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Acute Toxicity of the Fungicide Copper Oxychloride to Tadpoles of the Bullfrog *Rana catesbeiana*

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Copper compound pesticides are used in agriculture worldwide due to their fungicide and algaecide properties. Studies on the toxic effect of copper on aquatic biota have mainly focused on fish and macroinvertebrates (Johnson and Finley 1980, Reardon and Harrel 1990, Nussey et al. 1996 and Lombardi et al. 2000), and toxicity to amphibians has been investigated in some recent studies (Grillitsch and Linder 2000).

Amphibians are very useful in environmental assessment programs due to their natural characteristics of living in both aquatic and terrestrial habitats (Greenhouse 1976 and Beiswenger 1988). Therefore, it is important to determine their sensitivities to a variety of toxic substances to provide technical information for their use in the bio-management of pollutants in environmental programs. Moreover, frog aquaculture has increased over the last few years, especially with the cultivation of the bullfrog *Rana catesbeiana*, and the use of pesticides in agriculture may pose a risk to such commercial activity, since good water quality is required for spawning and growing frogs.

The aim of this study was to determine the acute toxicity of copper to tadpoles of the bullfrog *R. catesbeiana* using a bioassay carried out with the fungicide copper oxychloride.

MATERIAL AND METHODS

Tadpoles of *Rana catesbeiana* (1.55 ± 0.047 g) were purchased from a commercial frog culture located in Natal - Brazil. They were transported to a bioassay laboratory and acclimatized for 48 hr in a 100-L glass aquarium. Commercial dry pellets with 40% protein content were supplied during this period as tadpole feed.

Reconstituted standard water was used for acclimatizing tadpoles and conducting tests. It was prepared according to standard published methods (APHA et al. 1998), which describe the preparation of soft

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water by adding required salts ($NaHCO_3$, $CaSO_4.2H_2O$, $MgSO_4$ and KCl) to deionized fresh water. The air temperature inside the laboratory was maintained at 23°C, and the photoperiod was a 12-h L:D cycle.

The fungicide used in this study was copper oxychloride (Cuprogarb 500™ - wettable powder with 99% purity), purchased from Oxiquímica Ind. e Com. Ltda. Stock solutions were prepared by dissolving a measured quantity of active ingredient (500 g of copper/kg) in distilled water. A series of five nominal concentrations of Cu^{++} (toxic range determined by preliminary tests: 2.0, 4.0, 8.0, 16.0 and 32.0 mg L^{-1}) was prepared by adding a measured volume from the stock solution to test containers. One container was kept as the unexposed control group. Test containers were set up by placing 1 L of test solution in 2-L glass beakers. Each beaker was covered with a plastic film to prevent evaporation. Air pumps and individual stone air diffusers provided for aeration. Ten tadpoles were randomly distributed into each test container. No food was supplied during the experiment.

The bioassay was carried out with three simultaneous replicates according to the static method, i.e. with no replacement of solution during the 96 hr of exposure. Dissolved oxygen (mg L^{-1}), temperature (°C), pH and electric conductivity ($\mu S\ cm^{-1}$) were recorded individually in each test container at exposure times of 24, 48, 72 and 96 hr, and the total ammonia (mg L^{-1}) was determined by standard methods (APHA et al. 1998) only at the end of the experiment.

Mortalities were recorded at 24, 48, 72 and 96 hr of exposure and the dead organisms were removed regularly from the test solutions. The criterion for death was established as the total lack of movement, determined when tadpoles fail to respond to gentle touching with a glass rod. The median lethal concentrations (LC_{50}) were estimated by use of the Gwbasic 3.10 software, according to the statistical method "Trimmed Spearman Karber" (Hamilton et al. 1977). The safe concentration level of copper was estimated by the quotient of $LC_{50\text{-}96hr}$/ 100, according to the same methods used in earlier studies for establishing safe concentrations for crustaceans (Natarajan et al. 1992; Lombardi et al. 2000).

At the end of the test, all tadpoles were grouped by concentration and reserved for analysis of copper bioaccumulation. The whole body of the tadpoles was used in the measurements of copper bioaccumulation, since their small size did not allow for individual analysis of the different parts of the body. Samples were dried and digested in a 2.5-mL mixture of (2:1) nitric acid (64%) and perchloric acid (96%), according to the adapted method reported by Sarruge and Haag (1974). All samples were analyzed on a GBC 932 AAS. The standard was Merck aqueous

copper metal (99.99%), and 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 the determination of only the accumulated copper contents.

RESULTS AND DISCUSSION

The physical and chemical parameters analyzed during the bioassays showed no differences among the range of 5 concentrations, nor between concentration ranges and control groups. Therefore, it was possible to calculate the respective averages for these parameters: temperature (23.71 ± 0.35 °C), pH (7.28 ± 0.08), electric conductivity (0.15 ± 0.05 mS cm^{-1}), and dissolved oxygen (6.70 ± 0.42 mg L^{-1}, with 94.83 ±1.34% of saturation). All of these parameters were in accordance to the acceptable levels reported by Culley (1991) for the maintenance of tadpoles of *R. Catesbeiana* under culture conditions. The total ammonia was 6.23 ± 1.77 mg L^{-1}, which corresponds to 0.11 mg L^{-1} of un-ionized ammonia (NH_3-N). It may be considered high according to Schuytema and Nebecker (1999) that found out safe levels of 0.03 to 0.18 mg L^{-1} of NH_3-N to some species of amphibians. In addition, APHA et al. (1998) reccomend the maintenance of NH_3-N under 0.02 mg L^{-1} in bioassays.

The reason for this high concentration of ammonia in the present bioassay may be attributed to the static method used for carrying out the test, which allowed the increase of ammonia during the 96 hr of exposure time, since there was no replacement of the test solution during this period. Nevertheless, it does not seem to have interfered in the evaluation of copper toxicity, since the control group did not show any mortality, even when submitted to such a high ammonia level. Our laboratory reported similar findings for the acute toxicity test for copper carried out with postlarvae of the freshwater prawn *Macrobrachium rosenbergii* (Lombardi et al. 2000). It was shown that although copper toxicity was not altered during the 96-hr static test, a test design with regular renewal of the test solution would be better suited for carrying out bioassays, provided that solution replacement can prevent the possibility of interference due to ammonia toxicity.

Mortalities above 50% were registered only after 72 hr of testing. The LC_{50} values determined for copper were 13.45 and 2.83 mg L^{-1} at 72 and 96 hr of exposure, respectively. Lower $LC_{50\text{-}96hr}$ for copper (0.05 and 0.06 mg L^{-1}) were observed to freshwater prawns on experiments carried out with the same fungicide as in the present study (copper oxychloride) (Lombardi et al. 2000). It is difficult to explain any difference in copper sensitivity among aquatic organisms, since the studies that are available in the literature are not standardized in terms of species,

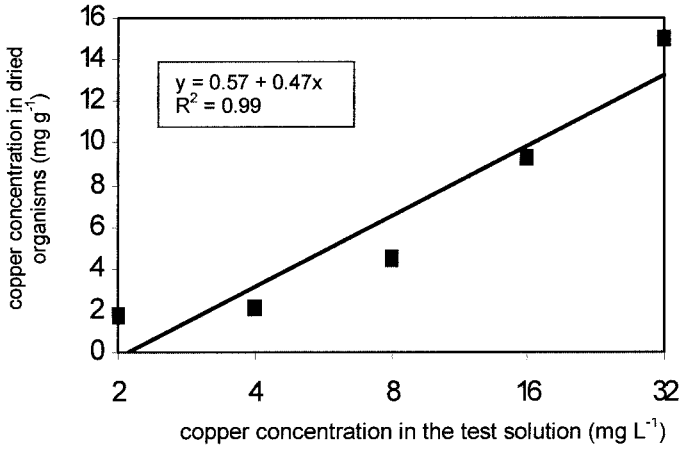

Figure 1. Copper bioaccumulation in *R. catesbeiana* during 96 hr of copper oxychloride exposure

organism size, chemical formulation of toxicants, physical and chemical parameters of water, etc.

Published reports indicate that most of the studies on copper toxicity have been carried out with copper sulfate. This may explain the different levels of copper sensitivity among some aquatic vertebrates, especially between those reported to fish and that determined in the present study. Lower $LC_{50\text{-}96hr}$ values were reported by Johnson and Finley (1980) to a few species of fish: 0.14 mg L^{-1} to *Salmo gairdneri*, 0.84 mg L^{-1} to *Pimephales promelas*, 0.89 mg L^{-1} to *Lepomis macrochirus*, and 1.38 mg L^{-1} to *Carassius auratus*. On the other hand, Reardon and Harrel (1990), and Nussey et al. (1996) found copper LC_{50} values of 2.68 – 7.88 mg L^{-1} to *Morone saxatilis* and 2.61 – 2.78 mg L^{-1} to *Oreochromis mossambicus*, respectively, values which are very similar to that determined to *R. catesbeiana* in the present study. The findings reported here are not at variance with earlier preliminary studies from our laboratory, where similar $LC_{50\text{-}96hr}$ were determined to larger tadpoles of *R. catesbeiana* (Ferreira et al. 2000). It seems that tadpoles of different sizes may not express different sensitivities to copper.

The analysis of copper in the bodies of tadpoles showed a bioaccumulation pattern directly proportional to the toxic concentration. Copper accumulation in the tadpoles increases with the rise in concentrations of copper in the experimental habitat (Figure 1). However, there is no information about saturation point and detoxification process in copper bioaccumulation. Thus, further studies

on acute and chronic exposures to copper are warranted to clarify this point.

A safe concentration level of copper for *R. catesbeiana* was determined to be 0.03 mg L^{-1}, which is slightly higher than the 0.02 mg L^{-1} value reported by the Brazilian Environmental Agency (CONAMA, 1986) as the safe limit for protecting aquatic life.

REFERENCES

APHA, AWWA, WPCF (1998) Standard Methods for the examination of Water and Wastewater, 20 ed. APHA – American Public Health Association, AWWA – American Water Works Association, and WPCF – Water Pollution Control Federation, Washington, DC

Beiswenger RE (1988) Integrating anuran amphibian species into environmental assessment programs. U.S. Forest Service. Gen Tech Rep RM 166: 159-165

CONAMA (1986) Resolução nº 20, de 18 de junho de 1986. Ministério do Desenvolvimento Urbano e Meio Ambiente. Conselho Nacional do Meio Ambiente. DOU Executivo 30/07/86: p. 11.356

Culley Jr DD (1991) Bullfrog culture. In: Neimann-Sorensen A, Tribe DE, Nash CE (eds), World animal science C4: Production of aquatic animals: crustaceans, mollusks, amphibians and reptiles, Elsevier, Amsterdam, p 185-205

Ferreira CM, Acencio MMP, Guimarães HB, Soares SR, Lombardi JV, Saldiva PHN (2000) Determinação da toxicidade do cobre para girinos de *Rana catesbeiana* Shaw, 1802. In: Encontro Brasileiro de Patologistas de Organismos Aquáticos & Encontro Latino Americano de Patologistas de Organismos Aquáticos. 3-6 Oct 2000, Florianópolis, Brasil. Abstracts... Associação Brasileira de Patologistas de Organismos Aquáticos, p 225

Greenhouse G (1976) The evaluation of toxic effects of chemicals in fresh water by using frog embryos and larvae. Environ Pollut 11: 303-315

Grillitsch B, Linder G (2000) Ecotoxicology of Metals in Amphibians and Reptiles. In: Sparling D, Linder G, Bishop C (eds) Ecotoxicology of Amphibians and Reptiles. SETAC Press, Pensacola FL, pp 325-460

Hamilton MA, Russo RC, Thurston RV (1977) Trimmed Spearman-Karber method for estimating median lethal concentrations in toxicity bioassays. Environ Sci Technol 11: 714-719

Johnson WW, Finley MT (1980) Handbook of acute toxicity of chemicals to fish and aquatic invertebrates. Res Publ 137. Fish Wildlife Service, Washington, DC

Lombardi JV, Machado-Neto JG, Brossi-Garcia AL, Marques HLA, Kubo E (2000) Acute Toxicity of the Fungicide Copper Oxychloride to the Freshwater Prawn *Macrobrachium rosenbergii* De Man. Bull Environ

Contam Toxicol 65: 383-390
Natarajan E, Biradar RS, George JP (1992) Acute toxicity of pesticides to giant freshwater prawn *Macrobrachium rosenbergii* (De Man). J Aqua Trop 7: 183-188
Nussey G, Van-Vuren JHJ, Du-Preez HH (1996) Acute toxicity tests of copper on juvenile Mozambique tilapia, *Oreochromis mossambicus* (Cichlidae), at different temperatures. South African J Wildl Res 26: 47-55
Reardon I, Harrel RM (1990) Acute toxicity of formalin and copper sulfate to striped bass fingerlings held in varying salinities. Aquaculture 87: 255-270
Sarruge JR, Haag HP (1974) Análises químicas em plantas. ESALQ/USP, Piracicaba
Schuytema GS, Nebecker, AV (1999) Comparative effects of ammonium and nitrate compounds on Pacific treefrog and African clawed frog embryos. Arch Environ Comtam Toxicol 36: 200-206