Histopathological changes in the gills of zebrafish (*Danio rerio*) and bullfrog tadpoles (*Lithobates catesbeianus*) caused by the use of formaldehyde

Diego Sales Santos1, Fernanda Menezes França1, Adriana Sacioto Marcantonio2, Cristina Viriato3, Ana Maria Cristina Rebello Pinto Fonseca Martins4, Cintia Badaró-Pedroso1, and Cláudia Maris Ferreira[[1]](#footnote-1)

1 Fisheries Institute – APTA/SAA, Av. Francisco Matarazzo, 455 – São Paulo – SP – Brazil. E-mail: diegosales.zoologia@hotmail.com - ORCID: 0000-0002-4344-2111, fernanda\_ranicultura@yahoo.com.br - ORCID: 0000-0002-6097-5366, cintia.pedroso@sp.gov.br - ORCID: 0000-0002-0219-7957.

2 APTA Regional – SAA, Av. Prof. Manoel César Ribeiro, 1920, 12411-010 Pindamonhanga, SP, Brazil. E-mail: adriana.sacioto@gmail.com - ORCID: 0000-0002-2897-577X.

3 Institute of Biosciences of Botucatu, São Paulo State University (UNESP), Distrito de Rubiao Junior, s/n, Caixa Postal - 510; CEP: 18618970; Botucatu - SP – Brazil. E-mail: cristinavf@outlook.com - ORCID: 0000-0002-5208-9639.

4 Biological Institute – APTA/SAA, Av. Conselheiro Rodrigues Alves, 1251, 04014-002 São Paulo, SP, Brazil. E-mail: crispfm@gmail.com - ORCID: 0000-0002-4061-0298.

**Abstract**

Formaldehyde is a carcinogenic and aggressive agent mainly to epithelial tissues. However, for rearing aquatic organisms its use is common for the treatment of fungi and parasites, and the use of incorrect doses can harm the health and life of these animals. The fish species *Danio rerio* and the tadpoles of the species *Lithobates catesbeianus* are internationally recognized for use in ecotoxicological tests. The objective of this study was to identify the main effects caused by formaldehyde on the gills of these two experimental models used in ecotoxicology, verifying the susceptibility of both species. Acute and chronic tests with formaldehyde were conducted for experiment. The Median Lethal Concentration of formaldehyde for tadpoles of it was 9.17 mg L-1. We found that the formaldehyde in this study caused injuries to the gills of both fish and tadpoles, with a loss and displacement of epithelium, vascular congestion, lamellar fusion, hypertrophy and hyperplasia of epithelial cells, lamellar aneurysm, in addition to the proliferation of mucus-secreting cells and chloride cells. Despite structural differences, the histological changes caused by chronic exposure to formaldehyde in sublethal concentrations were similar in both organisms and we recommend reviewing its use in prophylaxis and in prolonged treatments with this chemical.

Keywords: Anurans, branchial arches, formalin, histological damage, Osteichthyes

**Introduction**

The main threats to the aquatic ecosystem are sources of pollution, loss of biodiversity and habitat destruction (Linde-Arias et al., 2008; Thushari and Senevirathna, 2020). The aquatic ecosystem is considered the most susceptible to pollution, due to natural sources or as a result of human activity such as the discharge of domestic, industrial and agricultural effluents, which can occur intentionally or accidentally (Zagatto and Bertoletti, 2008). These changes in the aquatic environment directly affect the health of fish and amphibians, and even small changes are sufficient to trigger stressful stimuli in animals (Randall and Tsui, 2002; Gomez Isaza et al., 2020).

The *Danio rerio* species, commonly called zebrafish, is a small tropical freshwater fish, of Asian origin that has long been used as an ornamental fish. Because it has great tolerance to environmental variations and is easy to breed in captivity, it has proved to be a good organism for scientific studies (Spitsbergen and Kent, 2003; Liu et al., 2021). This fish withstands variations in temperature, pH and hardness, but in another hand, it is also very sensitive to a high number of substances. In 2001, the Sanger Institute started sequencing the zebrafish genome and found that this species has a genome with a high degree of similarity with the genomes of humans and mice (Barbazuk et al., 2000). For these reasons, this species is used worldwide as a toxicological model (Teraoka et al., 2003). The species *Lithobates catesbeianus*, known as the bullfrog, is native to northeastern North America. It is a robust and resistant species when compared to other frogs and is prolific and disseminated worldwide (Frost, 2016). Due to these characteristics and its easy acquisition and maintenance in the laboratory, it is also considered an excellent experimental model for use in ecotoxicological tests. Moreover, approximately 70% of anuran species have a life cycle linked to aquatic environments, a fact that makes them good indicators of water quality (França et al., 2015).

The principle of the 3 R's of animal experimentation proposed by Russel and Burch (1959) recommends “*replace*”, which translates into replacing sentient animals, that is, those that are capable of experiencing pain; “*reduction*”, which means reducing the number of animals used, without jeopardizing the reliability of the results; and “*refinement*”, which means the decrease in the incidence or severity of applied procedures (CONCEA, 2014). Corroborating this line of thought there are international recommendations such as the NBR ABNT 15088 of 2016 in Brazil (method of experimenting with fish), which recommends the reducing use of highly complex organisms in aquatic toxicology tests. In this sense, the tests with *D. rerio* and *L. catesbeianus* are considered a cutting-edge alternative and are increasingly used to replace the tests carried out with mammals.

In the cultivation of aquatic organisms, some chemicals and prophylactic products are used to maintain the health of the crops (Noga, 2010). Among these, formaldehyde (CH2O) is indicated by the US Food and Drug Administration (FDA) in the treatment of fungal, bacterial and parasitic diseases, even though it is a chemical agent considered to be carcinogenic by the International Agency for Research on Cancer (IARC, 2004). Formaldehyde is a colorless gas with an irritating odor and one of the most common and abundant aldehydes in the environment used in the production of resins and furniture, pulp, paper, as well as in the production of plastics and coatings, in textile finishing and in the manufacture of industrial chemicals and as a disinfectant and preservative product (ECOTOX, 2006). Its residue can potentially contaminate air, soil and water (Bueno-Guimarães et al., 2001). It has attracted great attention because of threats to ecological security when it enters the ecosystem (Li et al., 2020). Formaldehyde is an extremely reactive chemical compound that interacts with DNA, RNA, polysaccharides and glycoprotein proteins (Leal et al., 2018; De Swaef et al., 2015). It is a basic chemical compound whereas formalin is a 37% aqueous solution of formaldehyde (Devaraj et al., 2021). Formalin is used in the treatment of diseases caused by protozoa, fungi and bacteria in fish. Actually, it is one of the main chemicals used in aquaculture for the prophylaxis and treatment of various pathogens (De Swaef al. 2015; Resendes et al., 2018). However, there is evidence that this product may be aggressive to the gills of fish and amphibians (Bueno-Guimarães et al., 2001; Ramos et al., 2014). In these animals, the gills have several functions and are very important for gas exchange, osmoregulation processes, acid-base balance and excretion of nitrogen compounds. The gills can, thus, be an excellent biomarking tool for environmental damage and indicators of water quality (Machado and Fanta, 2003; Evans et al., 2006).

The objective of this study was to identify the main effects caused by formaldehyde to the gills of two experimental models used in ecotoxicology: zebrafish (*D. rerio*) and bullfrog tadpoles (*L. catesbeianus*), verifying the susceptibility of both species to this chemical.

**Materials & Methods**

The study was developed under ethical conditions and according to institutional, national and international guidelines on the use of animals in the research and authorized by the Ethics Committee of our institution under number 1535. For the experiments, two species of aquatic organisms were used: zebrafish (*D. rerio*) and bullfrog tadpoles (*L. catesbeianus*) (Gosner, 1960 - stages 31 to 36). The tests were divided into two stages, the acute test in the first and the chronic test in the second. Both experiments followed the same protocol adapted to the peculiarities of each species.

The technical standards for conducting the trials followed the recommendations according to pre-established protocols (APHA, 2005; ABNT, 2016; ASTM, 2014). The formaldehyde (P.A.) stock solution (Synt™) at a concentration of 100 g L-1 was prepared on the day of each intoxication, in a 500 mL flask, 125 mL of formaldehyde and 375 mL of distilled water. These concentrations are nominal concentrations based on the purity of the obtained formaldehyde and were prepared with the aid of pipettors (muitipette®E3) Eppendorf®. The dilutions were made with micropipettes (100-1000 µL and 20-200 µL) of the same trademark.

During the experiments the dead animals were counted, removed daily from the aquariums and discarded. At the end, all surviving animals were anesthetized with eugenol (7 mL L-1) and euthanized by deep anesthesia, to remove the gills that were preserved in 10% buffered formalin. Subsequently, the samples were dehydrated, diaphanized, embedded in a paraffin block, cut into fragments with a thickness of 4.5 µm and stained with Hematoxylin and Eosin (H&E) for producing histological slides.

* 1. *Experiments with zebrafish (D. rerio)*

The experiments of acute and chronic toxicity with *D. rerio* fish were carried out and published by Resendes et al. (2018). Respecting the principle of the 3 R's, we used the branchial tissue samples that were collected by these authors, but we clarify that these samples were not analyzed. We emphasizing that Resendes et al. (2018) only reported toxicity data (*i.e.* mortality) and does not include histopathological analysis of the effects of formaldehyde. The Median Lethal Concentration at 96 h (LC50-96h) for formaldehyde obtained by these authors was 45.73 mg L-1 and the chronic exposure concentrations were: LC50-96h/100 (T-1) - 0.45 mg L-1; LC50-96h/10 (T-2) - 4.57 mg L-1 and LC50-96h/2 (T-3) - 22.86 mg L-1, in addition to the negative control (without adding the product).

At 96 hours of experimentation and at the end of the experiment (192 h), eigth fish from each treatment were sacrificed by deep anesthesia to remove the gills which were conserved in 10 % buffered formalin and analyzed in the present study (n = 64).

*Experiments with bullfrog tadpoles (L. catesbeianus)*

*Acute test*

After preliminary tests, the acute toxicity test lasted 96 hours, with a completely randomized design in a semi-static system (24 hours), with six treatments and four replicates. The treatments were: 4, 8, 12, 16, and 20 mg L-1 of formaldehyde plus the control group (without adding the product). The aquariums were filled with the public water supply, dechlorinated overnight with aeration.

The analysis of water quality variables (temperature, dissolved oxygen, electrical conductivity and pH) were performed using the AK87 Akso™ oximeter and the Hl98129™ equipment. At the beginning of the experiment, the tadpoles were weighed and were not fed during exposure to the chemical agent. A density of 1 tadpole per liter was used, totaling 8 tadpoles per aquarium.

To verify the possible significant differences between the physical and chemical variables of water, one-way analysis of variance (p < 0.05) was used (Zar, 1999). Median Lethal Concentration was estimated using Software Gwbasis 3.0 according to the statistical method “Trimmed Spearman Karber” (Hamilton et al., 1977).

*Chronic test*

The test was semi-static, with solutions renewed after 96 h, and the exposure period was 192 h, following the same experimental design as Resendes et al. (2018) including four treatments with four simultaneous replicates, namely: LC50-96h/100 (T-1), LC50-96h/10 (T-2) and LC50-96h/2 (T-3), as well as the negative control (without adding the product).

Sixteen aquariums were randomly distributed under the work benches, each containing 12 L of water, 1 tadpole per litter, constant aeration, containing a total of 192 tadpoles.

The parameters of water quality (pH, temperature and dissolved oxygen, Hardness and Total Ammonia) were evaluated before and after the water was renewed at 96 h. The tadpoles were weighed before the beginning of the experiment and fed with Laguna crushed feed (Socil® 40 % PB) every two days, in the proportion of 0.5 % of the biomass of the aquarium.

At 96 h of experimentation and at the end of the experiment (192 h), eigth tadpoles from each treatment were sacrificed by deep anesthesia to remove the gills which were preserved in 10 % buffered formalin (n = 64).

*Histopathological analyses*

Histopathological analyses of the gills were performed following a standard protocol for this technique. The gills, being very small, were wrapped in gauze and packed in identified boxes, dehydrated in an increasing battery of alcohol baths (70%, 80%, 95% and absolute), diaphanized in xylol, embedded in paraffin and stored in an oven for 18 h at 52 °C. After this period, they were embedded in paraffin and subjected to 5 µm thick horizontal cuts with the aid of the Hyrax Zeiss® microtome. The cuts were placed on slides with gel, for better fixation, and deparaffinized in a xylol and alcohol baths for later staining with Hematoxylin and Eosin. The last bath included immersion in xylene and decreasing gradient of alcoholand the fragment was then mounted on a slide with Etellan™ and was covered with a cover slip. The observation and recording of the images were made under ana1™ CARL-Zeiss Axio Scope light microscope and ZEN™ image capture software.

**Results**

*Acute and chronic tests for tadpoles L. catesbeianus*

 The physical and chemical parameters of the water remained within the values considered acceptable for the maintenance of this type of organism in toxicological tests for *L. catesbeianus* species (Cribb et al., 2013; Lombardi et al., 2002) and were stable during the performance of the test, with no statistically significant differences between them. The mean values and standard deviation observed were: temperature 22.62 ± 0.8 ºC, pH 7.51 ± 0.1, electrical conductivity 121.06 ± 9.3 µS cm-1 and, dissolved oxygen 7.8 ± 0.8 mg L-1. The average weight of the tadpoles used in the acute test was 3.67 ± 0.27 g.

The Median Lethal Concentration (LC50-96h) of formaldehyde for *L. catesbeianus* tadpoles was 9.17 mg L-1. Figure 1 shows the accumulated mortality of the animals during the experiment.



**Figure 1** Accumulated mortality of *Lithobates catesbeianus* tadpoles submitted to different formaldehyde concentrations during the acute toxicity test (LC50-96h) expressed as a percentage (%). C - Control (without adding the product); T1 - 4 mg L-1; T2 - 8 mg L-1; T3 - 12 mg L-1; T4 - 16 mg L-1 and T5 - 20 mg L-1

In the chronic test the formaldehyde concentrations used were: LC50/100 = 0.09 mg L-1, LC50/10 = 0.92 mg L-1 and LC50/2 = 4.58 mg L-1. The average values and standard deviation of the physical and chemical parameters of the water were: pH 7.05 ± 0.16, dissolved oxygen 7.03 ± 1.13 mg L-1, temperature 24.5 ± 0.56 ºC, total ammonia (NH4) 2.7 ± 0.7 mg L-1, Hardness 1.19 ± 0.29 °dH. The average weight of the animals was 3.38 ± 0.39 g. There was no mortality during the chronic test.

*Histopathological analyses*

The gills of *D. rerio* have the standard formation of teleost fish, that is, they are external with four delicate pairs of branchial arches, containing small tracks located in the internal curvature of the arch, and numerous filaments that have an axis of hyaline cartilage lined with multilamellar squamous branchial epithelium made up of mucous, hydrochloric and columnar cells (Figure 2).

**Figure 2** Macroscopic view of *Danio rerio* gills. A - Four pairs of branchial arches (Nikon SMZ 745T - 2X magnification). B - Detail of a branchial arch (Nikon SMZ 745T - 5X magnification)

The gill apparatus of *L. catesbeianus* tadpoles is supported by four pairs of gill arches. Each branchial arch inserts ventrally into the gill tufts and dorsally into the gill filters. The gill has primary and secondary tufts with numerous finger-like ramification and are highly vascularized and is responsible for gas exchange; they are involved in a lot of mucus (Bueno-Guimarães et al., 2001; Viriato et al., 2021). This structural pattern was confirmed in this study (Figure 3).



**Figure 3** Macroscopic view of gill tadpoles of *Lithobates catesbeianus* Gosner (1960) stages 31 to 36. **A** - Four pairs of gill arches (Nikon SMZ 745T - 2X magnification). **B** - Detail of a gill arch showing tufts with numerous finger-like ramification (Nikon SMZ 745T - 5X magnification).

In *D. rerio* exposed to sublethal concentrations of formaldehyde, morphological alterations in the gills were observed. Structural disorganization of normal tissue with the loss and displacement of epithelium and rupturing of epithelial cells were observed in all treatments that contained formaldehyde. In T-1 treatment were observed lamellar fusion, hypertrophy and hyperplasia of epithelial cells and the presence of melanomacrophages. In T-2 and T-3 treatments were observed high formation of lamellar aneurysm, inflammation and proliferation of mucus-secreting cells and chloride cells (Figure 4).



**Figure 4** Comparative photomicrograph of zebrafish (*Danio rerio*) gills exposed for 192 h to formaldehyde. (A) Negative Control - epithelial cell layer constitutes the primary lamella (ec) and cartilaginous support tissue (ct) showing normal morphology at 400x magnification; (B) T-1 (0.45 mg L-1) - Lamellar fusion (white arrow), hyperplasia (H), hypertrophy (\*) and melanomacrophages (arrowhead) at 200x magnification; (C) T-2 (4.57 mg L-1) aneurysm (arrow) of the secondary lamellae at 200x magnification; (D) T-3 (22.86 mg L-1) - Inflammation and aneurysm (arrows) at 400x magnification. H&E staining.

In *L. catesbeianus* tadpoles exposed to sublethal concentrations of formaldehyde, morphological alterations in the gill tufts were also observed. The vessels were highly congested and lymphocyte infiltration were present in all treatments that contained formaldehyde. In T-1 treatment were observed congested venous sinus and epithelial cell hyperplasia. In T-2 and T-3 treatments were observed areas of inflammation and the presence of melanomacrophages and also cell necrosis in the highest concentration of formaldehyde (Figure 5).



**Figure 5** Comparative photomicrograph of gill tufts of bullfrog tadpoles (*Lithobates catesbeianus*) exposed for 96 h to formaldehyde. (A) Negative control – gill tuft (T) with ramifications and cartilaginous support tissue (ct) showing normal morphology at 100x magnification; (B) T-1 (0.09 mg L-1) **-** Congested venous sinus, epithelial cell hyperplasia (arrows) at 100x magnification; (C) T-2 (0.92 mg L-1) – Inflammation of tuft tissue and melanomacrophages (arrows) at 100x magnification; (D) T-3 (4.58 mg L-1) - Inflammatory reaction, congested vessels, cell necrosis and melanomacrophages (arrows) at 100x magnification. H&E staining.

**Discussion**

Formaldehyde is a ubiquitous compound classified as carcinogenic to humans and is tumorigenic and teratogenic for producing effects on reproduction (IARC, 2014), but even so it is indicated for the parasitic treatment and fungal control of aquatic organisms (De Swaef et al., 2015; Noga, 2010). In Europe and Japan it is not approved for usage in aquaculture because of its association with cancer and tumor development (Devaraj et al., 2021). A few scientific articles have reported the acute and chronic toxicity of this chemical: Santana et al. (2015) working with *L. catesbeianus* tadpoles obtained an LC50-96h of formaldehyde at 10.53 mg L-1, Hohreiter and Rigg (2001) determined 48.8 mg L-1 for rainbow trout (*Oncorynchus mykiss*) and 21.78 mg L-1 for American catfish (*Ictalurus punctatus*), and Resendes et al. (2018) obtained 45.73 mg L-1 for *D. rerio*. In the present study we determined 9.17 mg L-1 for *L. catesbeianus* tadpoles and we confirm the Santana et al. (2015) datas, indicating that the bullfrog is a species that is very sensitive to formaldehyde compared to most fish.

Various concentrations of formaldehyde have been tested for chemotherapeutic efficacy and its possible harmful effects in the treatment of aquatic animals; however, the vast majority demonstrate hyperplasia of the branchial cells, genotoxic damage and animal mortality, indicating the toxicity of this chemical (Martins, 2004; Santana et al., 2015; Resendes et al., 2018). According to Martins (2004), 37% formaldehyde can be used in the form of a short bath (up to 60 min) in the concentration of 55.5 mg L-1 to 92.5 mg L-1 and in long-term baths (24 h) at a concentration of 3.7 mg L-1 to 5.55 mg L-1. However, Paixão et al. (2013) working with these concentrations with the fish species *Hemigrammus* (a characid widely used in aquariums) using formaldehyde baths for 60 minutes reported 100% mortality. The authors suggest that this may have been due to the fact that wild fish are more susceptible to stress and less rustic than those in cultivation. Thus, greater care must be taken in the storage and treatment of wild native fish for export, as the stress response becomes more pronounced and adaptation to the new condition can be compromised. Martins (2004), states that formaldehyde, which came to replace malachite green in the treatment against parasites and fungi, has been highlighted for its effectiveness; if applied correctly. According to Cruz et al. (2005) formaldehyde’s toxicity preliminary tests are essential for the definitive use of this treatment.

Exposure of aquatic organisms to the lethal and sublethal concentration of contaminants in their environment can lead to different biochemical, physiological and histological changes in vital tissues (Hermenean et al., 2015; Ogbeide et al., 2019). The damage of the gills due to toxic agents causes a chain of destructive events, which can lead to respiratory problems (Magare and Patil, 2000). The branchial epithelium is one of the main surfaces of contact with the environment and constitutes one of the organism’s first lines of defense, and consequently, one of the first organs to be affected. Despite being a consensus in the scientific community, branchial lesions are poorly documented, especially when referring to amphibians.

In general, in freshwater organisms, water enters through the gills and the excess is eliminated by the kidneys. The teleostean gill is thus the most important osmoregulatory organ (Motais and Garcia-Romeu, 1972), although nowadays there are new mechanisms by which fish can change functional branchial area and diffusion distance (Wood and Eom, 2021).

In fish the branchial structure is very similar between different species. In both fish and anuran larvae, the gills are derived from the same embryonic pharyngeal arches (Saltys et al., 2006). The same study also reported that in *Xenopus* larvae, innervation of the gills closely resembles that observed in zebrafish. In amphibians there is considerable diversity between anurans in the structure and types of their branchial attachments. The structure of the tadpoles' larval branchial apparatus is closely associated with the mechanisms of pumping and oral feeding that undergo profound changes to form the adult hyoid apparatus, which supports the laryngeal structures and serves as a base for the tongue (Bandara et al., 2012). The gill apparatus of both species (*L. catesbeanus* and *D. rerio*) are supported by four pairs of gill arches. In bullfrog tadpoles, gill tufts are highly vascularized and is responsible for gas exchange, which corresponds to lamellas function in zebrafish.

The most common reactions in response to exposure to chemical agents are cellular growth and increased mucus production (Wong and Wong, 2000). In this study, for both species, the main histopathological changes varied as the formaldehyde concentration increased, that is, the severity of the lesions increased with the increase in formaldehyde concentrations and exposure time. Comparatively, we observed a greater tolerance to formaldehyde by fish (*D. rerio*). The most common gills injuries observed in both species (*L. catesbeanus* and *D. rerio*) were inflammation and presence of melanomacrophages. The highest concentration to which the tadpoles were exposed is equivalent to an intermediate concentration of exposure of these fish; but regardless of tolerance the lesions were present. Interstitial edematous areas, epithelial desquamation, epithelial hyperplasia and hypertrophy and lamella fusion in some animals are lesions that suggest defense mechanisms, as they reduce the vulnerable surface area of the gill or the chemical diffusion barrier (Karlsson-Norrgren et al., 1985; Erkmen and Kolankaya, 2000). These responses hinder the access of the toxic agent to the blood and impairing gas exchange (McDonald and Wood 1993). In turn, breathing difficulties may be responsible for inducing vasodilation. Studies by Bueno-Guimarães et al. (2001), Pahor-Filho et al. (2015) and Ramos et al. (2014) prove this.

The present work has demonstrated that the organisms exposed to the chemical agent, formaldehyde, produced an altered cellular and histological responses that can be described as injurious. Despite the structural differences, the histological changes in both organisms were similar and we suggest, therefore, a review of the recommendations for its use in the prophylaxis and treatment of aquatic organisms.

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1. Corresponding author: Claudia Maris Ferreira - Fisheries Institute – APTA – SAA, Av. Francisco Matarazzo, 455, 05001-900 São Paulo, SP, Brazil - E-mail: cmferreira@sp.gov.br ORCID 0000-0001-9549-4178. Phone number +55 11 964916071 [↑](#footnote-ref-1)