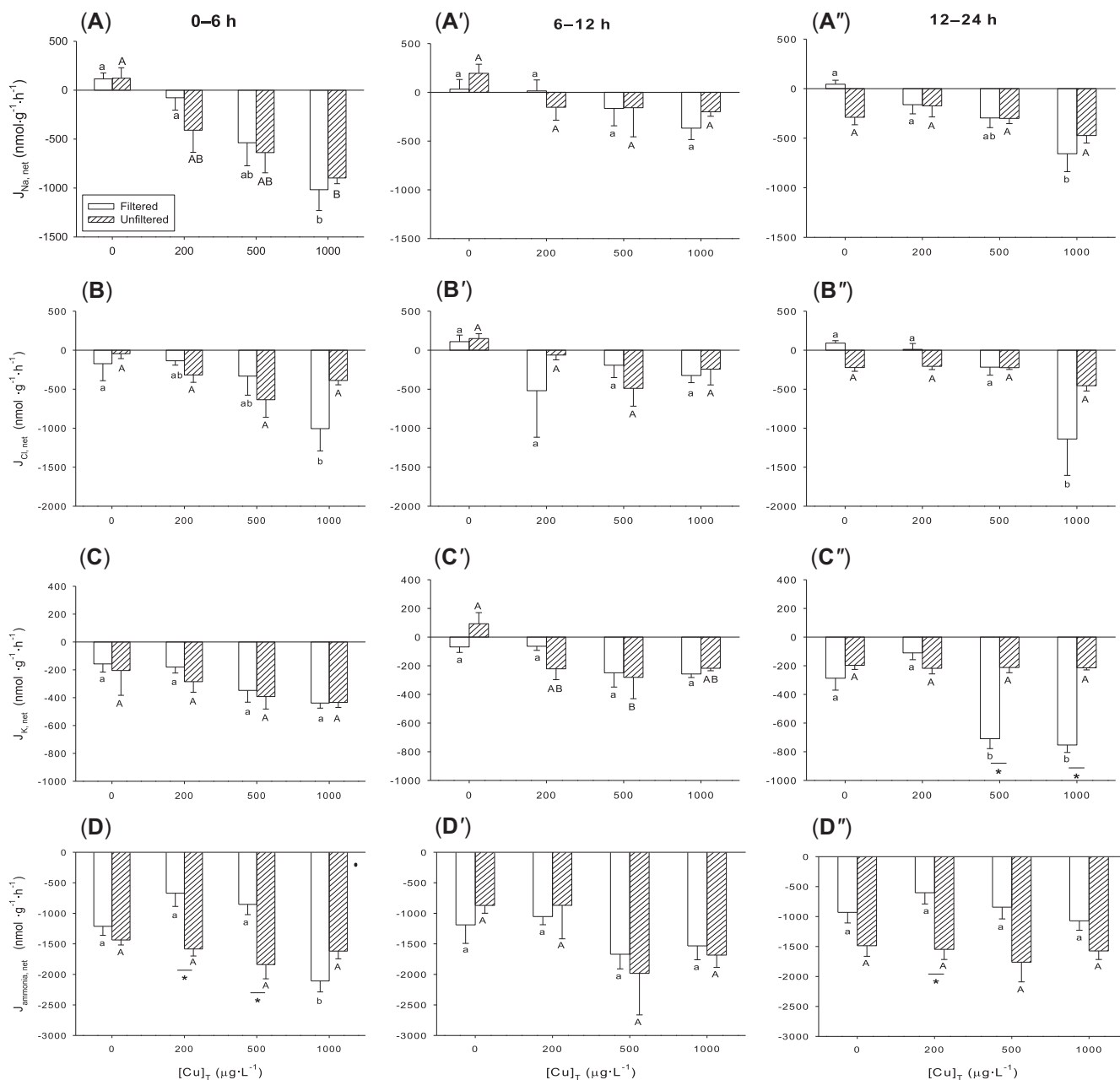


FIGURE 4: The 0–6 h, 6–12 h, and 12–24 h net flux rates of Na^+ (A, A', and A''), Cl^- (B, B', and B''), K^+ (C, C', and C''), and ammonia (D, D', and D'') in *Paracheirodon axelrodi* exposed to varying nominal Cu concentrations in filtered (plain bars) and unfiltered (dashed bars) Rio Negro water. Different lowercase or uppercase letters indicate significant differences across different Cu concentrations for filtered and unfiltered water, respectively, and asterisks indicate significant differences between filtered and unfiltered waters for a given Cu concentration (two-way analysis of variance with post-hoc Tukey's honest significant difference test). Mean \pm standard error ($n=6$, except for 12–24 h at $500 \mu\text{g L}^{-1}$ Cu in both treatments where $n=5$ and at $1000 \mu\text{g L}^{-1}$ Cu in filtered water where $n=3$, due to fish mortality). When comparing Figures 3 and 4, note that the range of Cu concentrations tested here in Rio Negro water (Figure 4) is higher than that tested in Rio Solimões water (Figure 3). Note also that the filtered data have been presented in a previous publication (Crémazy et al. 2016).

both filtered and unfiltered waters and 3 fish died at $1000 \mu\text{g L}^{-1}$ Cu in the filtered water.

96-h acute toxicity tests

In light of the Cu speciation and flux test results, the 96-h acute toxicity tests were performed only in Rio Solimões waters. Figure 5 shows the percentage mortality of *P. axelrodi* after a 96-h exposure to unfiltered and filtered Rio Solimões. As

a function of the measured total Cu concentration (Figure 5A), the 96-h LC50 value in unfiltered water (620 95% confidence interval = [450–780] $\mu\text{g L}^{-1}$) was significantly higher than in filtered water (190 [140–250] $\mu\text{g L}^{-1}$, showing a lower fish mortality when particles are present. As a function of the measured dissolved Cu concentration (Figure 5B), the 96-h LC50 value in unfiltered water (150 [120–180] $\mu\text{g L}^{-1}$) was no longer significantly different from the value in filtered water (190 [140–250] $\mu\text{g L}^{-1}$). By way of comparison, the 96-h LC50 value

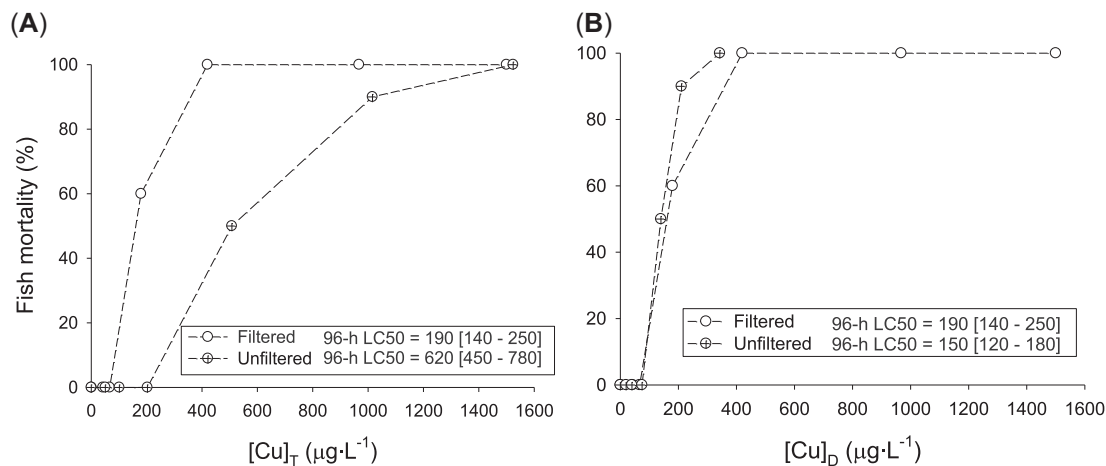


FIGURE 5: Percentage of mortality of *Paracheirodon axelrodi* exposed for 96 h in filtered (open circles) and unfiltered (crossed circles) Rio Solimões, as a function of measured (A) total and (B) dissolved Cu concentration. The dashed lines are connecting lines. The 96-h median lethal concentration (96-h LC50) estimates and their 95% confidence intervals are given in the figure boxes. Note that the 96-h LC50 values differed significantly as a function of total Cu concentration, but not as a function of dissolved Cu concentration. Each data point represents 1 treatment of 10 fish.

measured in filtered Rio Negro water by Crémazy et al. (2016) was approximately 6-fold higher at 1100 [890–1300] µg L⁻¹.

DISCUSSION

Particle effects on Cu aqueous geochemistry

Suspended solids in the present study were defined as particles with a size greater than 0.45 µm, which is the classical operational cut-off between the dissolved and the particulate fractions (Sigg et al. 2000), although 0.22 µm is also commonly employed (Allard et al. 2002; Aucour et al. 2003). Copper potentially bound to colloids (size < 0.45 µm and molecular wt > 10 kD) was not assessed in the present study. The measured TSS values in Rio Solimões and Rio Negro are in accord with previously reported values in the literature (Moreira-Turcq et al. 2003; Seyler and Boaventura 2003). Notably, in nearby sampling areas, Moreira-Turcq et al. (2003) measured TSS values of approximately 86 mg L⁻¹ in the Rio Solimões (4-yr average, min = 18 mg L⁻¹, max = 214 mg L⁻¹) and approximately 5.2 mg L⁻¹ in the Rio Negro (2-yr average, min = 3.0 mg L⁻¹, max = 8.1 mg L⁻¹), with high inter-seasonal and inter-annual variability. These suspended solids have been characterized as numerous minerals, including mainly quartz and clays (smectite, mica/illite, kaolinite, chlorite) and minor feldspars in the Rio Solimões, and as mainly kaolinite, quartz, minor gibbsite, and traces of mica/illite in the Rio Negro (Allard et al. 2002).

Our filtration tests showed that the majority of Cu (~70%) in the Rio Solimões was bound to particles, whereas the fraction bound to particles in the Rio Negro was undetectable. The ISE analyses were in approximate quantitative agreement with the above Cu measurements in both river waters, showing approximately 70% Cu-particle binding in Rio Solimões, and negligible binding in Rio Negro water. Seyler and Boaventura (2003) showed similar proportions in Rio Solimões under natural conditions (a few µg L⁻¹ dissolved Cu), with 80% of Cu being transported by particles > 0.22 µm. Furthermore, the

Cu partitioning coefficients we calculated for this river ($K_d \sim 32,000$, or $10^{4.5}$ L kg⁻¹) were in the same order of magnitude as previously reported values in temperate rivers, under similar pH and TSS (Lu and Allen 2001). In the present study, we observed an increased partitioning of Cu in the solid phase when total Cu concentration increased, which could be explained by the aqueous Cu speciation in the tested waters. Indeed, WHAM speciation modeling indicated that the percentage of Cu-DOM complexes in filtered Rio Solimões decreases by a factor of approximately 4 within the study range of [Cu]_T. This progressive saturation of DOM binding sites with the increase of Cu concentration, increases the percentage of Cu²⁺ that can bind to suspended solids (cf. Figure 1C). The undetectable particulate Cu in the Rio Negro could be attributed to a lower abundance of metal binding sites in the solid phase versus the dissolved phase, with a notably high DOM concentration for a very low TSS level. In addition to low TSS levels, the lower proportion of particulate Cu in the Rio Negro may also arise from the distinct nature of their suspended particles. Indeed, different solid phases may exhibit various affinities for dissolved metals (i.e., various K_d). Further studies would be needed to fully characterize how suspended particles in the 2 rivers affect metal speciation, and in turn, Cu bioaccumulation in aquatic organisms. Overall, our analyses of Cu geochemistry in the 2 river waters suggest that only the particles of the Rio Solimões, and not the particles in the Rio Negro, should significantly affect Cu acute toxicity to the cardinal tetra.

Effects of particles on Cu acute toxicity to the cardinal tetra

Effects of Cu alone. The gills of freshwater fish actively take up Na⁺ and Cl⁻ from the water to compensate for diffusive losses of these ions, thereby normally maintaining homeostasis of plasma Na⁺ and Cl⁻ levels (Evans et al. 2005). Thus, the observed NaCl net balance of *P. axelrodi* in the Cu-free media

was expected. In these waters, observed K^+ loss under control conditions is commonly observed in fasted fish and supports evidence that this element is mostly acquired from the food (Wood and Bucking 2011), whereas ammonia loss represents normal metabolic waste excretion (Wilkie 2002).

In the presence of Cu, the Na^+ and Cl^- losses observed in both filtered and unfiltered river waters during the 0–6 h period are in accordance with general knowledge on Cu mechanisms of acute toxicity to freshwater organisms. Indeed, impairment of Na^+ homeostasis (with accompanying impairment of Cl^- homeostasis) is generally considered to be the key Cu toxicity target in acutely exposed fish, with mortality occurring when approximately 30% of its exchangeable plasma Na^+ pool is lost (Grosell et al. 2002). In this regard, the occurrence of mortality in the cardinal tetra, reported in the present study for Rio Solimões water and in Crémazy et al. (2016) for filtered Rio Negro water, is consistent with the observed rate of Na^+ loss, if we assume a typical exchangeable Na^+ pool in this fish (i.e., $\sim 45 \mu\text{mol g}^{-1}$, Paquin et al. (2002)). Thus, we can conclude that failure of Na^+ homeostasis is the likely key target of Cu acute toxicity in the cardinal tetra, as commonly acknowledged for freshwater organisms. In the present study, the NaCl losses were almost always attenuated with time, although significant effects were still observed over the last flux period for the highest Cu concentration. This time effect may have a dual origin. At lower, sublethal Cu concentrations, it may reflect the ability of the cardinal tetra to physiologically acclimate to Cu exposure, whereas at higher concentration, it may simply result from the preceding decline in internal concentrations, and therefore in the decreased gradients for loss, rather than recovery (McDonald and Wood 1993). Although less frequent, effects on K^+ balance and ammonia excretion have also been associated with Cu exposure in freshwater fish, but as for Cl^- loss, they are considered secondary to the main Na^+ effects (Grosell 2011). In the present study, Cu effects on K^+ and ammonia fluxes in cardinal tetras were rare and no clear relationship was observed.

Effects of particles. In Rio Negro water, there was no effect of particles on Cu-induced NaCl imbalance, which was not surprising considering how little Cu^{2+} binds to the suspended solids in this water, in the tested Cu concentration range. A possibly important difference in these unfiltered versus filtered tests was observed during the longest exposure to the highest Cu concentration tested in the Rio Negro (12–24 h at $1000 \mu\text{g L}^{-1}$), where 3 out of 6 fish died in the filtered water and none in the unfiltered water. However, with the exception of K^+ disturbance, this higher mortality was not associated with a statistically larger ion imbalance in the fish that survived. It is thus difficult to attribute a cause to this mortality difference in these flux tests (which were not designed to record lethal endpoints). Overall, we conclude that there is no convincing evidence that particles in the Rio Negro have a significant impact on Cu toxicity to cardinal tetras. In this river, DOM, rather than particles, is the main protective agent against Cu toxicity (Crémazy et al., 2016). This great DOM protection leads to a lower Cu toxicity in the Rio Negro than in the Rio Solimões,

despite the lower cation protection expected from the Rio Negro's lower Ca^{2+} , Mg^{2+} , and Na^+ concentrations (Niyogi and Wood 2004). Indeed, we previously estimated a 96-h LC50 value of $1090 [887–1290] \mu\text{g L}^{-1}$ for *P. axelrodi* exposed to Cu in filtered Rio Negro.

In the Rio Solimões, effects of particles were observed in our flux and toxicity tests, as expected from the much higher TSS levels and consequently larger partitioning of Cu to the solid phase in this water compared to the Rio Negro. The flux tests showed some evidence of particulate protection against Cu toxicity in cardinal tetras. Indeed, Cu-induced Cl^- losses in cardinal tetras were fully alleviated in the presence of Rio Solimões particles (Figure 3A). For Na^+ , however, the effect of particles on $J_{Na,net}$ was not significant (Figure 3B). The uncoupling of particle effects on Na^+ and Cl^- fluxes may appear surprising, as net Cl^- losses generally follow net Na^+ losses through the constraints of electroneutrality and acid-base balance. Yet, this may not always be the case. As explained by Wood (1992), these net fluxes represent the arithmetic sum of active influx (positive) and passive efflux (negative) components, and these may be differentially affected by toxicants, with resultant consequences for the fluxes of acid-base equivalents. In the present study, protection from the decreased Cu bioavailability may have been counter-balanced by direct negative effects of particles on Na^+ loss (whereas negative particle effects on Cl^- loss may occur at higher TSS levels). Indeed, in the absence of Cu, Rio Solimões particles increased Na^+ loss during the 12–24 h flux period (Figure 3A"). Note that because the fish were pre-exposed to the experimental conditions for 12 h prior to the test, this effect actually occurred after a >24-h exposure to particles. When Cu was present though, Na^+ losses were still slightly higher in the unfiltered than in the filtered waters, but the difference was no longer significant.

Short-term sublethal effects of suspended solids have been reported to occur for TSS ranging from 10 to $10\,000 \text{ mg L}^{-1}$, depending mostly on fish sensitivity, but also on the type of suspended solid tested (e.g., size, shape, mineral type, contamination status; Martens and Servizi 1993; Au et al. 2004). In most of these studies, the primary effects of particles were physical impairments at the gill surface, such as lifting of the epithelium, epithelial hyperplasia on lamellae, and fusion of adjacent lamellae (Au et al. 2004; Wong et al. 2013). These gill damage effects have been associated with impacts on fish respiration and, less frequently, on fish ionoregulation (Newcombe and Macdonald 1991; Au et al. 2004).

The BLM, a widely used regulatory and risk assessment tool that generates site-specific water quality criteria based on local water chemistry, employs dissolved Cu concentration as an input parameter (Niyogi and Wood 2004), and therefore does not directly consider the effects of particles. Indeed, the BLM simply assumes that metal bound to particles is not bioavailable because it has been removed from solution, and therefore cannot contribute to toxicity. Underestimation of toxicity by the BLM has been reported in a few studies with high suspended solids (Erickson et al. 1996; Ma et al. 2002; Natale et al. 2007). In addition to direct physiological damages caused by particles, the

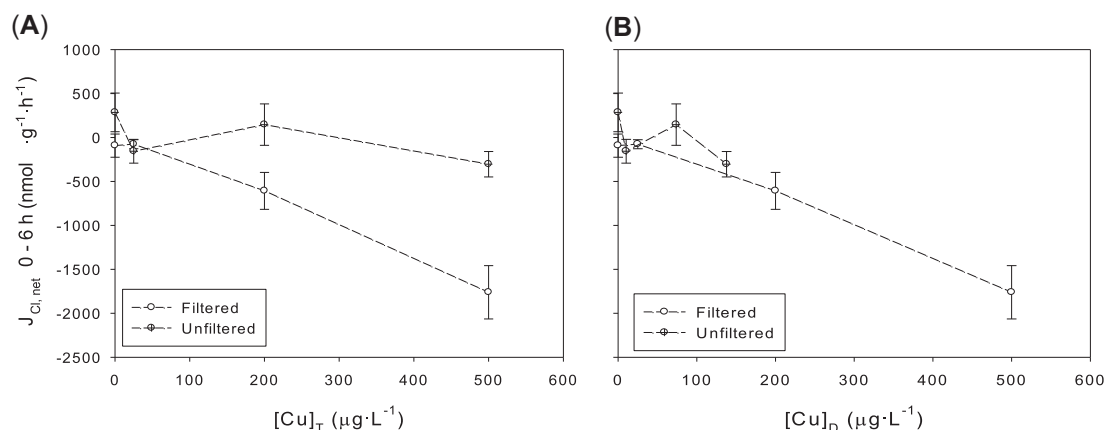


FIGURE 6: The 0–6 h net flux rates of Cl^- in *Paracheirodon axelrodi* as a function of (A) the total and (B) the dissolved Cu concentration in filtered (open circles) and unfiltered (crossed circles) Rio Solimões water. The dashed lines are connecting lines. Mean \pm standard error ($n = 6$).

bioavailability of some particulate metal forms has also been speculated to explain the deviation from BLM predictions (Tao et al. 2000). In the Rio Solimões, concerns that the TSS may introduce an overprotection bias in metal toxicity predictions have been raised (Dal Pont et al. 2017). However, in the present study, the limited particle effects observed on the fish ion balance were not accompanied by a higher fish mortality in the 96-h acute toxicity tests. Indeed, the coincidence of the concentration-response relationships of the filtered and unfiltered Rio Solimões waters, when expressed as a function of the dissolved Cu concentration (Figure 5), shows that the particle protection simply reflects the decrease in the dissolved Cu pool in the Rio Solimões, as expected in the BLM framework. It should be mentioned that a higher replication of the toxicity tests would have probably produced tighter dose-response curves, which may have been able to discern a potentially small particle effect. Nevertheless, this conclusion was supported when doing the same type of analysis on the 0–6 h Cl^- net fluxes (where the clearest Rio Solimões particle effects were observed). Indeed, although the Cu-induced Cl^- losses were larger in filtered than in unfiltered Rio Solimões water for a same $[\text{Cu}]_T$ (Figure 6A), these flux curves coincided when expressed as a function of $[\text{Cu}]_D$ (Figure 6B).

In summary, the present study emphasizes the importance of TSS as a modulating factor of Cu fate and effects in the Rio Solimões. Indeed, within the range of Cu concentration tested in the present study, the majority of Cu was associated with the suspended solids, whereas Cu-DOM complexes were 3 times less abundant at the 96-h LC50. The present study suggests that the large particulate protection against Cu toxicity is adequately characterized by bioavailability-based models, such as the BLM. Nevertheless, this statement needs some qualification, because the present study also showed that particles may affect fish ionic balance. Considering the high temporal variability in TSS loads in the Rio Solimões (3x higher TSS have been observed [Moreira-Turcq et al. 2003]), it is possible that Cu toxicity will sometimes deviate from BLM predictions, due to direct physiological effects of particles. Overall, we recommend that the role of suspended solids in metal toxicity be systematically evaluated, when assessing metal environmental risk in natural rivers with high TSS loads. For example, the

extent to which the acidity of the Rio Negro may promote metal desorption from Rio Solimões particles at the rivers mixing zone is a question of particular interest.

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Data Accessibility—Data and calculation tools are available by contacting the corresponding author (anne.cremazy@unb.ca).

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