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Median lethal concentration of formaldehyde and its genotoxic potential in bullfrog tadpoles (*Lithobates catesbeianus*)

JULIANA M. SANTANA[1], ADRIANA DOS REIS[1], PATRÍCIA C. TEIXEIRA[1], FÁBIO C. FERREIRA[2] and CLÁUDIA M. FERREIRA[1]

[1]*Fisheries Institute, APTA, SAA, São Paulo, São Paulo, Brazil*
[2]*State University of São Paulo, UNESP, Santos, São Paulo, Brazil*

In order to avoid that contaminated frog farms animals escaping in the environment and become potential vector of emergent diseases, studies with disinfection protocol are strictly necessary. The formaldehyde is one of the compounds tested in fungal disinfection protocols and also used in aquaculture. This study aimed to determine the median lethal concentration ($LC_{50–96h}$) of formaldehyde in bullfrog tadpoles and to evaluate the possible genotoxic effects in acute exposition. Accordingly, the animals were exposed to formaldehyde in the concentrations of 6, 9, 12, 15, and 18 mg L^{-1}, and after 96 h blood samples were drawn for the micronucleus (MN) test. The $LC_{50–96h}$ was 10.53 mg L^{-1}, and the MN frequency increased in proportion to the formaldehyde concentrations, with an estimated frequency in the negative control being 1.35 MN/individual. We concluded that formaldehyde is genotoxic to tadpoles of bullfrogs in the tested concentrations, and the choice of this chemical should be contemplated before its use in animals in captivity.

Keywords: Chytridiomycosis, bullfrog farm, amphibians, micronucleus.

Introduction

Amphibian populations are declining and some groups are disappearing completely from their natural habitats in various parts of the world, where they are considered as one of the most endangered vertebrate classes today.[1,2] The most important factors contributing to this process are the loss of biodiversity with habitat destruction or alteration, the introduction of invasive and exotic species, climate change, increased UV-B radiation, chemical contamination, deformities and defects, and emerging infectious diseases such as those caused by the fungus *Batrachochytrium dendrobatidis* (Bd) (chytridiomycosis) and Ranavirus, which causes ranavirus infection.[3–7]

Some frog species are also used in food, where their breeding is called "frog farming."[8] The bullfrog (*Lithobates catesbeianus*) is a commercially bred species that has been suggested as one of the main vectors of chytridiomycosis.[5,9] However, a recent study[10] indicates the susceptibility of this animal depending on the virulence of different strains. In any case, the bidirectional flow comprising the entrance of wild animals and escape of infected exotic animals in the culture environment is of concern.[11]

Chytridiomycosis has been classified by the International Office of Epizootics (OIE) and the Groupon Amphibian Diseases as one of the most important pathogens in international trade in amphibians, second only to ranavirus infection.[12] The fungus Bd survives in water, where it has two life cycles, the zoospore (free-swimming), which has flagella, and sporangium (fixed to the substrate).[4] Bd is found in keratinized structures of infected animals and lodged in the skin of adults and in the mouth of tadpoles,[13] causing depigmentation. However, other diagnostic methods must be used, since the lack of pigmentation is also caused by exposure to chemical contaminants or low temperatures.[12,14–16]

Protocols for fungal disinfection have been tested *in vivo* as well as *in vitro* in combating Bd cultures,[17–19] but the potential toxicity of some fungicides against different amphibian species makes it difficult to standardize an effective fungicide method. The ideal disinfectant must act quickly, be safe for humans exposed, generate minimal impact on the equipment used, have low environmental contamination potential, and be readily available for use, and besides, any amount of waste generated must not have harmful effects on the amphibian community.[20]

Address correspondence to Juliana M. Santana, Fisheries Institute, APTA, SAA, São Paulo, São Paulo 11045, Brazil; E-mail: julianamacedo.bio@gmail.com
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Formaldehyde is one of the fungicides used in aquaculture, and has a variety of uses because it is highly reactive, colorless, stable, pure in commercial form, and low cost. It serves as a curing agent, disinfectant, fungicide, and preservative,[20–22] However, in 2004 it was reclassified by the World Health Organisation (WHO) and International Agency for Research on Cancer (IARC) as carcinogenic to humans.[23] The aim of this study was to determine the median lethal concentration of formaldehyde (Vetec®) for bullfrog tadpoles (*L. catesbeianus*) and assess its genotoxic effects using the micronucleus (MN) test as a biomarker.

Materials and methods

The work was performed in the Aquatic Toxicology Laboratory of the Fisheries Institute, APTA/SAA, São Paulo, Brazil. Since it is an exotic species, it is not found in abundance in the Brazilian natural environment. Therefore, the animals (*L. catesbeianus* tadpoles) were obtained from a commercial frog farm in the state of São Paulo. The bullfrog was chosen as the test animal because it is an amphibian already adapted to captivity, and is widely used in ecotoxicological studies, adding to the fact that there are restrictions in Brazilian law that hinder the use of wild animals in scientific research.

The tests were conducted in accordance with the standard guidelines of pre-established protocols.[24–26] The animals were acclimated for seven days, and they were maintained in a room (22 ± 1.0°C), with a controlled 12/12-h photoperiod. The tadpoles were fed once daily with 1% of the biomass of aquarium, using ground feed (40% protein crude, 12% fiber, and 8% ether extract). Preliminary tests were performed in a semi-static system (24 h), and the results served as a basis for determining the doses of the definitive test, and the concentrations of formaldehyde (Vetec®, Brazil) determined were 6, 9, 12, 15, and 18 mg L^{-1}, whereas the control group had no added formaldehyde.

At the beginning of definitive tests, the tadpoles were weighed on a digital scale, averaging 5.99 ± 1.49 g, and had a stage of development between 31 and 36.[27] This stage was selected because it facilitated the extraction of caudal vessel blood from the animals, providing enough sample to carry out the MN test for evaluation of genotoxicity.

Throughout the study, the animals were kept in aquariums filled with 8 L of solution with constant aeration. The density was 1 tadpole L^{-1}, and there were four replicates per treatment, following the recommended standard protocol,[24] totaling eight tadpoles per replicate. The acute toxicity test lasted for 96 h, using tap water dechlorinated for overnight, and the solutions were renewed every 24 h (semi-static system) due to the high volatility of the product. For this reason, the stock solution was prepared on the same day for each intoxication.

There was no food supplied during exposure to the chemical agent, thus reducing organic input in aquariums and minimizing possible changes in the concentration of formaldehyde and interference with its action.[28] The analysis of water quality variables (temperature, dissolved oxygen, electrical conductivity, and pH) were performed at baseline (before the tests) and at 48 and 96 h using a HORIBA™ multiparameter probe, and dead animals were counted and removed daily.

The genotoxic effect was evaluated in the surviving animals at 96 h of exposure (three animals per replicate – 12 tadpoles per treatment) by withdrawing blood by puncture of the caudal vessel using lidocaine cream as a local anesthetic. The slides were made in duplicate and analyzed in a blind test. Each slide was fixed with absolute methanol for 10 min, air-dried by immersing in 5 N hydrochloric acid (HCl) for another 10 min, and then stained by the Feulgen/fast green method adapted for *L. catesbeianus* tadpoles.[29] MN frequency was determined by evaluating 2,000 erythrocytes per slide ($n = 53$) using a light microscope with oil immersion objective (100×).

Statistical analysis

The water quality data were subjected to analysis of central tendency and dispersion, evaluating the coefficient of variation of each parameter.[30] The LC_{50-96h} of formaldehyde was estimated by the software Gwbasis 3.0, according to the Trimmed Spearman Karber statistical method.[31] The MN count, depending on the formaldehyde concentration, was fitted by a generalized linear model (GLM) assuming the negative binomial distribution as a function of the response variable distribution. The negative binomial distribution was defined by μ parameter representing the mean, and k as a measure of dispersion. The variance was given by $\mu + \frac{\mu^2}{k}$, and the systematic part of the negative GLM binomial was given by $E(Y) = \boldsymbol{\mu} = e^{\beta_0 + \beta_1 X}$, where β_0 and β_1 are the parameters of the intercept and the slope of the model respectively. The percentage explanation of the model was calculated as

$$\frac{\text{Null deviance} - \text{Residual deviance}}{\text{Null deviance}} \times 100.$$[32]

Results and discussion

The water quality data showed mean values of pH 5.60 ± 0.40, temperature 17.41 ± 0.46°C, 6.38 ± 0.39 mg L^{-1} dissolved oxygen, and 96.74 ± 6.53 μS cm^{-1} electrical conductivity. According to statistical analysis, there were no significant differences in the physical and chemical parameters analyzed at five concentrations of formaldehyde as well as between these means and the control group. These

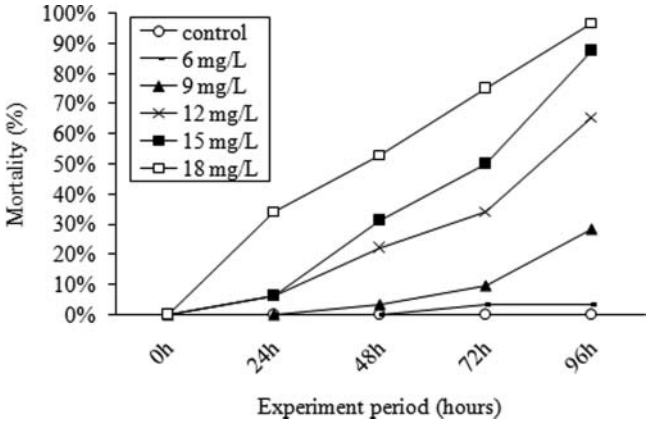

Fig. 1. Cumulative mortality (%) of *Lithobates catesbeianus* tadpoles exposed to various concentrations of formaldehyde during the acute toxicity test (LC_{50-96h}).

variables remained within the values indicated for conducting toxicity tests in this species.[33]

The estimated LC_{50-96h} of formaldehyde for *L. catesbeianus* tadpoles was 10.53 mg L^{-1}. There was no mortality in the negative control, and the highest concentration of formaldehyde caused almost 100% mortality, leaving only one surviving tadpole at 96 h, and 50% of the deaths at this concentration were recorded at 48 h. The cumulative mortality (%) of *Lithobates catesbeianus* tadpoles is showed in Figure 1.

In a review article on formaldehyde,[28] the LC_{50-96h} reported for bullfrog was 9.52 mg L^{-1} (data fitted), similar to that found in our tests. The authors also pointed out that the toxicity of this chemical is more for smaller animals, and that frog tadpoles are more sensitive than most species of fish and aquatic invertebrates. Other studies determined the LC_{50-96h} of formaldehyde for other aquatic organisms in a static system as 429.68 mg L^{-1} for Nile tilapia (*Oreochromis niloticus*),[34] 48.8 mg L^{-1} for rainbow trout (*Oncorynchus mykiss*), and 21.78 mg L^{-1} for the American catfish (*Ictalurus punctatus*).[28] The data indicate that the bullfrog is a very sensitive species compared with these fish species.

Formaldehyde is a compound classified as carcinogen for humans,[23] while in fish farming, it is indicated for the treatment of parasitic diseases and for fungal control[35–37] but the surviving organisms can have their health impaired.[22] A formaldehyde has been traditionally used on Australian trout farms as a treatment for *Ichthyophthirius multifiliis* exposed to 64 mg L^{-1} for 15 min.[38] The bath is used in the prophylaxis or treatment infestation of the ectoparasitic ciliate, *Chilodonella hexasticha*, and that of the monogenean gill fluke, *Lepidotrema bidyana*, treated successfully with an application of 30 mg L^{-1} formalin to Australian freshwater silver perch (*Bidyanus bidyanus*),[39] these concentration are similar to those indicated for the frog *Xenopus tropicalis* in the treatment of Bd infection, which is 25 mg L^{-1}. However, this concentration caused mortality in frogs within 48 h of initial treatment, indicating high toxicity.[15] The fungicide power of formaldehyde has been proven in vitro test to be 10 mg L^{-1} using cultured fungus Bd.[20]

To evaluate genotoxicity, 53 samples were subjected to the MN test, where fewer slides were examined with the highest formaldehyde concentration, owing to greater mortality.

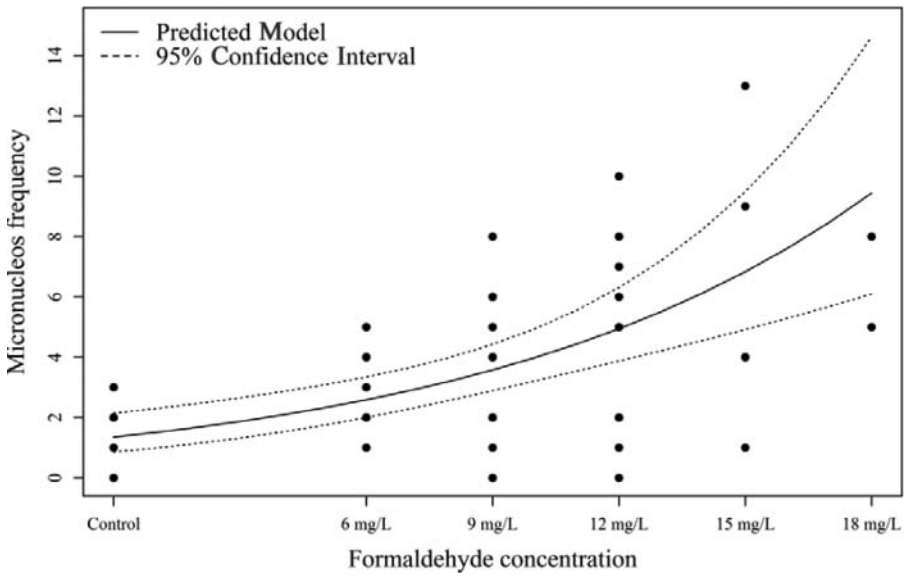

Fig. 2. Scatter plot of micronucleus frequency versus formaldehyde concentration (2,000 erythrocytes/slide) found in the acute toxicity test with *Lithobates catesbeianus* tadpoles.

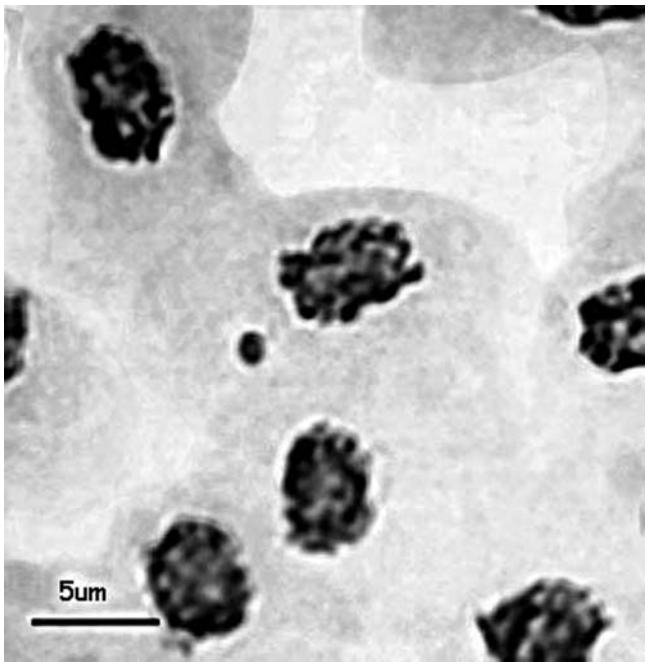

Fig. 3. Image of a micronucleated erytrocyte using a light microscope with oil immersion objective (100×).

There was a positive relationship between MN frequency and formaldehyde concentration (Fig. 2), and the negative binomial GLM explained 26.94% of variability in this regard. MN count increased with formaldehyde concentration, which was due to increasing cells/individual with higher numbers of MN, while frequency values in other tadpoles were close or equal to zero. This increase in variance justifies the use of GLM with the negative binomial distribution (dispersion coefficient, $k = 3.35$). According to the estimator of the intercept ($\hat{\beta}_0 = 0.303$, $P = 0.196$), in the absence of formaldehyde (control group), an average of 1.35 MN per tadpole was expected ($e^{0.303} = 1.35$); however, this value was not statistically different from zero (Fig. 3). Values between 0 MN and 6 MN were found in the negative control, and are similar to the values reported by other authors, 3 to 5 MN per individual, in the counts of 1,000 erythrocytes/slide of samples not exposed to contaminants (negative control).[40,41] The slope coefficient ($\hat{\beta}_1 = 0.108$, $P < 0.001$) suggests that the addition of 1 mg L^{-1} formaldehyde results in a mean increase of 11.4% in MN count per individual ($e^{0.108} = 1.114$).

There are few studies regarding the tolerance of amphibians to formaldehyde, including native animals, which can be affected by the waste produced in aquaculture, which in contact with other substances form complex mixtures, more toxic than the original ones. Meanwhile, the bullfrog has been an effective biomarker of genotoxicity, showing an increased incidence of MN and nuclear abnormalities when exposed to various contaminants.[42–44] In this study, we demonstrated the genotoxic potential of acute formaldehyde exposure in the bullfrog, which contributes to the scarce literature on the subject.

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