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Article

# Tilapia (*Oreochromis niloticus*) as a Biondicator of Copper and Cadmium Toxicity. A Bioavailability Approach

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The acute toxicity of copper and cadmium in *Oreochromis niloticus* was evaluated through a 96 h static assay. Precipitation of Cu and co-precipitation of Cd in the presence of Cu were noted, being indicative of differences in nominal and actual concentrations of metals. Under these conditions, $LC_{50\text{-}96h}$ was determined as 3.53 mg $L^{-1}$ Cu, 20.1 mg $L^{-1}$ Cd and 1.36 mg $L^{-1}$ (Cu + Cd). Besides the quantitative determinations of total dissolved metals in water, considerations on Cd/Cu interactions in aquatic media were presented, allowing the assessment for metals speciation. Data revealed that alkalinity, hardness, dissolved organic carbon and formation of inorganic complexes reduce metal availabilities, mainly in relation to Cu. In spite of this, the $LC_{50}$ for Cd was significantly reduced in the presence of Cu, matching environmental realistic values. Based on simulated fate of metals, hardness may impair a reduction of 18 and 2% in metal activities, respectively to Cu and Cd.

**Keywords**: *Oreochromis niloticus*, metals acute toxicity, bioindicator, chemical speciation, bioavailability

## Introduction

Cadmium and copper have been intensively investigated through fish bioassays in acute and chronic exposures.[1-11]

The contamination of aquatic ecosystems by these metals is a consequence of the rapid population growth, increased urbanization, expanded agricultural activities and exploitation of natural resources, threatening the biota in these ecosystems.[9,12,13]

Cadmium, a non-essential metal, has been investigated in environmental studies due to its toxicity to marine species, even when present in low concentrations. Exposure to this chemical element may disturb the central functions and physiological processes, thus leading to diseases in organisms.[4,14,15] Cadmium exposure may lead to adverse effects on fish growth, reproduction, liver (and other organs) functions and inhibition of calcium uptake by the gills, causing hypocalcemia, which represents the key mechanism of toxicity induced by this metal.[4,16]

Copper is an essential element which plays an important role in cellular metabolism of organisms. When present in higher concentrations, however, it may become toxic.[13] The effects of copper toxicity in fish include histopathological alterations in the liver and gills, growth reduction, oxidative stress damage to hepatic metabolism and inhibition of enzymes activity $Na^+/K^+$-ATPase, resulting in $Na^+$ homeostasis break down.[17,18] In this context, several experiments have been carried out with tilapia, generally involving the species *Oreochromis niloticus* and *Oreochromis mossambicus* used as indicator organisms in field surveys.[7,19]

In view of its easy handling, adaptation to confinement, laboratory maintenance, susceptibility to various pollutants and economic importance, the species *O. niloticus* has been widely used in environmental studies as well as in evaluating the toxicity of contaminants in aquatic ecosystems.[20-22]

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In the present work, the average lethal concentrations estimated ($LC_{50\text{-}96h}$) for $CuCl_2$, $CdCl_2$ and their combinations in *O. niloticus* were determined. Emphasis was given to the acute poisoning signs in fish under stress conditions to these chemicals during experimental exposures. To the best knowledge of the authors, the combined toxicity of mixed solutions of $CuCl_2 + CdCl_2$ to this species was demonstrated for the first time.

As fish under stress conditions produce metallothioneins (Mts) as a response for essential and non-essential metals uptake, another objective of this work was to predict concentrations of the investigated metals in order to assess for the dynamic of Mts formation in *O. niloticus* at sub-lethal levels inducing its formation in a given concentration interval. In addition, several aspects related to the fish bioassay structure and operation were found out. Finally, under the established conditions, the availability of both metals to fish were simulated by using an aquatic speciation model.

## Experimental

All research protocols in this work followed guidelines of the Environmental Protection Agency[23] and Associação Brasileira de Normas Técnicas (ABNT: NBR 15088)[24] for acute toxicity test with fish, handling animals gently and carefully to minimize stress. Regarding disposal, all organisms, including control, were humanely destroyed according to an appropriate manner. All effluents were properly purified before discharge.

Juvenile specimens of *O. niloticus* with an average weight of 10 g were collected at fish farms and transported to the laboratory in plastic bags containing up to 100 individuals. Prior to the experiments, fish were acclimated in 500 L water tanks during a 20 day period and offered 32% protein extruded feed. The water from these tanks was purified through a system of Dry-type Wet filtration, and flow rate was 3.0 L $h^{-1}$.

The water used for acclimation and experimentation came from a local potable network supply, following a minimum 48 h residence time to allow spontaneous dechlorination.

During the acclimation period, physico-chemical variables were daily checked and mortalities were taking into account. The water temperature was maintained as 26 ± 1 °C, by using an electronic 300 W heater, and a photoperiod of 12 h light and 12 h dark was set. These procedures were maintained for the whole experimental period, according to ABNT: NBR 15088.[24]

Experiments with copper chloride, cadmium chloride or combinations of both were carried out during a 96 h period, for determining the acute toxicity of the metals to *O. niloticus*. Assays were performed in static exposure system.

Concentrations for acute toxicity assays were selected in preliminary tests, during 48 h. Values were established in the range of the highest nominal concentration of toxic agent in which no lethality was observed (NOEC) and the lowest nominal concentration of toxic agent which caused 100% lethality of organisms (LOEC).[23]

Metal concentrations related to static acute toxicity tests for copper chloride and copper plus cadmium chlorides were: 0, 0.5, 1.0, 2.5, 5.0 and 10.0 mg $L^{-1}$ and for cadmium chloride 0, 1.0, 5.0, 10.0, 25.0 and 50.0 mg $L^{-1}$. All tests were performed in triplicate, involving the placement of 10 fish inside a 60 L resistant plastic aquarium filled with 40 L solution. In the experimental tanks, fish were subjected to a second 48 h period of acclimation. Fish were not fed 24 h prior to the start of acute toxicity tests.[23,24]

Temperature, pH, dissolved oxygen, ammonium, total dissolved copper and cadmium were monitored at the beginning of the tests and at every 24 h, whereas total hardness was checked only at the beginning and at the end of the assays in the controls.

A mobile multiple analyzer YSI Incorporated 556 MPS (Ohio, USA) was used for temperature, pH and dissolved oxygen quantification. Total metals were determined with a series 6000 inductively coupled plasma optical emission spectrometer (ICP OES) from Thermo Scientific (Waltham, USA). Total hardness was determined by ethylenediaminetetraacetic acid (EDTA) titration,[25] and ammonia ($NH_3$-N) obtained by calculation, taking into account the ammonium ion quantification ($NH_4$-N) determined by flow injection conductometry,[26] as well as pH and temperature of the water samples.[23] The $NH_3$ contents were estimated based on the reversible reactions between $NH_4$ and $NH_3$ assessed by the pH values and aquarium temperatures during water sample collections. Previously to chemical analysis, the water samples were filtered through a 0.45 μm filter. Concentrations of metals were quantified in terms of its most toxic fraction to fish, i.e., soluble metals. Alkalinity was estimated by the WHAM 7.0 model.[27] Through the mathematical model, metals speciation were calculated as well. To this end, three concentrations of metals, in isolated and in combined conditions, with the minimum and maximum values of hardness were selected. Ammonium ion and temperature were measured at the aquariums.

Feces and other waste were daily removed through siphoning. Dead fishes were removed from aquariums every 24 h. At the 96 h, the surviving fish were killed by hypothermia, by transferring them from the aquariums to an ice water bath.

Statistical analysis to assess for the dosage against effect on the biota for the specific metal and its combination involved analysis of variance with the SAS System. For differences among treatments the statistical method Trimmed Spearman-Karber[28] was used, determining the $LC_{50\text{-}96h}$, all calculations were performed by the statistical computer program $LC_{50}$ Programs JSPear Test, Montreal, Canada.

## Results and Discussion

The water quality data (Table 1) were similar among treatments, thus reducing the possibility of mortality due to the water quality alterations; these values were within the recommended ranges.[23]

Levels of ammonia were included in the above statement and in all situations, according to the temperature and pH of the bioassay conditions, the ammonia concentrations were much lower than those that caused mortalities for larvae and fingerlings of *O. niloticus*.[29]

Figures 1, 2 and 3 show the percentage mortalities obtained in each of the acute tests with $CuCl_2$, $CdCl_2.H_2O$ and $CuCl_2$ + $CdCl_2.H_2O$ during the exposure period of 96 h, respectively.

Statistical variance analysis indicates that mortality is explained by metal concentration, as the determination coefficients were high, $R^2$(Cu) = 0.9551, $R^2$(Cd) = 0.9522 and $R^2$(Cu + Cd) = 0.8885. The low $R^2$(Cu + Cd) can be due to the chemical interactions between the two metals in combination, as discussed below, indicating how complex this link can be.

According to Klemm *et al.*,[30] the mortality rates for the control treatments in the three assays were within acceptable limits, as control mortalities did not exceed 20%

**Table 1.** Range of variation for pH, temperature, D.O, total hardness and ammonia determined in the experimental aquariums used to establish the acute toxicity 96 h test with *O. niloticus*

| | | Control | $CuCl_2$ | $CdCl_2.H_2O$ | $CuCl_2 + CdCl_2.H_2O$ |
|---|---|---|---|---|---|
| Temperature / °C | Min | 24.45 ± 0.85 | 25.23 ± 0.05 | 23.57 ± 0.01 | 24.28 ± 0.04 |
| | Max | 25.44 ± 0.30 | 25.87 ± 0.02 | 25.50 ± 0.07 | 25.64 ± 0.07 |
| pH | Min | 6.91 ± 0.29 | 6.74 ± 0.55 | 6.64 ± 0.03 | 6.80 ± 0.00 |
| | Max | 7.42 ± 0.12 | 7.74 ± 0.07 | 7.20 ± 0.09 | 7.60 ± 0.00 |
| D.O / (mg $L^{-1}$) | Min | 7.38 ± 0.39 | 7.58 ± 0.11 | 6.77 ± 0.17 | 7.06 ± 0.92 |
| | Max | 8.19 ± 0.22 | 8.83 ± 0.29 | 8.28 ± 0.45 | 8.56 ± 0.20 |
| Ammonia / (mg $L^{-1}$) | Min | 0.02 ± 0.01 | 0.01 ± 0.00 | 0.01 ± 0.00 | 0.01 ± 0.00 |
| | Max | 0.08 ± 0.03 | 0.10 ± 0.02 | 0.04 ± 0.01 | 0.13 ± 0.03 |
| Total hardness / (mg $L^{-1}$ $CaCO_3$) | Min | 68.54 ± 0.25 | – | – | – |
| | Max | 84.30 ± 0.14 | – | – | – |

Min: minimum values of the parameters; Max: maximum values of the parameters; D.O: dissolved oxygen.

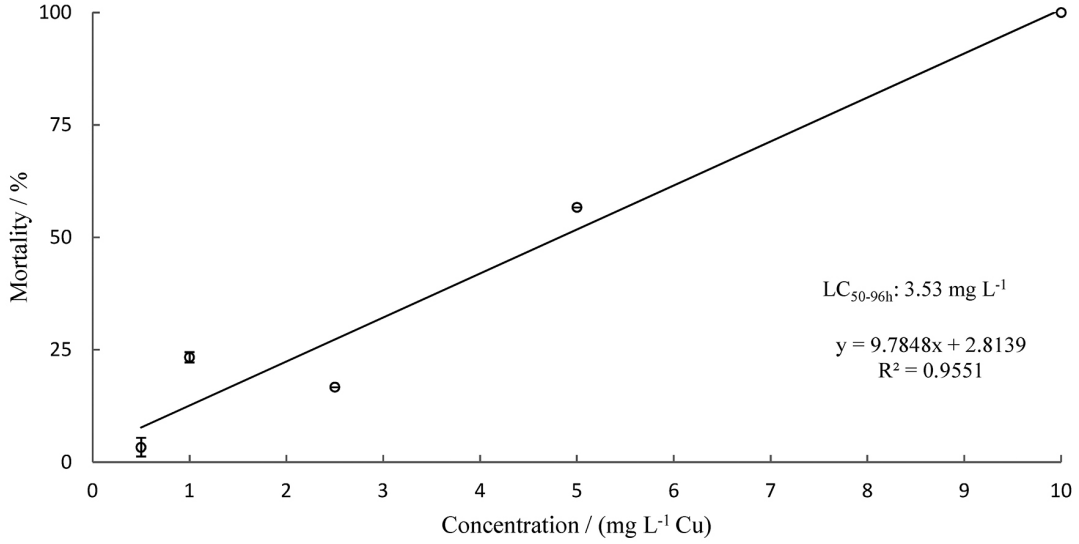


**Figure 1.** Mortality (%) of *Oreochromis niloticus*, as a function of metal concentration in the 96 h acute toxicity test with $CuCl_2$.

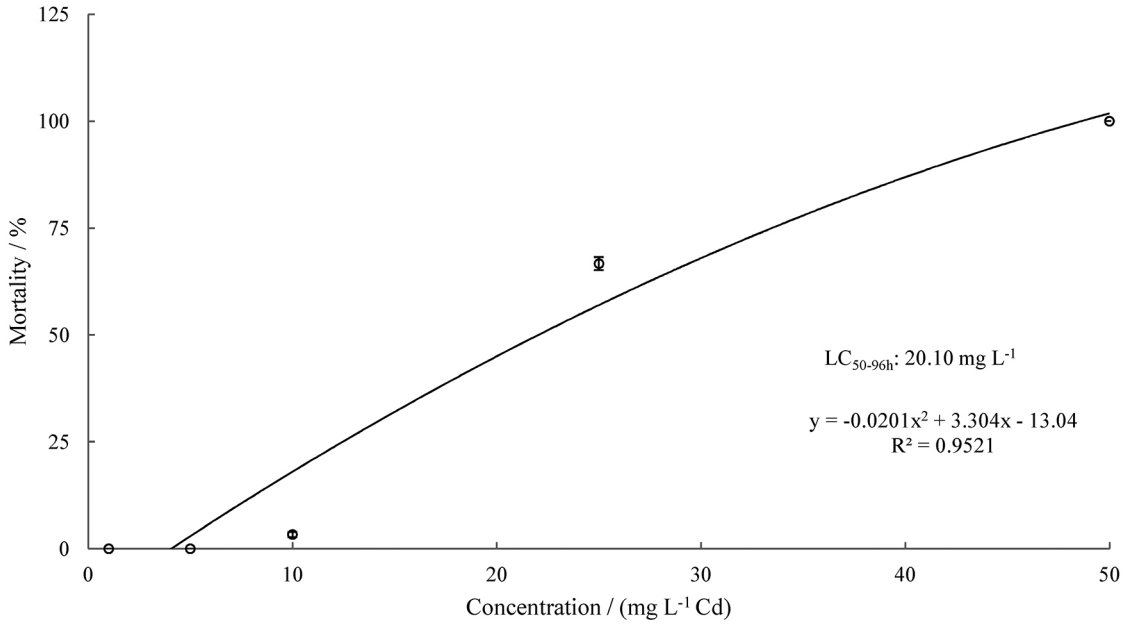


**Figure 2.** Mortality (%) of *Oreochromis niloticus*, as a function of metal concentration in the 96 h acute toxicity test with $CdCl_2.H_2O$.

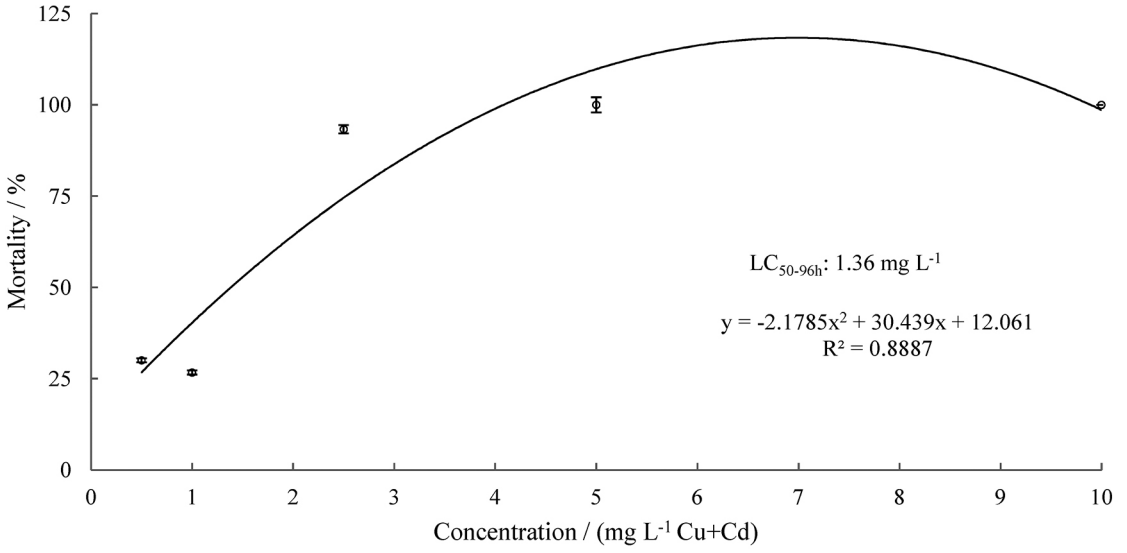


**Figure 3.** Mortality (%) of *Oreochromis niloticus*, as a function of metal concentration in the 96 h acute toxicity test with $CuCl_2$ + $CdCl_2.H_2O$.

for the entire 96 h period. In addition, the % of mortality standard deviations were low in the concentration ranges for all three treatments.

Figure 3 presents evidence of the effect resulting from the combination of $CuCl_2$ + $CdCl_2.H_2O$ solutions, which leads to higher mortality percentages in comparison with exposure to either metal in isolation. This assessment is confirmed by the lethal concentrations ($LC_{50\text{-}96h}$) for the tests of acute toxicity of these metals in different combinations. Lethal concentration ($LC_{50}$) values (95% confidence intervals) were 3.53 mg $L^{-1}$, with upper (UL) and lower (LL) limits of 4.42 to 2.82 mg $L^{-1}$ for copper chloride, 20.1 mg $L^{-1}$ with UL and LL of 23.3 to 17.4 mg $L^{-1}$ for cadmium chloride and 1.36 mg $L^{-1}$ with UL and LL of 1.59 to 1.16 mg $L^{-1}$ for copper chloride + cadmium chloride.

Evaluating the response of *O. niloticus* to $CuSO_4$ and $K_2Cr_2O_7$ in tests with single metals, Masutti *et al.*[31] found that this species has a higher sensitivity to copper than to potassium chromate, with $LC_{50\text{-}96h}$ ranging from 0.32 to 0.65 mg $L^{-1}$. Seddek[32] determined the toxicity of copper as $CuSO_4$ for this species, finding values for $LC_{50\text{-}96h}$ of 7.98 mg $L^{-1}$ Cu, significantly higher than the results of Masutti *et al.*[31] These differences can be linked to the chemical form of the metal since different salts were used and water chemistry. Cu toxicity is enhanced in low alkalinity waters and at low pH.[33,34]

Oransaye and Ogunbor[35] determined $LC_{50\text{-}96h}$ values of 1.0, 0.68 and 0.60 mg $L^{-1}$ $Cu^{2+}$ for fingerlings of *O. niloticus* after 4, 9 and 10 days, respectively, showing the response of toxicity with exposure time.

Results obtained in this study for $CdCl_2.H_2O$ in *O. niloticus* are close to those found by Annune *et al*.,[36] in which the $LC_{50\text{-}96h}$ were 19.3 mg $L^{-1}$ Cd, whereas Garcia-Santos *et al*.[37] found a $LC_{50\text{-}96h}$ of 14.8 mg $L^{-1}$ Cd for the same species. At a first glance, it could be concluded that *O. niloticus* is quite resistant to Cd, and therefore not a suitable bioindicator for assessing the presence and the availability of metal in the environment. Before confirming this statement, it should be important to mention that among water properties, hardness and alkalinity are important parameters in the potential toxicity of Cd. In the experimental conditions, fish acclimation was carried out with the same water as the control treatment, which constitutes a protection to fish when transferred to water in the presence of Cd. The 96 h period is not enough to disturb the protection mechanism of Ca against Cd uptake from the water by the gill and can explain the fish tolerance.[33] Certainly the $LC_{50}$ for Cd would be lower for low alkalinity waters (12.0 mg $L^{-1}$). On the other hand, the resistance of this species could be exploited in the investigation of the fate and interaction processes of this metal in aquatic organisms.[37]

It should be stressed that the above mentioned authors did not mention differences between nominal and measured dissolved metal concentrations (actual). Also, considerations of metal fate in relation to pH were not discussed in details. These assertions are justified, since differences can be expected in these concentrations, due to solubility products of metals, co-precipitation, adsorption on walls, among others.

As demonstrated in Figures 4 and 6, which represent the temporal variations of $Cu^{2+}$ and $Cu^{2+} + Cd^{2+}$, a sharp decline of $Cu^{2+}$ at the concentration 10.0 mg $L^{-1}$ Cu was observed. The reduction was caused by precipitation, due to the low solubility of copper hydroxide (Kps $Cu(OH)_2 = 1.60 \times 10^{-19}$) and pH values during the test, 6.74 to 7.74. According to Çoğun and Kargin,[13] high values of pH contribute to diminish the copper solubility.

Precipitation was not observed during the test with $CdCl_2.H_2O$, which proved to be stable during the experimental period (Figure 5). The solubility product of cadmium hydroxide (Kps $Cd(OH)_2 = 5.30 \times 10^{-15}$) is four orders of magnitude higher than that of cupric hydroxide, keeps metal stabilized in solution, even in higher concentrations and pH values used in the tests. Reductions in Cd concentration for metals in combination were due to the co-precipitation induced by Cu (Figure 6).

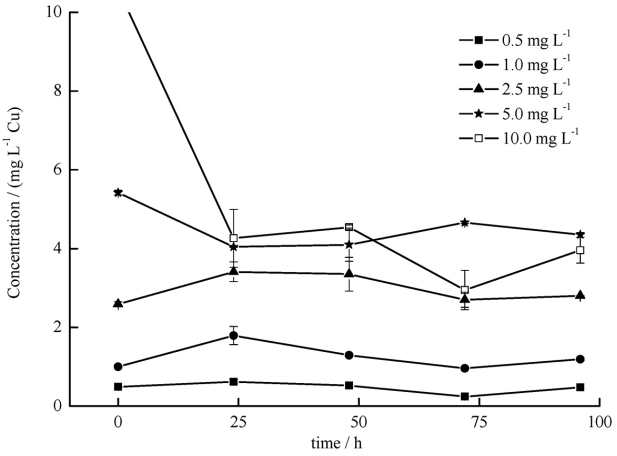


**Figure 4.** Temporal variation of copper concentrations obtained at every 24 h for the acute toxicity test in a 96 h period. Data refer to the metal actual concentrations of 0.5, 1.0, 2.5, 5.0 and 10.0 mg $L^{-1}$ Cu ($CuCl_2$) solutions.

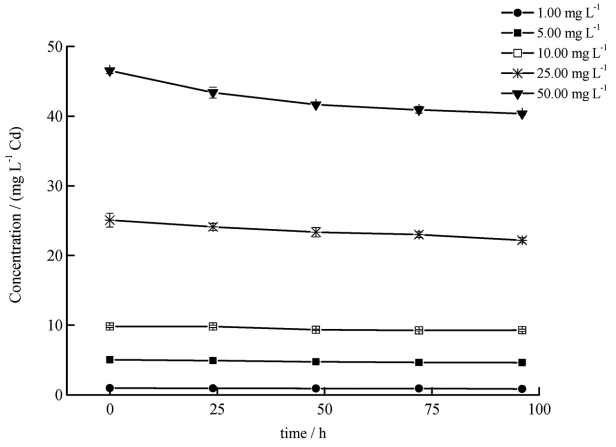


**Figure 5.** Temporal variations of cadmium concentration obtained every 24 h for the acute toxicity test in a 96 h period. Data refer to the metal actual concentrations of 1.0, 5.0, 10.0, 25.0 and 50.0 mg $L^{-1}$ Cd ($CdCl_2.H_2O$) solutions.

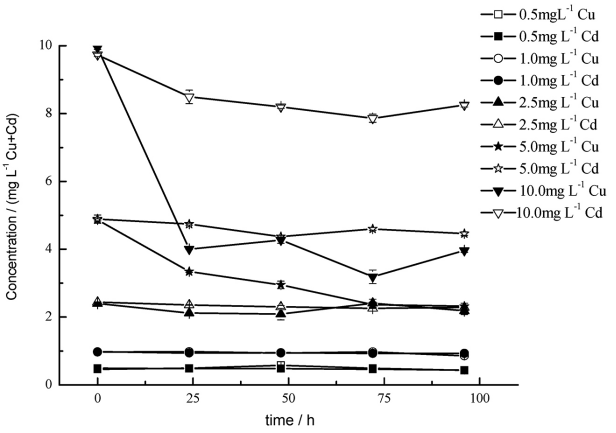


**Figure 6.** Temporal variations of copper and cadmium concentrations obtained every 24 h for the acute toxicity test in a 96 h period. Data refer to the metal actual concentrations of 0.5, 1.0, 2.5, 5.0 and 10.0 mg $L^{-1}$ (Cu + Cd), as $CuCl_2$ and $CdCl_2.H_2O$ salts in mixed solutions. In all situations, the ratio 1:1 for Cu:Cd ratio is maintained as 1:1 (m/m) (for details see the text).

Figures 4 to 6 denote the fate of Cu and Cd in the systems. Cu presented similar behavior at almost all concentrations, either alone or in combination. An exception was noted at 5.0 mg $L^{-1}$ Cu, where the precipitation was slightly higher when the metals were mixed. Cadmium, which was approximately stable up to concentrations as high as 25.0 and 50.0 mg $L^{-1}$, presented distinct behavior at 10.0 mg $L^{-1}$ for both situations. Cadmium concentration decreased when in combination with Cu, as explained earlier. Reductions observed for nominal concentrations of 25.0 and 50.0 mg $L^{-1}$ Cd, are due to either adsorption to walls, or absorption of metal by fish.

Differences in actual and nominal concentrations were significative for 10.0 mg $L^{-1}$ Cu, for both isolated and in combination with Cd (Figures 4 and 6). Highest percentage of mortality occurs at 10.0 mg $L^{-1}$ Cu (Figures 1 and 4) and nearly 60% of deaths were achieved for 5.0 mg $L^{-1}$ Cu (Figure 1). Although the nominal concentration was reduced to the half during the first 24 h period (Figure 4), the 100% mortality observed in this treatment demonstrated that fish intake of Cu during the 24 h period is determining for fish response to the environmental stress, while nominal concentration was being reduced. A similar fate of Cu in solution was verified for the two metals in combination (Figure 3). In this case, due to the additional effect, 100% mortality was verified at 5.0 mg $L^{-1}$ for Cu and Cd. In these situations, the kinetic of precipitation of Cu occurring in the first 24 h period persisted, still influencing fish toxicity.

Differences between expected and obtained concentrations show the importance of monitoring the chemical species of interest in a toxicity assay, considering that the actual concentrations of these chemicals must be used for obtaining the concentration factor, bioaccumulation and aquatic biomagnification data.

Due to all advantages in using this species for fish bioassays,[20-22] these characteristics can, in fact, rank this species as a useful bioindicator in metal interactions to fish, allowing to assess for important mechanisms responses, like the Mts formation induced by metal stress. However, there has been an increasing appreciation of the need for studying sub-lethal effects and some growing interest in the physiological mechanisms by which various pollutants affect fish populations in the wild. In this sense, dosages for experimental work should be based on the $LC_{50}$, which determines the optimum range for experiments, at levels that do not cause any acute toxicity.

Under the above described conditions, fish were affected with a formation of hypersecretion of mucus by the gills and skin at higher concentrations of Cu (5.0 and 10.0 mg $L^{-1}$) and Cd (25.0 and 50.0 mg $L^{-1}$) isolated and in combination (5.0 and 10.0 mg $L^{-1}$). According to Heath,[38] this is a protective mechanism against contamination, decreasing metal absorption through chelation and inhibition of diffusion. When excessive, this process can hamper the gas exchange, which explains why some fish appeared to be seeking oxygen at the air-water interface. This behavior is an additional component of the interference of metals with the respiratory mechanism and fish physiology.[38,39]

Another observed response was the presence of reddish spots in the body, especially in the contour of the lip in organisms exposed to copper isolated and in combination at concentrations of 10.0 mg $L^{-1}$ Cu and 10.0 mg $L^{-1}$ (Cu + Cd). This is due to the rapid loss of electrolytes from the body.[40]

### Chemical speciation - Wham 7.0 mathematical model

The fate of Cu and Cd in the acute toxicity bioassay were assessed by the WHAM model, version 7.0. On Table 2, it is summarized the data obtained from the model output files.

Among the wide possibilities of evaluation, treatments involving 5.0 and 10.0 mg $L^{-1}$ Cu, 25.0 and 50.0 mg $L^{-1}$ Cd and 5.0 and 10.0 mg $L^{-1}$ Cu + Cd for the entire period of 96 h were selected. In addition, the model was also applied for metals $LC_{50}$ concentrations (Table 2). The choices were based on metals equilibrium constants and on the Figures 4, 5 and 6, which allow the calculation of metals actual concentrations. The solubilities of metals with time were stable for lower concentrations. A slight variations on metal concentrations were also a function of re-establishment of solutions up to the initial 40 L volume, required for static bioassays (Figures 4, 5 and 6).

For the input files, the model was feed with the molar salts composition and $NH_4$, excreted by fish. The model was run twice, considering the minimum and maximum hardness values in the assays (Table 1). The dissolved organic carbon was fixed for all situations as $2.0 \times 10^{-3}$ g $L^{-1}$ humic acid in colloidal phase.[34]

It was verified that Cu and Cd interact with hydroxide, carbonate and chloride forming inorganic complexes with very low activities. These complexes were in general less concentrated to Cd than to Cu. Metal hydroxides varied from $10^{-10}$ to $10^{-7}$ mol $L^{-1}$ and $10^{-7}$ to $10^{-5}$ mol $L^{-1}$ for Cd and Cu, respectively. The same figures were followed for both metals in combination. For carbonates these activities were in the range of $10^{-10}$ to $10^{-6}$ mol $L^{-1}$ to Cd and $10^{-8}$ to $10^{-5}$ mol $L^{-1}$ to Cu. When combined, the range of variation was narrower for Cd, varying from $10^{-10}$ to $10^{-7}$ mol $L^{-1}$ and maintained for Cu. With chlorides, the range of molar activities varied from $10^{-9}$ to $10^{-5}$ mol $L^{-1}$ to Cd isolated and $10^{-11}$ to $10^{-6}$ mol $L^{-1}$ in metals combination conditions. Cu molar activity was $10^{-10}$ isolated and $10^{-9}$ in combination.

**Table 2.** Carbonate alkalinity and important data of Cu and Cd speciation chemistry calculated by WHAM 7.0 model in the 96 h static bioassay test, considering the minimum and the maximum hardness concentrations observed in the aquariums and $LC_{50}$ for Cu, Cd and Cu + Cd

| Bioassay treatment | Carbonate alkalinity / (eq $L^{-1}$) | | Fraction bound to colloidal / HA:Cu | | Fraction bound to colloidal / HA:Cd | | Activity $Cu^{2+}$ / (mol $L^{-1}$) | | Activity $Cd^{2+}$ / (mol $L^{-1}$) | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Minimum hardness | Maximum hardness | Minimum hardness | Maximum hardness | Minimum hardness | Maximum hardness | Minimum hardness | Maximum hardness | Minimum hardness | Maximum hardness |
| 0 hour | | | | | | | | | | |
| Cu5 | $2.94\ 10^{-4}$ | $3.77\ 10^{-4}$ | 0.03803 | 0.03585 | – | – | $9.35\ 10^{-6}$ | $7.62\ 10^{-6}$ | – | – |
| Cu10 | $2.07\ 10^{-4}$ | $2.75\ 10^{-4}$ | 0.02058 | 0.01952 | – | – | $2.84\ 10^{-5}$ | $2.27\ 10^{-5}$ | – | – |
| Cd25 | $2.87\ 10^{-4}$ | $3.57\ 10^{-4}$ | – | – | 0.01341 | 0.01277 | – | – | $1.77\ 10^{-4}$ | $1.75\ 10^{-4}$ |
| Cd50 | $2.71\ 10^{-4}$ | $3.37\ 10^{-4}$ | – | – | $8.08\ 10^{-3}$ | $7.79\ 10^{-3}$ | – | – | $3.18\ 10^{-4}$ | $3.16\ 10^{-4}$ |
| Cu5 + Cd5 | $2.72\ 10^{-4}$ | $3.48\ 10^{-4}$ | 0.03605 | 0.03392 | $8.52\ 10^{-3}$ | $8.34\ 10^{-3}$ | $1.03\ 10^{-5}$ | $8.41\ 10^{-6}$ | $3.66\ 10^{-5}$ | $3.61\ 10^{-5}$ |
| Cu10 + Cd10 | $2.14\ 10^{-4}$ | $2.83\ 10^{-4}$ | 0.02025 | 0.01916 | $5.23\ 10^{-3}$ | $5.21\ 10^{-3}$ | $2.62\ 10^{-5}$ | $2.10\ 10^{-5}$ | $7.25\ 10^{-5}$ | $7.17\ 10^{-5}$ |
| 24 hours | | | | | | | | | | |
| Cu5 | $3.45\ 10^{-4}$ | $4.38\ 10^{-4}$ | 0.04903 | 0.04609 | – | – | $4.43\ 10^{-6}$ | $3.66\ 10^{-6}$ | – | – |
| Cu10 | $3.45\ 10^{-4}$ | $4.38\ 10^{-4}$ | 0.04644 | 0.0437 | – | – | $4.49\ 10^{-6}$ | $3.71\ 10^{-6}$ | – | – |
| Cd25 | $3.27\ 10^{-4}$ | $4.07\ 10^{-4}$ | – | – | 0.01435 | 0.01367 | – | – | $1.70\ 10^{-4}$ | $1.68\ 10^{-4}$ |
| Cd50 | $3.51\ 10^{-4}$ | $4.37\ 10^{-4}$ | – | – | $9.26\ 10^{-3}$ | $8.94\ 10^{-3}$ | – | – | $2.94\ 10^{-4}$ | $2.91\ 10^{-4}$ |
| Cu5 + Cd5 | $3.43\ 10^{-4}$ | $4.34\ 10^{-4}$ | 0.05042 | 0.04720 | 0.01227 | 0.01196 | $4.46\ 10^{-6}$ | $3.68\ 10^{-6}$ | $3.59\ 10^{-5}$ | $3.53\ 10^{-5}$ |
| Cu10 + Cd10 | $3.23\ 10^{-4}$ | $4.11\ 10^{-4}$ | 0.04235 | 0.03967 | $9.66\ 10^{-3}$ | $9.49\ 10^{-3}$ | $6.25\ 10^{-6}$ | $5.13\ 10^{-6}$ | $6.26\ 10^{-5}$ | $6.17\ 10^{-5}$ |
| 48 hours | | | | | | | | | | |
| Cu5 | $3.08\ 10^{-4}$ | $3.92\ 10^{-4}$ | 0.04531 | 0.04262 | – | – | $7.15\ 10^{-6}$ | $5.86\ 10^{-6}$ | – | – |
| Cu10 | $3.07\ 10^{-4}$ | $3.90\ 10^{-4}$ | 0.04568 | 0.04293 | – | – | $7.15\ 10^{-6}$ | $5.86\ 10^{-6}$ | – | – |
| Cd25 | $3.35\ 10^{-4}$ | $4.17\ 10^{-4}$ | – | – | 0.01498 | 0.01423 | – | – | $1.65\ 10^{-4}$ | $1.63\ 10^{-4}$ |
| Cd50 | $3.43\ 10^{-4}$ | $4.27\ 10^{-4}$ | – | – | $9.39\ 10^{-3}$ | $9.05\ 10^{-3}$ | – | – | $2.85\ 10^{-4}$ | $2.82\ 10^{-4}$ |
| Cu5 + Cd5 | $3.42\ 10^{-4}$ | $4.32\ 10^{-4}$ | 0.05530 | 0.05167 | 0.01259 | 0.01228 | $4.17\ 10^{-6}$ | $3.44\ 10^{-6}$ | $3.32\ 10^{-5}$ | $3.27\ 10^{-5}$ |
| Cu10 + Cd10 | $2.41\ 10^{-4}$ | $3.06\ 10^{-4}$ | 0.01057 | $9.47\ 10^{-3}$ | $5.19\ 10^{-4}$ | $5.26\ 10^{-4}$ | $9.76\ 10^{-6}$ | $8.15\ 10^{-6}$ | $4.55\ 10^{-3}$ | $4.54\ 10^{-3}$ |
| 72 hours | | | | | | | | | | |
| Cu5 | $3.17\ 10^{-4}$ | $4.05\ 10^{-4}$ | 0.04165 | 0.03927 | – | – | $7.33\ 10^{-6}$ | $5.99\ 10^{-6}$ | – | – |
| Cu10 | $3.27\ 10^{-4}$ | $4.12\ 10^{-4}$ | 0.06692 | 0.06242 | – | – | $4.02\ 10^{-6}$ | $3.32\ 10^{-6}$ | – | – |
| Cd25 | $3.54\ 10^{-4}$ | $4.41\ 10^{-4}$ | – | – | 0.01552 | 0.01477 | – | – | $1.59\ 10^{-4}$ | $1.57\ 10^{-4}$ |
| Cd50 | $3.54\ 10^{-4}$ | $4.40\ 10^{-4}$ | – | – | $9.63\ 10^{-3}$ | $9.29\ 10^{-3}$ | – | – | $2.80\ 10^{-4}$ | $2.77\ 10^{-4}$ |
| Cu5 + Cd5 | $3.54\ 10^{-4}$ | $4.45\ 10^{-4}$ | 0.06285 | 0.05856 | 0.01399 | 0.01358 | $3.1710^{-06}$ | $2.63\ 10^{-6}$ | $3.51\ 10^{-5}$ | $3.46\ 10^{-5}$ |
| Cu10 + Cd10 | $3.39\ 10^{-4}$ | $4.29\ 10^{-4}$ | 0.04994 | 0.04666 | 0.01106 | 0.01081 | $4.5410^{-06}$ | $3.74\ 10^{-6}$ | $5.85\ 10^{-5}$ | $5.76\ 10^{-5}$ |
| 96 hours | | | | | | | | | | |
| Cu5 | $3.10\ 10^{-4}$ | $3.95\ 10^{-4}$ | 0.04208 | 0.03966 | – | – | $7.66\ 10^{-6}$ | $6.26\ 10^{-6}$ | – | – |
| Cu10 | $3.12\ 10^{-4}$ | $3.97\ 10^{-4}$ | 0.04640 | 0.04363 | – | – | $6.78\ 10^{-6}$ | $5.56\ 10^{-6}$ | – | – |
| Cd25 | $3.21\ 10^{-4}$ | $3.99\ 10^{-4}$ | – | – | 0.01510 | 0.01435 | – | – | $1.57\ 10^{-4}$ | $1.55\ 10^{-4}$ |
| Cd50 | $3.13\ 10^{-4}$ | $3.90\ 10^{-4}$ | – | – | $9.37\ 10^{-3}$ | $9.02\ 10^{-3}$ | – | – | $2.76\ 10^{-4}$ | $2.73\ 10^{-4}$ |
| Cu5 + Cd5 | $3.42\ 10^{-4}$ | $4.30\ 10^{-4}$ | 0.06437 | 0.05994 | 0.01349 | 0.01310 | $3.52\ 10^{-6}$ | $2.91\ 10^{-6}$ | $3.28\ 10^{-5}$ | $3.23\ 10^{-5}$ |
| Cu10 + Cd10 | $3.15\ 10^{-4}$ | $4.00\ 10^{-4}$ | 0.04183 | 0.03918 | $9.36\ 10^{-3}$ | $9.20\ 10^{-3}$ | $6.76\ 10^{-6}$ | $5.54\ 10^{-6}$ | $6.18\ 10^{-5}$ | $6.09\ 10^{-5}$ |
| $LC_{50}Cu$ | $3.21\ 10^{-4}$ | $4.07\ 10^{-4}$ | 0.0516 | 0.04844 | – | – | $5.68\ 10^{-6}$ | $4.67\ 10^{-6}$ | – | – |
| $LC_{50}Cd$ | $3.14\ 10^{-4}$ | $3.91\ 10^{-4}$ | – | – | 0.01600 | 0.01518 | – | – | $1.43\ 10^{-4}$ | $1.42\ 10^{-4}$ |
| $LC_{50}Cu + LC_{50}Cd$ | $3.45\ 10^{-4}$ | $4.32\ 10^{-4}$ | 0.10422 | 0.09626 | 0.01907 | 0.01819 | $1.94\ 10^{-6}$ | $1.62\ 10^{-6}$ | $1.01\ 10^{-5}$ | $9.96\ 10^{-6}$ |

H.A = humic acid.

According to the model calculation only one chloride complex was observed with Cu ($CuCl^+$) and two of them were formed with Cd ($CdCl^+$; $CdCl_2$).

During the whole experiment, alkalinity varied in the range of 2.07 to 3.45 $10^{-4}$ eq $L^{-1}$ for the low hardness water and from 2.75 to 4.45 $10^{-4}$ eq $L^{-1}$ to the higher hardness water. Without any exception, an increase in hardness led to an increase in alkalinity. The free ion activity of both metals diminished when both of these variables were increased in the observed experimental pH variation. Metals inorganic complexes compete with DOC (dissolved organic carbon), as an increase in these variables reduced the metal fraction bounded to the colloidal humic acid. The fraction of Cu bounded to the

colloidal humic acid was higher in comparison to Cd (Table 2). The bioavailability and toxicity of Cd was less affected by DOC than the other metals.[41]

This can confirm that toxicity decreases as hardness and alkalinity increase. This could be expected for both metals, although hardness had a minor effect on Cu solubility, but can protect fish in low alkalinity waters by Ca competition with divalent metals.[33] By considering the water properties and metals interactions with the water components, WHAM model revealed that the availability of the free $Cu^{2+}$ was reduced in 18% in the hardness interval of this experiment, whereas for $Cd^{2+}$ only a 2% reduction was verified, being Cu more affected than Cd.[42] For both metals, the free ions concentration reductions were stable from 0 to 96 h (Table 2), indicating good experimental conditions. The intensity of interactions of both metals differs, resulting that the Cd free ion is one order of magnitude higher than that of Cu free ion. It can also be observed that the fraction bounded to the humic acid was reduced as metals concentrations increased. This is probably to the saturation of active sites in the colloidal phase.[43]

If one take into account the above described interactions for the metals at $LC_{50}$ concentrations it can be seen that the free Cu ion ($Cu^{2+}$) was 10.2% for low hardness water and 8.4% for the higher hardness. Cadmium availability of the free ion ($Cd^{2+}$) varied only from 82 to 81.6% under the same water conditions. When combined, the free ion availability would be in the range of 9.2 to 7.7% for Cu and 84.1 to 83% for Cd. In other words, in the hardness range in which these assays were carried out, the metal fractions available on $LC_{50}$ concentrations were only 0.35 to 0.28 mg $L^{-1}$ Cu ($LC_{50}$ = 3.53 mg $L^{-1}$ Cu); 16.48 to 16.40 mg $L^{-1}$ Cd ($LC_{50}$ = 20.10 mg $L^{-1}$ Cd). For the two metals combined, 0.12 to 0.10 mg $L^{-1}$ Cu and 1.14 to 1.13 mg $L^{-1}$ Cd ($LC_{50}$ = 1.36 mg $L^{-1}$ Cu + Cd). These values are more realistic for Cu, but still not for Cd isolated. Although it is stated that Cd toxicity diminished as hardness increased, pH influence is biphasic increasing toxicity below pH 7.0 and diminished again if it goes further down (pH 5.50), the water chemistry do not alter the speciation of Cd as it does for Cu.[42] It should be denoted that under the assay conditions, in combination the toxicity of Cd to tilapia was well reduced.

Although this particular species appears to be highly tolerant to Cu and Cd, with $LC_{50}$ values out of the environment realism, the fate of metals in the medium is a characteristic of the specific site, which can impair and conditioning the free ion activity, which is the one available to the biota. Inasmuch metals do not occur alone in a natural environment, an additive effect like the one mentioned in this paper should be considered.

## Conclusions

The static bioassay system was stable during the entire period of the experiment, observed by the physico-chemical variables data measured at every 24 h. In this sense, fish response was only due to the effects of the studied chemicals.

The fate of metals depends not only on their inherent characteristics, but also on the abiotic medium composition. The low solubility product of Cu hydroxide in the pH experimental range acts upon Cd at 10 mg $L^{-1}$ (Cu + Cd) through a co-precipitation when combined.

When considering both actual metal concentrations and bioavailable metal fractions to the biota, assessed by the mathematical speciation model, the $LC_{50}$ can reach environmental realistic concentrations. This could not be stated in the Cd isolated assay, but only when this metal is in combination with Cu.

The acute toxicity of Cd and Cu to tilapia differs significantly, being higher for Cu, an essential element, than for Cd, which has no recognized biological function. In combination (Cu + Cd), the $LC_{50}$ is decreased nearly two fold compared to that observed for Cu.

To state if tilapia is a good or not so good bio-indicator of Cd and Cu toxicity there is a need to consider not only the nominal metal concentrations, but also the characteristics of the abiotic medium which implies metals bioavailability.

## Supplementary Information

Supplementary Information (water chemistry interactions conditioning metals availability) is available free of charge in PDF file at http://jbcs.sbq.org.br.

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