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Toxicity of Waterborne Copper in Premetamorphic Tadpoles of *Lithobates catesbeianus* (Shaw, 1802)

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Abstract Toxicity parameters of Copper to pre-metamorphic larvae of *Lithobates catesbeianus* were evaluated in laboratory conditions. The acute toxicity (as LC-50 96 h) was 3.96 mg Cu^{2+} L^{-1} (95% confidence interval: 3.21–4.89); the bioconcentration of the metal after 96 h exposure followed an exponential increase. The potential genotoxicity effect evaluated with Micronucleus Test showed a reduced sensitivity of the animals to the assayed concentrations of the metal, exhibiting only a modest increase in the frequency of erythrocytes micronuclei, meanwhile larvae exposed to cyclophosphamide (positive control) showed significant increases. The Condition Factor was significantly reduced while the Hepatosomatic Index remained unaltered.

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Copper (Cu) is a heavy metal commonly found in aquatic ecosystems due to its extensive use in agriculture and water treatments, in industrial activities, in aquaculture considering its algicide properties as well as a therapeutic agent to control ectoparasitic infestations and bacterial diseases. The organisms are exposed in the aquatic ecosystems to different classes of pollutants. Polycyclic aromatic hydrocarbons (PAHs) and heavy metals represent two important classes of aquatic contaminants (Gravato et al. 2006); Cu is known to pose a serious threat to aquatic organism likewise. It is recognized as an essential metal in plants and animals. It is an accumulative environmental pollutant, which exert a wide range of adverse effects on aquatic organisms. The bioaccumulation of Cu is important as a bioindicator of environmental pollution at sublethal concentrations. Its toxicity to biota has been shown to be reduced by induction of the expression of metal binding proteins that may play a role in the tolerance of animals to the metal (USEPA 2007a, b). However, the mechanisms of its toxicity still remain unclear. Cu is known to exert its toxicity partly due to the formation of reactive oxygen species (ROS) (Bopp et al. 2008).

Premetamorphic larvae specimens of the bullfrog *Lithobates catesbeianus* (syn. *Rana catesbeiana*) exposed to different concentrations of Copper were studied under environmentally controlled laboratory conditions. The aims of the present study were to determine toxicity parameters of the metal and to examine its bioconcentration and genotoxicity effects by means of the Micronucleus Test carried out on peripheral red blood cells.

Materials and Methods

Tadpoles were obtained from a commercial dealer of La Plata City, Buenos Aires, Argentina. All animals used in the experiments came from a laboratory stock kept in an aquaria containing tap-water. The stage of the animals was premetamorphic (25–26; Gosner 1960). Wet mean (±SEM) body weight and length of all animals used in this study was 3.2 ± 0.1 g and 6.3 ± 0.1 cm, respectively ($N = 201$); the humidity of the animals oscillated between 86.5% and 90%.

The physicochemical parameters of the water used in the assays was determined on samples taken from the aquaria; most of them (pH, alkalinity, dissolved oxygen, conductivity) were measured according to Standard Methods (APHA–AWWA–WPCF 1998); the measurement of hardness was carried out with a Merck reagents kit and total Cu^{2+} concentration was measured by atomic absorption spectrometry (detection limit 0.1 mg L^{-1}).

Stock animals were fed ad libitum once a day with dough made of pulverized fish food and tasteless gelatin of the following gross mean composition (in g/100 g): lipids, 12.7; proteins, 39.7; carbohydrates, 28.6; humidity, 7.8 and ash, 11.2.

Acute toxicity assays were conducted on tadpoles located in 18 L glass aquaria containing Luján City tap-water, at a rate of 2.1 g of organisms/L. Animals were acclimated during 5–7 days prior to Copper addition; during this period they were fed ad libitum. Tests were run under a 12:12 h light–dark photoperiod regime, at constant temperature (21 ± 2°C) and with permanent aeration. Nominal Copper exposure concentrations were 0 (controls in tap-water), 0.75, 1.50, 2, 3, 4, 6, 8, 10 or 12 mg Cu^{2+} L^{-1}; solutions were prepared fresh daily from a stock solution of 5 g Cu^{2+} L^{-1} [as $CuSO_4 \cdot 5H_2O$ (analytical grade) Mallinckrodt] in distilled water. The assays were static, with daily renewal of the media; animals were not fed during the assays. A total of 126 tadpoles were used.

Following Cu additions, the number of dead animals was determined every day for 96 h; dead tadpoles were removed from the containers. Median lethal concentrations (LC-50) at 24, 48, 72 and 96 h were calculated by the Trimmed Spearman–Karber method with 95% confidence limits (USEPA Program version 1.5) and expressed as mg Cu^{2+} L^{-1}.

The study of bioconcentration of the metal was conducted with tadpoles exposed to the following series of solutions: 0, 2, 4, 6, 8, and 10 mg Cu^{2+} L^{-1} for 96 h. Assays were carried out under the same conditions described for acute toxicity trials. At the end of the assays, larvae were weighed (ww, wet weight), measured and washed with distilled water. Then they were individually dried at 60°C until constant weight (dw, dry weight). Aliquots of approximately 20 mg from each dry sample were digested for 12–20 h with 1 ml of HNO_3 65% v/v at 55°C until total digestion. The obtained solutions were diluted to 5 ml with ultrapure water and kept in cold (4–8°C) for subsequent analysis for the determination of Copper concentration. The results were expressed as μg Cu^{2+} mg dw^{-1} (means ± SEM).

Total Cu^{2+} concentration in testing media samples and the metal content in tissues were determined by flame atomic absorption spectrometry using a Jarrell Ash unit; the calibration was with a curve of an appropriate scale of standards, including a blank. Actual Copper levels in the assay media were regularly monitored and were at the range of 75 ± 15% of nominal values. The concentration in the water of control tadpoles was below the analytical detection limit.

The Micronucleus Test (MN) was performed under the same conditions described for acute toxicity assays. A total of 75 tadpoles were used. Copper concentrations were 0 (controls; in tap-water), 2 and 3 mg Cu^{2+} L^{-1}. A positive control series was run with cyclophosphamide monohydrate (Sigma–Aldrich, CAS No. 6055-19-2) 40 mg L^{-1} solutions. After the exposure (96 h) animals of each group were narcotized by immersion in ice-cold water, their length and weight were determined for Condition Factor (CF) calculation [(body weight × 100)/body $length^3$] and blood samples collected by cardiac puncture into heparinized syringes. Two blood smears for each larvae were prepared on clean sliders and fixed with ethanol 96% for 10 min, air dried and stained with Acridine Orange (Sigma–Aldrich, CAS No. 158550) following the method described by Schmid (1975) and Ueda et al. (1992). For micronuclei analysis, erythrocytes were examined using a Zeiss HBO50 epifluorescence microscope at a 400× magnification. For each tadpole 1,000 erythrocytes from each slide were examined; results were expressed as number of cells with micronuclei per 2,000 cells. The MN identification followed the criteria of Grisolia (2002).

After blood removal, the whole liver was excised and weighed for Hepatosomatic Index determination (HSI: liver weight/body weight × 100).

The statistical significativity of the bioaccumulation study results was analyzed by the Kruskal–Wallis test. The results of the MN number, the CF and HIS values (shown in Table 2) were carried out by ANOVA. Normality was confirmed by modified Shapiro Wilks test and homoscedasticity by Bartlett's test; for the cases that did not fulfill the required conditions, Kruskal–Wallis test was also used. All statistical analyses were conducted with the Infostat Program (2004 version) and GraphPad InStat (3.01) at the 95% level ($p < 0.05$).

Results and Discussion

The correlation between weight and length of all the tadpoles used in this study is shown in Fig. 1 which indicated a best fit, $y = 0.1902\ e^{0.4249x}$; $R^2 = 0.6773$ ($N = 201$).

It is well known that the toxicity of Copper is strongly dependent on several biotic factors (age, stage, size and condition of the organisms) as well as on water quality of the media. In our experimental design those factors were kept constant along the assays thus the registered changes cannot be attributed to them.

The physicochemical parameters of the water used in the incubation media were (means ± SEM; in parenthesis, number of measurements): pH, 8.51 ± 0.02 (37); hardness, 94.37 ± 4.45 mg $CaCO_3$ L^{-1} (40); alkalinity, 345 ± 18 mg $CaCO_3$ L^{-1} (27); dissolved oxygen, 6.37 ± 0.18 mg L^{-1} (43); conductivity, 939 ± 23 µS cm^{-1} (32); Cu^{2+}, < 0.1 mg L^{-1} (77). No changes were observed for these values overtime.

The 48, 72 and 96 h LC-50 s are shown in Table 1.

Ferreira et al. (2003, 2004) determined for the same species at stages 31–36, a value slightly lower than ours pointing out a higher sensitivity of *L. catesbeianus* to Copper at earlier developmental stages. They conducted their bioassays using a commercial fungicide formulation of copper oxychloride.

The acute toxicity of Copper has been determined in a number of anuran at larval stages. The comparison of our results with those from other laboratories show important discrepancies; with similar hardness of our assay media, the 96 h LC-50 range were as diverse as 324 (*Bufo melanostictus*), 1,700 (*Xenopus laevis*) and 5,380 µg L^{-1} (*Microhyla ornata*) (Devillers and Exbrayat 1992; Schuytema and Nebeker 1996).

The bioconcentration of Copper after 96 h exposure is presented in Fig. 2. The response was biphasic; the animals exposed to 2 mg Cu^{2+} L^{-1} did not show accumulation of the toxic; however, between 4 and 10 mg Cu^{2+} L^{-1} a kinetic of exponential accumulation was observed; the equation that adjusted all the data was $y = 0.1027x^2 - 0.3413x + 0.3139$; $R^2 = 0.9271$.

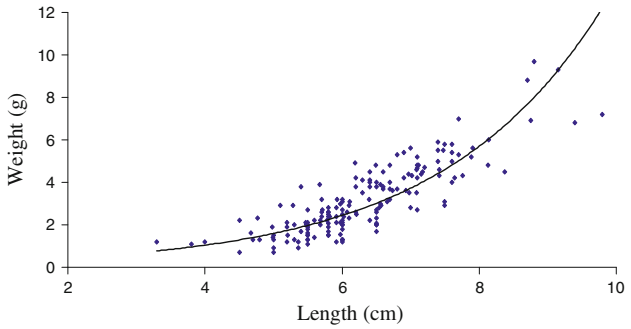

Fig. 1 Correlation between body weight and length of *Lithobates catesbeianus* pre-metamorphic larvae used in the assays

Table 1 Mean lethal concentration (LC-50) of Copper (as mg Cu^{2+} L^{-1}) for premetamorphic larvae of *Lithobates catesbeianus* determined after different exposure periods

Exposure time (hour)	LC-50	95% Confidence limits
48	9.49	8.20–10.98
72	6.89	5.46–8.70
96	3.96	3.21–4.89

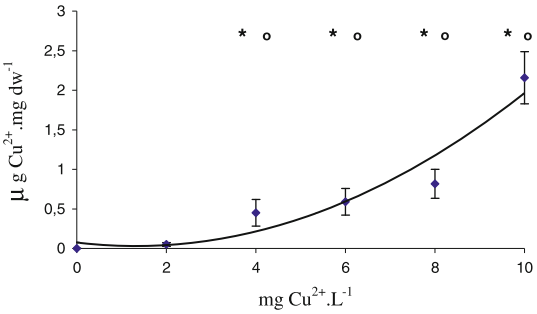

Fig. 2 Intralarval Cu (µg Cu^{2+}·mg dw^{-1}) content (means ± SEM) in *Lithobates catesbeianus* pre-metamorphic larvae after 96 h exposure in media with different concentrations of the metal. *Asterisk* indicates significant differences compared to the control and *Circle* compared to the larvae to 2 mg Cd^{2+} L^{-1} ($p < 0.0001$)

MN frequency and morphometric parameters are presented in Table 2. In the last 10 years, different authors referred that Micronucleus assays prove to be suitable tools to detect DNA damage induced by Copper in aquatic organisms (Sanchez-Galan et al. 1999; Cavas et al. 2005). Peripheral blood erythrocytes in control animals exhibited a certain number of spontaneous MN. This fact was also observed in larvae of *Hyla pulchella* (Lajmanovich et al. 2005) and *Rana catesbeiana* (Campana et al. 2003). The cells of the larvae after 96 h exposure to the metal showed an increase in the frequency of MN with significant differences relative to controls with 2 mg Cu^{2+} L^{-1}. A single MN was predominant in the analyzed cells. Increases accounted for approximately 50%. Two–three micronuclei were found in some samples of animals exposed to cyclophosphamide. Other authors have stated that the amount of MN in the blood of amphibian larvae exposed to heavy metals considerably increase after a more prolonged exposure period than ours. In this respect, it is interesting to consider the results reported in pre-metamorphic stages of *Rana catesbeiana* by Krauter (1993). He found that the maximum number of MN occurred at two separate times, suggesting the presence of two erythrocyte population (coming from two hemopoietic sources, liver and kidneys).

It is interesting to mention that Wirz et al. (2005) used older stages caged tadpoles of *Rana catesbeiana* as

Table 2 Number of MN registered in erythrocytes, Condition Factor (CF) and Hepatosomatic Index (HSI) of premetamorphic larvae of *Lithobates catesbeianus* after 96 h exposure to solutions of 2 and 3 mg Cu^{2+} L^{-1} and to cyclophosphamide (CP) 40 mg L^{-1}

Condition	MN/2,000 cells	CF	HSI
Control (18)	5.06 ± 0.79°	1.26 ± 0.11	4.43 ± 0.42
2 mg Cu^{2+} L^{-1} (22)	7.68 ± 0.67*,$^{\circ}$	0.87 ± 0.09*,$^{\circ}$	4.69 ± 0.36
3 mg Cu^{2+} L^{-1} (16)	6.06 ± 0.79°	0.86 ± 0.11*,$^{\circ}$	4.68 ± 0.62
CP (10)	10.50 ± 0.65	1.25 ± 0.16	3.34 ± 0.21

Data are means ± SEM. Number of animals between parentheses

* Significant differences ($p < 0.05$) compared with the control

$^{\circ}$ Significant differences ($p < 0.05$) compared with CP

pollution bioindicator organism, showing they were useful for assess the effects of genotoxic agents in the water of a Brazilian river and thus in determining the quality of a natural water body.

The results showed that in our test-system, the used stages exhibited a mild genotoxic impact of Copper as evaluated on the blood cells. On the contrary by the response of animals to cyclophosphamide, were significantly different from controls in tap water, showing a 100% increase in number of MN.

Finally, the Condition Factor of the Cd-exposed groups of tadpoles significantly decreased by 30% compared to the controls. These changes revealed that the acute exposure to the metal produced an important stress in the animals. Conversely, CF in animals exposed to cyclophosphamide did not show differences; this behavior can be interpreted as an evidence of differences in the toxicity mechanisms between both toxic elements. HSI of exposed larvae remained unaltered suggesting that the hepatic detoxification mechanisms are still not fully functional.

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