Bull. Environ. Contam. Toxicol. (2004) 73:465–470

DOI: 10.1007/s00128-004-0452-7

Bulletin of Environmental Contamination and Toxicology

Effects of Copper Oxychloride in *Rana catesbeiana* Tadpoles: Toxicological and Bioaccumulative Aspects

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Received: 27 May 2004/Accepted: 13 July 2004

A variety of bioassays have been carried out in amphibians using these animals as a model in order to evaluate the consequence of aquatic pollution in different environments (Greenhouse, 1976; Schuytema et al., 1991; Schuytema and Nebeker, 1999; Djomo et al., 2000; Bueno-Guimarães, et al. 2001; Lombardi, et al. 2002). During the life history of amphibians, they interact with water, soil and air, from which they acquire all their requirements to survive and perpetuate. Due to human interference, the biosphere sometimes does not provide adequate conditions for a safe habitat. Since these organisms are very sensitive to environmental chemical contaminants, they could serve as sentinel animals in environmental biomonitoring programs to measure the degree of contamination in an ecosystem (Schuytema et al., 1991; Bueno-Guimarães et al., 2001).

Aquatic organisms are continually subjected to long-term exposure to low concentrations of pollutants rather than high ones which would cause rapid mortality. Therefore, it is important to study the effects of pollutants on such organisms, in order to determine their adaptive responses to environmental contaminants (Abel, 1998). Embryonic, larval and adult anurans have been shown to be sensitive to a number of environmental pollutants, such as hydrazine and copper (Greenhouse 1976; Miller and Mackay, 1983; Grillitsch and Linder, 2000). In aquatic ecosystems, the absorption of toxic chemicals promotes increased concentrations through the food web (biomagnification). Heavy metals are easily bioaccumulated in the adipose and muscle tissues of aquatic organisms, and they may be unsafe for human consumption (Ferreiro, 1980; Rand and Petrocelli, 1985; Abel, 1998).

According to NAS (1977), copper has numerous and varied biological effects in animals as an essential element as well as a toxicant. In addition copper residues are produced by many industrial and agricultural activities and can cause a serious hazard to aquatic ecosystems (Damato et al., 1989; Cooke et al., 1993; Grillitsch and Linder, 2000).

The purpose of the present study was to determine the acute toxicity of copper to late-stage tadpoles of *Rana catesbeiana* and to examine bioaccumulation

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aspects in chronic exposures.

MATERIALS AND METHODS

Tadpoles of the bullfrog *Rana catesbeiana* (measures of mean 6.13 g and 8.97 cm), stage 31 to 36 (Gosner, 1960), were acquired from the Fishery Institute of São Paulo State brood stocks. The animals were held under laboratory conditions (22 ± 1°C, 10:14 LD photoperiod) for 48 hours in a common aquarium containing 60 liters prior to experimentation. Reconstituted standard water was used for both tadpole acclimatization and experimental tests. It was prepared according to standard methods (APHA et al., 1998), which state the composition of soft waters by adding the required salts ($NaHCO_3$, $CaSO_4.2H_2O$, $MgSO_4$ and KCl) to deionized water. The animals were fed commercial frog dry powder (Nutremix-Brazil).

Dissolved oxygen (mg L^{-1}), temperature (°C), pH and electric conductivity (μS cm^{-1}) were monitored daily. The product used in this study was the fungicide copper oxychloride (Cuprogarb 500™-wettable powder with 99% purity) from Oxiquímica Ind. e Com. Ltda, Brazil. This commercial formulation of copper was used in the present experiment due to its wide employment in the agriculture as a fungicide, which provide a significant source of copper pollution to the aquatic environment. A stock solution was prepared by dissolving a measure quantity of the active ingredient (500 g of metallic copper Kg^{-1}) in distilled water.

In order to determine acute toxicity (in terms of median lethal concentrations - LC_{50}), a series of three nominal concentrations (2.0, 4.0 and 8.0mg L^{-1}) was prepared by diluting proportional amounts (considering Cu^{++} equivalents) of the stock solution in water added to each 12-L test glass aquarium. These concentrations were based on the results reported by Lombardi et al. (2002). One container was kept as an unexposed control group. Each aquarium was internally coated with a plastic bag and covered with a plastic film to prevent the adsorption and volatilization of copper, respectively. Air pumps and individual stone air diffusers provided aeration.

The bioassay was carried out as a static toxicity test with continuous exposure for 96 hours. A total of 144 tadpoles were randomly distributed among the 12 aquaria containing different copper concentrations in triplicate. Mortality was recorded at 24, 48, 72 and 96 hours of exposure. The LC_{50s} were estimated by the Trimmed Spearman-Karber statistical method (Hamilton et al., 1977).

Once the LC_{50} had been determined, a chronic experiment was carried out through the maintenance of 320 tadpoles in a 312 hours exposure renewal system, with an initial density of 2 animals/L in each test aquarium (n=16) following the same protocols of the previous test. The bioassay was conducted using four simultaneous replicates for copper concentrations (0; 2.4 mg L^{-1}= $LC_{50\text{-}96h}$; 1.2 mg L^{-1} = $LC_{50}/2$, and 0.2mg L^{-1} = $LC_{50}/10$) according to Rudek and Rozek

(1992) suggestions. The solutions were changed at each 96 hours during the experimentation (re-intoxication). Dead animals were removed and the mortality was recorded every day.

Samples of tadpoles were taken during the chronic experiment and pooled according to the level of copper exposure for the measurement of copper bioaccumulation. The animals were dried, and digested in a 2.5 mL mixture of (2:1) nitric acid (64%) and perchloric acid (96%), according to an adapted method reported by Sarruge and Haag (1974). All samples were analyzed in a GBC 932 Atomic Absorption Spectrophotometer. The standard was Merck aqueous copper metal (99.99%). The lower limit of detection was 0.01 μg/g dry weight. The natural concentration of copper (estimated from the control group) was subtracted from the test groups for calculating only accumulated copper levels.

RESULTS AND DISCUSSION

The physical and chemical parameters analyzed during the bioassay showed no differences among the aquaria within the range of copper concentrations examined nor relative to control tanks. The mean values for these parameters were: temperature (22.7 ± 0.9 °C), pH (7.04 ± 0.6), electric conductivity (109.9 ± 2.9 μS cm^{-1}), dissolved oxygen (7.1 ± 0.2 mg L^{-1}) and total ammonia (8.3 ± 1.2 mg L^{-1}) which correspond to 0.03 μg L^{-1} of un-ionized ammonia (NH_3-N). The initial hardness and alkalinity of each aquarium were 14.91 mg $CaCO_3$ L^{-1} and 23.76 mg $CaCO_3$ L^{-1}, respectively. The values for these parameters were in accordance with the levels reported by Culley (1991) as being acceptable for the maintenance of tadpoles of *R. catesbeiana* in culture systems, and also with those described by Schuytema and Nebeker (1999) in bioassays conducted with *Pseudacris regilla* and *Xenopus laevis* embryos.

The median lethal concentrations (LC_{50s}) for copper for the different exposure times were found to be 4.3 mg L^{-1} (48h), 2.8 mg L^{-1} (72h) and 2.4 mg L^{-1} (96h). Lombardi et al. (2002) carried out copper bioassays with *R. catesbeiana* tadpoles at an earlier developmental stage and observed an $LC_{50\text{-}96h}$ of 2.8 mg L^{-1}, which is quite similar to that determined in the present study. In accordance with their life stage, tadpoles of *R. catesbeiana* are relatively tolerant to copper, mainly when compared with another amphibians: *Bufo areanarum* embryos ($LC_{100\text{-}96h}$ = 0.24 mg L^{-1}) (Herkovits et al., 2000) or to other aquatic organisms, which show lower LC_{50} values for copper, such as the freshwater prawn *Macrobrachium rosenbergii* (0.05 mg L^{-1}) and the fish species *Salmo gairdneri* (0.14 mg L^{-1}), *Pimephales promelas* (0.84 mg L^{-1}), *Lepomis macrochirus* (0.89 mg L^{-1}) and *Carassius auratus* (1.38 mg L^{-1}) (Johnson and Finley, 1980 and Lombardi et al., 2000). We opted to use pre-metamorphic stage, because the organisms in such life stage are intensively submitted to the employment of copper formulation, directly in the culturing water, as a treatment commonly used to prevent fungus diseases.

Mortality was very high at copper concentrations of 1.2 mg L^{-1} and 2.4 mg L^{-1}, especially after 120 hours of chronic exposure (Figure 1). This could have been

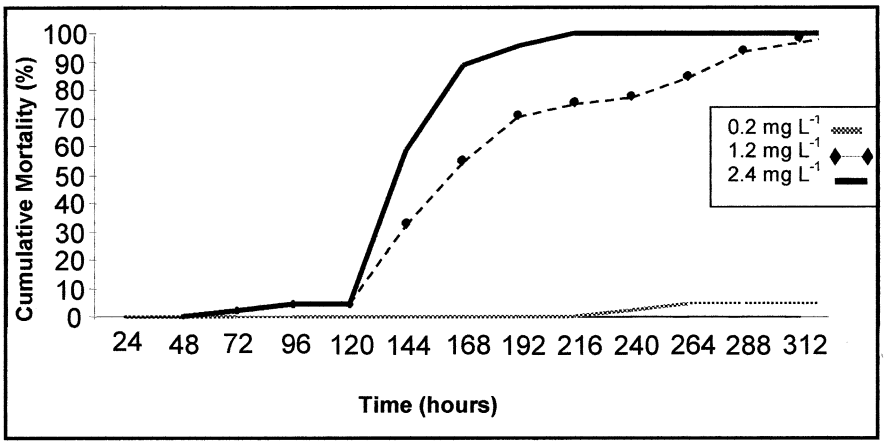

Figure 1. Cumulative mortality observed during the chronic copper toxicity test in tadpoles of *R. catesbeiana.*

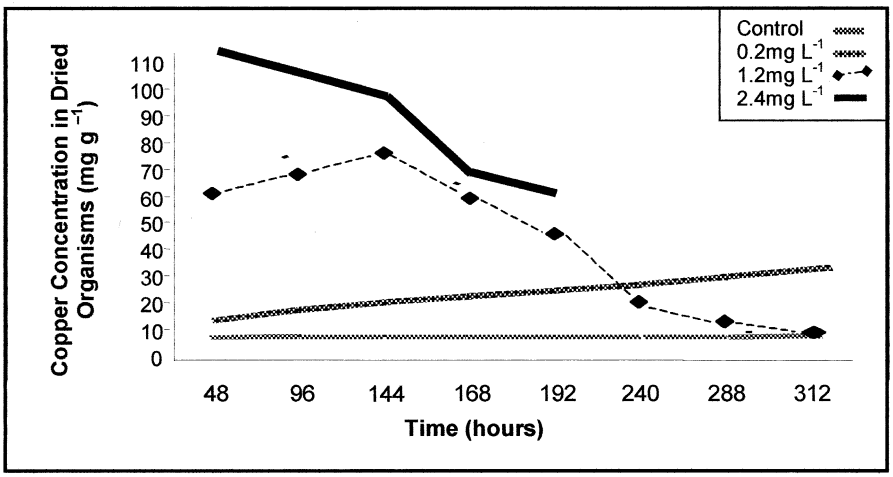

Figure 2. Chronic test for copper bioaccumulation in tadpoles of *R. catesbeiana.*

attributed to re-intoxication which may have occurred when the solutions were replaced every 96 hours. There was no mortality in the control group.

The determination of copper bioaccumulation in the body of tadpoles showed that copper absorption was dose-dependent, at least for the first 144 hours of exposure (Figure 2). In addition, copper biaccumulation seemed to be inversely proportional to exposure time, but only at the higher concentrations of copper (1.2 mg L^{-1} and 2.4 mg L^{-1}). Meanwhile, large quantities of excrement were observed on the bottom of the aquaria, which was related to exposure to higher concentrations of copper. It may explain the phenomenon described above, that

is, the inversely proportional relationship, since digestive metabolism can be considered an efficient mechanism for metal detoxification in aquatic organisms (Schuytema et al., 1991).

Additional information about copper absorption and the bioconcentration process in tadpoles should be investigated further since this metal is widely used as a disinfectant, fungicide and/or on the treatment of some frog farms.

Acknowledgments. We grateful to SAS Evangelista, LCS Evangelista and MMP Acêncio for help and assistance during experimentation.

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