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Histopathological changes in *Lithobates catesbeianus* tadpoles used as biomarkers of pesticide poisoning

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ABSTRACT

The use of biological indicators has increased in recent years with the aim of investigating environmental pollution in aquatic environments that are vulnerable to the constant use of pesticides. Some biomarkers can help assess the health status, indicating physical, metabolic, and behavioral changes under acute and sublethal poisoning. The mixture of the active ingredients cyproconazole and picoxystrobin is a widely used fungicide for the control of pests in cotton, rice, coffee, sugarcane, corn, soybean, and wheat. The objective of this study was to verify the occurrence of possible histopathological lesions in the liver and kidneys of bullfrog tadpoles (*Lithobates catesbeianus*) caused by a fungicide commercial formula composed of picoxystrobin and cyproconazole. The animals were subjected to different concentrations of the fungicide to determine the median lethal concentration ($LC_{50\text{-}96h}$ = 0.05 mg L^{-1}), that is, the lethal dose for 50% of the animals in 96 h. After determining the value of $LC_{50\text{-}96h}$, the animals were subjected to three sublethal concentrations ($LC_{50\text{-}96h/2}$, $LC_{50\text{-}96h/10}$, and $LC_{50\text{-}96h/100}$). Through histological biomarkers, it was verified that this fungicide changed the morphology of the animals' kidney and liver tissues in a chronic way, impairing the functioning of organs that are essential for their survival and metamorphosis, which can result in an imbalance in the biodiversity of aquatic ecosystems.

Keywords: amphibian; ecotoxicology; kidney; liver; cyproconazole; picoxystrobin.

Alterações histopatológicas utilizadas em girinos de *Lithobates catesbeianus* como biomarcadores de intoxicações causadas por pesticidas

RESUMO

O uso de indicadores biológicos tem aumentado nos últimos anos, com o intuito de investigar a poluição ambiental em ambientes aquáticos que são vulneráveis ao constante uso de pesticidas. Alguns biomarcadores podem ajudar a avaliar o estado de saúde, indicando alterações físicas, metabólicas e comportamentais de intoxicações agudas e subletais. A mistura dos ingredientes ativos picoxistrobina e ciproconazol é amplamente usada como fungicida para o controle de pragas da cultura de algodão, arroz, café, cana-de-açúcar, milho, soja e trigo. O objetivo deste trabalho foi verificar a ocorrência de possíveis lesões histopatológicas em fígado e rins de girinos de rã-touro (*Lithobates catesbeianus*) causadas pela mistura dos fungicidas picoxistrobina e ciproconazol. Os animais foram submetidos a diferentes concentrações do fungicida para determinação da concentração letal mediana ($CL_{50\text{-}96h}$ = 0,05 mg L^{-1}), ou seja, a dose letal para 50% dos animais em 96 horas. Após a determinação do valor da $CL_{50\text{-}96h}$, os animais foram submetidos a três concentrações subletais ($CL_{50\text{-}96h/2}$, $CL_{50\text{-}96h/10}$ e $CL_{50\text{-}96h/100}$). Através dos biomarcadores histológicos, a pesquisa verificou que esse fungicida alterou a morfologia dos tecidos renais e hepáticos dos animais de maneira crônica, prejudicando o funcionamento de órgãos que são fundamentais para sua sobrevivência e metamorfose, o que pode resultar em um desequilíbrio para a biodiversidade dos ecossistemas aquáticos.

Palavras-chave: anfíbios; ecotoxicologia; rim; fígado; picoxistrobina; ciproconazol.

INTRODUCTION

A systemic fungicide that contains picoxystrobin (from the strobilurin group — mitochondrial respiration electron flow inhibitors) and cyproconazole (from the triazole group — ergosterol biosynthesis inhibitors) is used in preventive sprays for the control of aerial diseases of cotton, rice, coffee, sugarcane, corn, soybean, and wheat crops. In Brazil, there are some commercial formulas that contain these two active ingredients, such as Aproach® Prima. This pesticide was classified as moderately toxic to humans (toxicological class II), but in 2019, it was reclassified as product

unlikely to cause acute injury (toxicological class V) through evaluations (ANVISA, 2019). Regarding the classification of the potential for environmental hazard, it belongs to class II, which is very dangerous to the environment (MAPA, 2022). There is no maximum limit allowed for this pesticide in Brazilian waters, but it has already been found and recorded in inland waters (CETESB, 2021). Therefore, this fungicide was chosen to carry out ecotoxicological tests, due to its use in the cultivation of flooded rice, which is the main crop in the region of Vale do Paraíba, São Paulo, Brazil, and which possesses a large interface with the aquatic environment.

According to data from the product package insert, the recommended dose for rice cultivation is 300–400 mL ha^{-1}. The insert also presents data on acute toxicity in rats, rabbits, and guinea pigs as well as chronic effects only on rats, mice, and dogs, without mentioning its impact on aquatic organisms. In rabbits, skin and mucosal contact causes eye and skin irritation and ingestion causes increased liver weight, liver hypertrophy, histopathological changes, and liver damage. In severe exposures, diarrhea, vomiting, kidney failure, impaired consciousness, and respiratory distress were reported, according to the product package insert data.

Anuran amphibians occur on all continents and have a wide geographic distribution, except for Antarctica, and are abundantly observed in tropical forests (Amphibia, 2008; Dufresnes, 2019). Some specific characteristics, such as strong dependence on the environment in which they live, aquatic and terrestrial life cycles, epidermis permeable to water and electrolytes, shelled eggs, being ectothermic, and the fact that they occupy different positions in the food chains, make this group very vulnerable to environmental changes (Verdade et al., 2010). These changes can be briefly described as the presence of contaminants in water, changes and fragmentation of habitats, introduction of exotic species, climatic effects, emerging diseases, or the use of chemical pollutants. Due to these characteristics and the observed population decline of certain species, these animals have been recognized as bioindicators of environmental quality (Pinto-Vidal et al., 2022) and are increasingly used in ecotoxicological studies (Mikó et al., 2017; Nkontcheu et al., 2017).

Bullfrogs *(Lithobates catesbeianus*) have been used in ecotoxicological tests (Viriato et al., 2021; Grott et al., 2022; Pontes et al., 2022). They represent an excellent experimental tool, as they are rustic, tolerant to diseases and infections, can be found in commercial farms, and, at the same time, are sensitive to exposure to pollutants (Ossana et al., 2013; França et al., 2015; Miaud et al., 2016).

The evaluation of biological responses and the understanding of how the environment affects amphibian communities are the major challenges for research in the area of aquatic toxicology (Giagnessi and Reigner, 2006; Jha, 2008). Toxicity tests are required by law in Brazil and in other parts of the world, with the objective of evaluating the potential environmental risk of contaminants, since chemical analyses alone do not allow this type of evaluation in a satisfactory manner (Costa et al., 2008).

One method that enables the assessment of the influence of contaminants on aquatic organisms is the observation of histopathological changes, using them as a biomarker of the effects of exposure to toxic substances, which can help establish a causal relationship between exposure to toxic substances and the occurrence of changes. Thus, the aim of this study was to determine acute toxicity and observe possible toxic effects of the picoxystrobin and cyproconazole fungicides mixture through histopathological changes in the liver and kidneys of bullfrog tadpoles (*L. catesbeianus*).

MATERIAL AND METHODS

This work was authorized by the Ethics Committee of the Fisheries Institute under opinion 14/2016. For both acute and chronic toxicity tests, dilutions of the fungicide were carried out directly from the commercial formula Aproach® Prima composed of picoxystrobin [methyl (E)-3-methoxy-2-{2-[6-(trifluoromethyl)-2-pyridyloxymethyl] phenyl} acrylate] (200 g L^{-1}) and cyproconazole [2RS,3RS;2RS,3SR)-2-(4-chlorophenyl)-3-cyclopropyl-1-(1H-1,2,4-triazol-1-yl) butan-2-ol] (80 g L^{-1}). With 60 days of development after hatching, the *L. catesbeianus* tadpoles at stage 26 of Gosner's table (1960), from the same location, were removed from the breeding tanks and acclimated for 7 days in aquariums with dechlorinated water and a photoperiod of 12:12 h, along with artificial aeration and with a density of 1 tadpole per liter of water.

The physical and chemical parameters of the water were monitored daily from the beginning of the acclimation period until the end of the final acute toxicity tests and demonstrated the following mean values: temperature 22.8 ± 0.9°C, pH 7.2 ± 0.2, dissolved oxygen 7.2 ± 0.6 mg L^{-1}, and electrical conductivity 124 ± 2 µS cm^{-1}. Acute toxicity tests were performed according to standardized methods proposed by ASTM E729-96 (2014). The average weight of the animals used to carry out the acute toxicity tests was 1.23 ± 0.25 g. To determine the concentrations used in the test, preliminary assessments were carried out to define the ranges between the total absence of effect and 100% effected (mortality), which allowed the calculation of the limits to be used in the definitive test. The definitive test was conducted in a static system for 96 h, using 3 L aquariums with the animals randomly distributed, at the maximum density of 1 tadpole L^{-1}, totaling six treatments plus the control group conducted with four replicates until obtaining the concentration that caused 100% mortality. The treatments and respective nominal concentrations used in the definitive test were as follows: C (control): 0, T1: 0.009, T2: 0.018, T3: 0.036, T4: 0.072, T5: 0.144, and T6: 0.288 mg L^{-1}. Mortality was recorded every 24 h until the end of the trial and dead individuals were counted to calculate the median lethal concentration value ($LC_{50\text{-}96h}$). The observed $LC_{50\text{-}96h}$ was compared according to the classification of the Globally Harmonized System Classification and Labeling of

Chemicals (United Nations, 2011), where it is established that the compounds can be classified into three categories after exposure for 96 h: high toxicity (LC_{50} < 1 mg L^{-1}), moderate toxicity (LC_{50} between 1 and 10 mg L^{-1}), and low toxicity (LC_{50} > 10 mg L^{-1}). After the acute toxicity tests, and once the $LC_{50\text{-}96h}$ was determined, the chronic toxicity test was initiated. To calculate the $LC_{50\text{-}96h}$, the trimmed Spearman-Karber statistical method (Hamilton et al., 1977) was used. The experiment was conducted in a semi-static system, lasting 14 days, with solutions being renewed every 96 h, and the animals were fed 24 h before this renewal. The density applied was 1 tadpole L^{-1}, using 160 tadpoles randomly collected in the acclimatization tank and transferred to 16 aquariums with a capacity of 10 L equipped with artificial aeration. Three sublethal concentrations ($LC_{50\text{-}96h}/2$ = 0.025 mg L^{-1}, $LC_{50\text{-}96h}/10$ = 0.005 mg L^{-1}, and $LC_{50\text{-}96h}/100$ = 0.0005 mg L^{-1}) and a negative control group (also with four simultaneous replicates) were used for the tests of this step. Daily, the animals that died were removed and the volume of solution in the aquarium was adequate to maintain the initial density. The physical and chemical parameters of the water (pH, dissolved oxygen, electrical conductivity, and temperature) were measured before and after each solution change. At 7 and 14 days of exposure, eight tadpoles from each treatment were removed and anesthetized in eugenol (7 mL L^{-1}) and later euthanized by deep anesthesia to remove liver and kidney samples, which were preserved in 10% buffered formalin (n = 32). Then, the samples were processed in duplicate for histological analysis, according to the methodology of Gartner and Hiatt (1999).

To perform the histopathological analysis, liver and kidney fragments were dehydrated in a growing series of alcohol baths (70, 80, 95%, and absolute). Then, they were cleared in xylene, embedded in paraffin, and placed in an oven at 52°C for 18 h. After being incorporated into paraffin, the fragments were cut into 5-µm thick sections, with the aid of a Zeiss® microtome, and placed on slides. Next, they were placed in bath of xylene and decreasing alcohol concentrations for the removal of paraffin. Then, they were rehydrated and were subjected to staining with hematoxylin and eosin (H&E), ending with an immersion bath in an increasing series of alcohol and xylene. The tissue section was covered with Etellan® and a coverslip and observed in a Zeiss® Axio microscope, with direct light coupled to a Zeiss® AxioCam ERC5S camera to capture the images.

RESULTS

The median lethal concentration at 96 h ($LC_{50\text{-}96h}$) for *L. catesbeianus* tadpoles was 0.05 mg L^{-1}. The lower confidence limits were 0.03 mg L^{-1} and upper confidence limits were 0.07 mg L^{-1}. There was no mortality in the control group and the result of the $LC_{50\text{-}96h}$ indicates that, according to the GHS (United Nations, 2011), the fungicide tested is highly toxic (LC_{50} < 1 mg L^{-1}) for these animals. LC_{50} data were not found in the literature referring to the substances that make up the tested.

The mean values of the physical and chemical parameters of the water measured before and after the solution renewal, respectively, during the chronic toxicity test were as follows: temperature 22.9 ± 0.7°C and 22.9 ± 0.9°C; dissolved oxygen 7.0 ± 0.3 and 6.7 ± 0.5 mg L^{-1}; pH 7.3 ± 0.2 and 7.7 ± 0.4; and electrical conductivity 121.0 ± 2.0 and 123.0 ± 3.0 µS cm^{-1}. They remained within the proper conditions for the maintenance of these animals in toxicology experiments (Viriato et al., 2021).

No marked differences were observed in histopathological changes in the liver and kidney of tadpoles, in the samples collected at 7 and 14 days. However, in treatments of higher concentration ($LC_{50\text{-}96h}/2$ = 0.025 mg L^{-1}, $LC_{50\text{-}96h}/10$ = 0.005 mg L^{-1}, and $LC_{50\text{-}96h}/100$ = 0.0005 mg L^{-1}), we observed increase of melanomacrophages (MMs) number, with evidence of areas with large centers of MMs. It is interesting to note that vacuolization was already evident even in the control group and probably occurred due to nutritional causes (Figure 1).

Kidney changes are shown in Figure 2. In the control group, there were no significant histopathological changes, but in animals submitted to expose by fungicide mixture for 7 days, we can observe some low severity injuries. At the highest chronic concentration tested (0.025 mg L^{-1}), increase in interstitial spaces, areas of hyaline necrosis (tubulonephrosis) as well as hypotrophic tubules and infiltrated inflammatory cells were observed.

DISCUSSION

The liver and kidneys of amphibians perform a number of important functions related to the metabolism and excretion of substances. Damage to these organs caused by chemical pollutants can have negative consequences on the detoxification and homeostasis of animals (Monteiro et al., 2018). Mostly, nutritional causes are responsible for the biochemical or structural imbalance in these organs. However, for amphibians, the inappropriate use of pesticides and xenobiotics causes such severe negative effects on metabolism that it can have physiological consequences that are often irreversible (Guyton and Hall, 2002; Hipolito et al., 2004; Viriato et al., 2021).

The mixture of these two active ingredients is widely used in rural areas and the initial hypothesis of the present study is that they would cause serious damage to liver and kidney tissues. However, we found low severity injuries in the liver and a large amount of MMs.

The liver has MMs which are rounded cells capable of producing and storing pigment in its interior and are also found in other hematopoietic organs of amphibians, such as liver, spleen, and kidneys, as well as in other vertebrates, such as fish and reptiles (Fishelson, 2006). The function of MMs is to destroy, detoxify, or recycle endogenous and exogenous materials, acting with a bactericidal and an immunity role (Agius

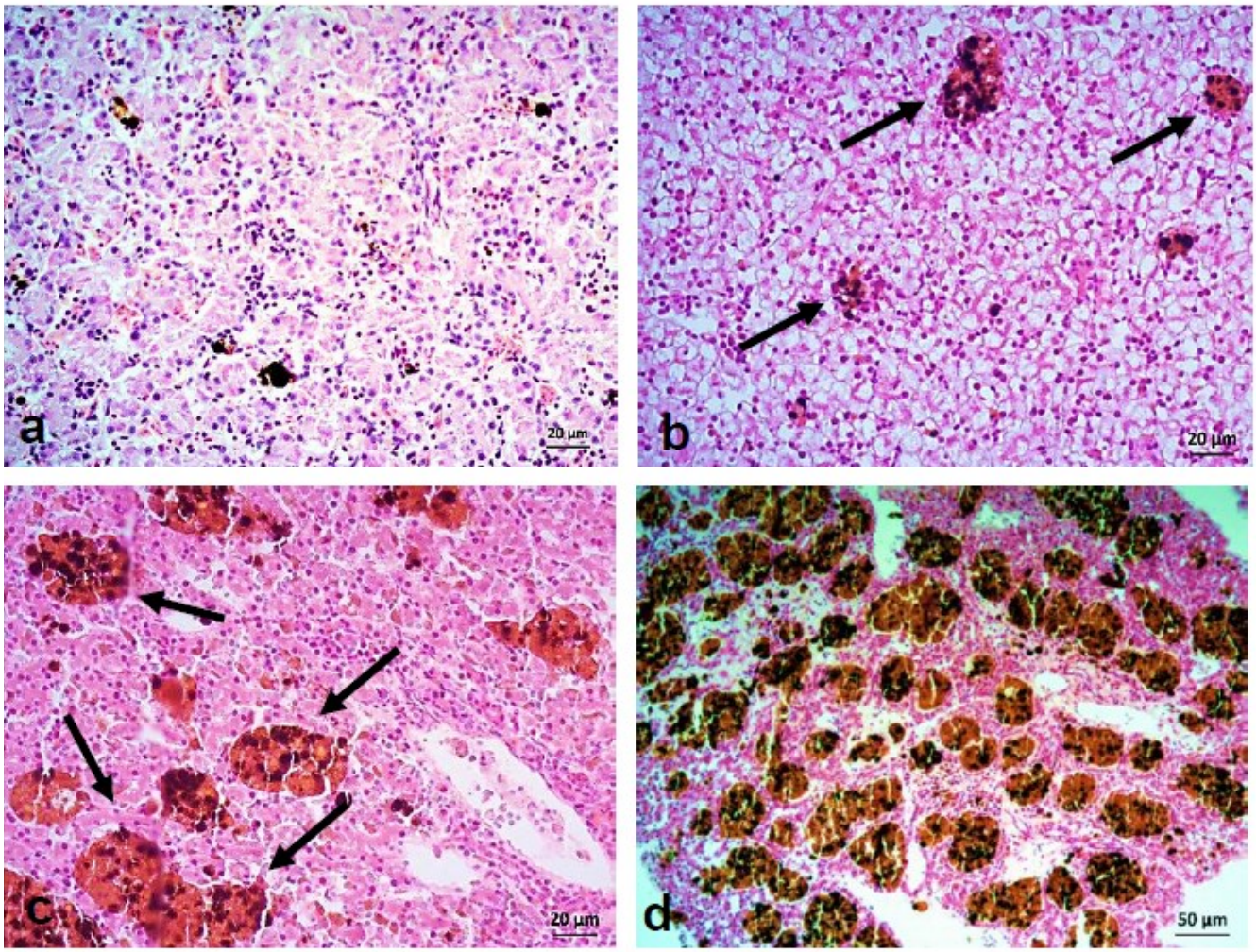

Figure 1. Photomicrograph of livers of bullfrog tadpoles (*Lithobates catesbeianus*) exposed for 7 days to picoxystrobin and cyproconazole fungicides mixture (H&E staining; $LC_{50\text{-}96h}$ 0.05 mg L^{-1}). (A) Control group: liver tissue with hepatocytes and few melanomacrophages. 200× magnification. (B) $LC_{50/100}$ (0.0005 mg L^{-1}): areas with centers of melanomacrophages (arrow) and vacuolization of hepatocytes. 200× magnification. (C) $LC_{50/10}$ (0.005 mg L^{-1}): large centers of melanomacrophages in brown (arrow). 200× magnification. (D) $LC_{50/2}$ (0.025 mg L^{-1}): detail of areas with a lot of brown melanomacrophage centers. 100× magnification.

and Roberts, 2003; Franco-Belussi et al., 2013). Their quantity, size, and distribution vary in different species, and depending on the animal's age and the presence of stress factors, they are often used as biomarkers of environmental contamination, including as a biomarker of the consequences of climate change in amphibians (Passantino et al., 2014; Santos et al., 2014). These cells can aggregate, forming the so-called melanomacrophage centers (Agius, 1981). They are macrophages that store pigments such as melanin, a pigment endogenously synthesized in vertebrates and invertebrates (Césarini, 1996) that has the function of absorbing and neutralizing free radicals, cations, and other potentially toxic agents, derived from the degradation of phagocytosed cell material (Zuasti et al., 1998); hemosiderin, granular substance derived from the catabolism of hemoglobin in erythrocytes; and lipofuscin, derived from the oxidation of fatty acids (Kranz, 1989). According to Fenoglio et al. (2005), the presence of MMs indicates a detoxification response, as they are important neutralizing agents against free radicals. Some studies show morphological changes in MMs when there is exposure to contaminants (Franco-Belussi et al., 2013; Çakici, 2015). Therefore, these cells are used as contamination biomarkers (Paetow et al., 2012; Santos et al., 2014). In fish, MM aggregates usually increase in number and size when exposed to toxic substances, playing an important role in resistance against infectious agents (viruses, bacteria, and parasites) (Agius and Roberts, 2003). There was no quantification of these structures, but they were accompanied by increasing and dose-dependent histopathological lesions, which can be seen in Figure 1.

Bach et al. (2018), analyzing the effects of exposure of *Leptodactylus latrans* tadpoles to the herbicide Roundup®

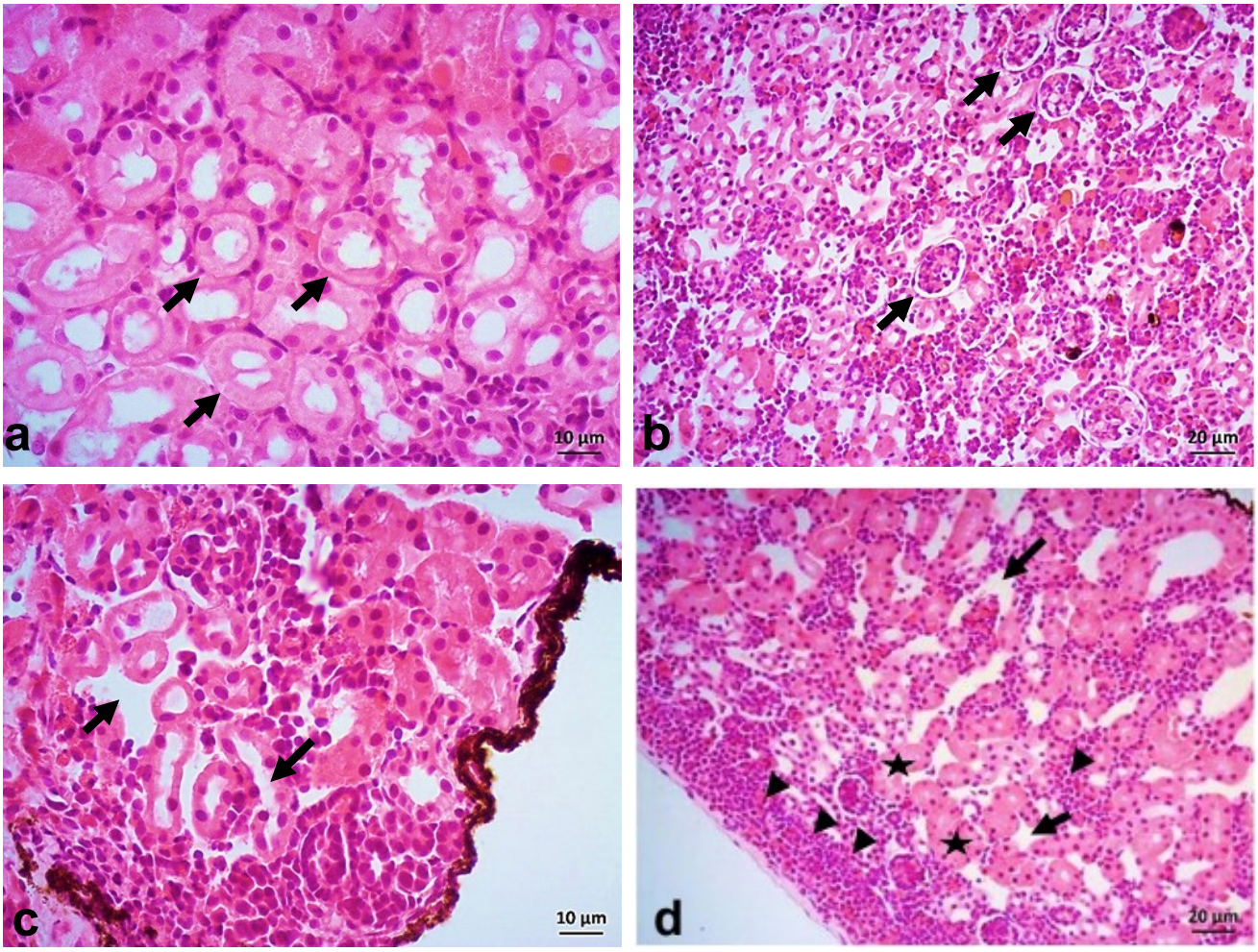

Figure 2. Photomicrograph of kidneys of bullfrog tadpoles (*Lithobates catesbeianus*) exposed for 7 days to the picoxystrobin and cyproconazole fungicides mixture (H&E staining; $LC_{50\text{-}96h}$ 0.05 mg L^{-1}). (A) Control group: renal tissue with glomerulus and Bowman's capsule (black arrow). 400× magnification. (B) $LC_{50/100}$ (0.0005 mg L^{-1}): increased interstitial space (black arrow). 200× magnification. (C) $LC_{50/10}$ (0.005 mg L^{-1}): areas of dilated interstitial space (black arrow). 400× magnification rim. (D) $LC_{50/2}$ (0.025 mg L^{-1}): increase in interstitial spaces (black arrow), areas of hyaline necrosis (tubulonephrosis) (star), infiltrated inflammatory cells (monolymphocytic and eosinophilic), glomerulonephritis (arrowhead). 200× magnification.

Ultramax based on glyphosate, observed a picture similar to what was found herein, with an increase in MMs and liver histopathological lesions, including lipidosis and liver congestion. Similar results were observed by Boncompagni et al. (2004) analyzing the effects of exposure of *Rana esculenta* tadpoles to hexavalent chromium. They observed a reduction in the volume of hepatocytes, marked eosinophilia, and an increased number of MMs (exudative macrophages or Kupffer cells). Jayawardena et al. (2017) also observed the presence of inflammatory infiltrates, karyocytomegaly, and Kupffer cells, as well as hypertrophy in the adult liver of *Euphlyctis hexadactylus* submitted to a mixture of heavy metals (Cu, Cd, Cr, Zn, and Pb); however, with an exposure period longer than that applied herein (i.e., 28 days).

The low severity alterations observed such as dissociation of the hepatic trabeculae, monolymphocytic and eosinophilic hepatitis, dilation of blood vessels and sinusoids, blood congestion, and hemorrhagic areas could also be observed after 7 days of exposure to the pesticide, indicating rapid metabolic kinetics of aggression. During the detoxification process, the cells that are being attacked are damaged, causing lesions as well as cell and tissue disorganization. Such changes can harm the frogs' metamorphosis process, since the animal is losing part of its normal metabolic function which uses nutrients to produce enzymes, proteins, and other elements necessary to carry out the process. Damaged cells lose part of their normal metabolic function, such as the production of proteins and amino acids, not being able to utilize nutrients, which can lead to deficiencies.

The kidneys of *L. catesbeianus* are elongated organs that extend from the cloaca region and nearly to the heart. Histologically, they contain numerous renal corpuscles, and their Bowman capsule consists of a simple squamous cell layer (Manso et al., 2009). These kidneys play important roles related to metabolism and excretion of substances and injuries caused by chemical pollutants

or xenobiotic agents, which may have negative consequences on the animal's detoxification and homeostasis processes (Medina et al., 2016). In this study, areas of tissue necrosis and renal tubule hypertrophy were observed, similar to the results found by Monteiro et al. (2018), who exposed bullfrog tadpoles to chromium.

The different degrees of injury observed at different concentrations may be due to the rate of metabolization of the pigments hemosiderin and lipofuscin, which may have changed between treatments at different concentrations and at different times of collection (day 7 and day 14). Hemosiderin is derived from the hemoglobin of erythrocytes that migrate to the spleen when they end their half-life and are degraded. Lipofuscin, on the other hand, arises from indigestible residues from cellular metabolism that gradually polymerize and form insoluble molecular complexes. Studies carried out by Medina et al. (2016) using cadmium showed mixed inflammatory infiltrates in the kidneys of *L. catesbeianus* with a predominance of polymorphonuclear cells, hypertrophied glomeruli, hydropic degeneration, tubular necrosis, expansion of the glomerular mesangium, presence of amorphous eosinophilic intraluminal material (amorphous substance in the cytoplasm), and several granulomas, alterations that were also found herein.

On the other hand, it is a fact that there was no mortality during chronic exposure as a result of picoxystrobin and cyproconazole fungicides mixture; however, the structural changes, even if it has low severity injuries, demonstrated by the histological findings prove the involvement of the organisms' vital organs. In summary, it can be concluded that this pesticide changed the morphology of the kidney and liver tissues of bullfrog tadpoles, in a short period of exposure, interfering directly with fundamental processes for the maintenance of its homeostasis. In amphibians, the liver and kidney perform several physiological functions, including energy and protein metabolism, urea synthesis, secretion of bile salts, biotransformation, and detoxification in response to toxic or infectious agents in a similar way to other vertebrates. This detoxification may manifest as signs of an organic disorder such as nutritional deficiency, poisoning, infections, or parasitism, through changes in its cellular, biochemical, and morphological structure (Crawshaw and Weinkle, 2000).

CONCLUSION

The picoxystrobin and cyproconazole fungicides mixture induced the structural changes during chronic exposure, as demonstrated by the histological findings, indicating the involvement of the organisms and vital organs. For this reason and for the important effects in a short period of exposure, this pesticide should be used with caution.

CONFLICT OF INTERESTS

Nothing to declare.

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AUTHORS' CONTRIBUTION

Marcantonio, A.S.: Conceptualization, Data curation, Project administration, Supervision, Formal Analysis, Funding acquisition, Investigation, Methodology, Validation, Visualization, Writing — original draft, Writing — review and editing. França, F.M.: Formal Analysis, Investigation, Methodology, Validation, Visualization, Writing — original draft. Santos, D.S.: Investigation, Methodology. Martins, A.M.C.R.P.F.: Investigation, Validation, Visualization, Writing — original draft. Hipólito, M.: Validation, Visualization, Writing — original draft. Schalch, S.H.C.: Writing — original draft. Viriato, C.: Visualization, Writing — original draft. Ferreira, C.M.: Formal Analysis, Investigation, Methodology, Validation, Visualization, Writing — original draft, Writing — review and editing.

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