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Original Article

Evaluation of the acute and sublethal toxicity of Mancozeb in Pacamã (*Lophiosilurus alexandri*)

Avaliação da toxicidade aguda e subletal do Mancozebe em Pacamã (*Lophiosilurus alexandri*)

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Abstract

The toxic potential of dithiocarbamates fungicides widely used in world agriculture is well known, among which Mancozeb is one of the most used. This study aimed to evaluate the toxicity of Mancozeb, determining the LC50% of the product and the behavioral and histological changes observed in fish of the Pacamã species through acute and sublethal toxicity tests. The first experiment was carried out on Pacamã fingerlings exposed to dosages of 0.5, 1, 2, 4, and 8mg/L of Mancozeb under the form ManzateWG®, for a total period of 96 hours in the acute experiment, and in the second experiment, fish were subjected to concentrations of 1/10 of those used in the acute experiment (0.05, 0.1, 0.2, 0.4 and 0.8mg/L, respectively), for 15 days in total. The 50% lethal concentration of ManzateWG® was calculated at the end of the acute experiment, presenting a value of 2.29mg/L at 96h for Pacamã fingerlings. A behavioral assessment was carried out through daily observation of the fish during both experiments, and an increase in mucus production was observed, as well as atypical social behavior in those exposed to the toxic agent. Histopathological evaluation was performed on livers collected after the end of the sublethal experiment, and the main hepatic alterations observed were cytoplasmic vacuolization, inflammatory infiltrate, and necrosis. Mancozeb has toxic potential and is capable of generating behavioral changes, as well as increasing the risk of liver damage in Pacamãs exposed to this compound.

Keywords: biomarkers, ecotoxicology, fish, LC50.

Resumo

O potencial tóxico dos fungicidas ditiocarbamatos amplamente usados na agricultura mundial é bem conhecido, e entre estes, o Mancozebe é um dos mais utilizados. Esse estudo teve como objetivo avaliar a toxicidade do Mancozebe, determinando a CL50% do produto e as alterações comportamentais e histológicas, observadas em peixes da espécie pacamã através de testes de toxicidade aguda e subletal. O primeiro experimento foi realizado em alevinos de pacamã expostos a dosagens de 0,5, 1, 2, 4 e 8mg/L de Mancozebe sob a forma ManzateWG®, por um período total de 96 horas no experimento agudo, e no segundo experimento os peixes foram submetidos a concentrações de 1/10 daquelas utilizadas no experimento agudo (0,05, 0,1, 0,2, 0,4 e 0,8mg/L, respectivamente), a exposição no segundo experimento foi de 15 dias. A concentração letal a 50% do ManzateWG® foi calculada ao fim do experimento agudo, apresentando um valor de 2,29mg/L às 96h para alevinos de pacamã. Avaliou-se o comportamento por meio da observação diária dos peixes, notando-se o aumento da produção de muco assim como comportamento social atípico naqueles indivíduos expostos ao agente tóxico. A análise histopatológica foi conduzida nos fígados coletados após a conclusão do experimento subletal, identificando as principais alterações hepáticas como vacuolização citoplasmática, infiltrado inflamatório e necrose. O Mancozebe apresenta potencial tóxico e é capaz de gerar alterações comportamentais, assim como aumentar o risco de lesões hepáticas em pacamãs expostos a este composto.

Palavras-chave: biomarcadores, ecotoxicologia, peixe, CL50.

1. Introduction

Around the world, pesticide poisoning has been identified as the cause of mortality in several species of fish. Animals that occupy high trophic levels also suffer the effect of biomagnification, where they consume organisms with high levels of pesticides in their tissues and organs, magnifying their exposure to such compounds (Yancheva et al., 2016; Stoyanova et al., 2019).

The number of agrochemicals available on the Brazilian market has grown exponentially over the years (Souza et al., 2020), and dithiocarbamate fungicides, such as Mancozeb,

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have been widely used in agriculture and soil treatment (Weis et al., 2019; Botelho et al., 2020). The assessment of environmental damage caused by pollution resulting from pesticide residues can be done through tests carried out with different organisms exposed to different concentrations of toxic substances dispersed in water; these assays are called toxicity tests (Sisinno and Oliveira-Filho, 2013). This evaluation is also performed through the histopathological evaluation of fish tissues exposed to toxic agents, and through these tests it is possible to identify new biomarkers (Yancheva et al., 2016). Biomarkers are alterations caused in the animal organism due to its exposure to a substance, and there are several types of markers; they are relatively inexpensive methods that can be useful to demonstrate the effects of xenobiotic agents in animals (Jesus et al., 2020).

However, studies on the toxicity of Mancozeb in fish are still scarce (Saha et al., 2016), especially in species native to Brazil, such as Pacamã. Thus, more studies are needed on how Mancozeb affects fish and local aquatic ecosystems. This study aimed to evaluate the acute and sublethal toxicity of Mancozeb in Pacamã fingerlings, by determining the 50% lethal concentration (LC50) and evaluating behavioral and histopathological alterations.

2. Material and Methods

The study was carried out in two stages, consisting of a short-term experiment (acute test) and a longer experiment (sublethal test).

The study was performed with fingerlings of Pacamã (*Lophiosilurus alexandri)*, with an average weight of 7.7±0.87g and an average length of 10.0±0.42cm, clinically healthy. The fish were kept in acclimatization for 10 days, according to the recommendations of the Organization for Economic Cooperation and Development's (OECD, 2019) acute toxicity test standards for fish.

The fungicide used was Manzate®WG, a commercial product based on Mancozeb, composed of 750 g/kg (75% m/m) of Mancozeb (manganese ethylene bisdithiocarbamates with a polymer complex of zinc salts) and 250 g/kg (25% m/m) of inert ingredients.

2.1. Experimental design and animals

Both experiments took place in the animal pathology laboratory located within the university and were approved by the Animal Ethics Committee, under protocol number 0002/270619.

2.1.1. Acute toxicity test

The acute experiment was carried out over a total period of 96 hours, with observation and quantification of mortality once a day (every 24 hours). For this purpose, 6 glass aquariums with 50 liters of dechlorinated water were used in a static system with artificial aeration. Five different concentrations of fungicide and a control (without fungicide) were used. Fish feeding was also suspended 24 hours before the beginning of the experiment.

The exposure was carried out on day zero, using concentrations of 0.5, 1, 2, 4, and 8mg/L of Manzate®WG, respectively. The fish were allocated to aquariums, making a total of 7 fish per aquarium/treatment, without repetition, according to the OECD protocol (2019). The groups were named T1, T2, T3, T4, and T5 according to the concentration the group was exposed to, being T1 the lowest concentration and T5 being the highest; the control group was called such.

Water quality parameters such as dissolved oxygen, pH, temperature, conductivity, and total ammonia (through colorimetric kits) were measured at the beginning of the experiment and also every 24 hours.

Observations were made twice a day regarding the behavior of the animals for 2 minutes/group under physical stimulation (light agitation of the water using a sanitized metal rod), observing social and swimming activity, and mucus production. The occurrence of mortality was observed once a day, in the afternoons, and dead animals were removed from the aquariums whenever necessary. At the end of the experiment, the surviving animals were euthanized by anesthetic deepening with benzocaine.

Using the daily mortality rate, the LC50 was calculated. The 50% lethal concentration (LC50%) used was based on the product formulated as Manzate®WG.

2.1.2. Sublethal toxicity test

The sublethal test consisted of exposing the animals to different sublethal concentrations of Mancozeb, which were 0.05, 0.1, 0.2, 0.4, and 0.8mg/L, corresponding to 1/10 of each dose previously used in the acute test, in addition to a control group not exposed to the pesticide. The experiment was carried out over a period of 15 days, with organ and tissue collection at the end of this period. Just like in the previous experiment, exposure to the product was unique and performed on day zero. 42 Pacamã fingerlings were used, distributed in 6 fifty-liter aquariums, with 7 individuals per aquarium/treatment, the groups were named in the same way as the first experiment. There was no suspension of feeding, the animals continued to be fed with fish larvae, once a day, throughout the test period.

Water quality parameters (pH, DO, and temperature) were measured as described in the acute test, while total ammonia was measured weekly. Mortality was checked twice a day, and dead fish were removed. After the end of the experiment period, the surviving fish were collected from the aquariums and euthanized by anesthetic deepening, then we proceeded with the collection of the liver for histopathological evaluation.

After collection, the liver samples were fixed in a 10% neutral buffered formalin solution and subsequently processed following a standard tissue dehydration protocol. Slides stained with Hematoxylin-Eosin (HE) were prepared from the samples and analyzed using light microscopy. The sections were analyzed under a white light microscope, and the identified alterations were individually classified based on the degree of lesion frequency in the tissue. For this purpose, the following scoring per occurrence/distribution of alteration per slide was used: 0 – alteration not observed; 1 – mild (up to two occurrences of alteration per slide); 2 – moderate (three to five occurrences of alteration per

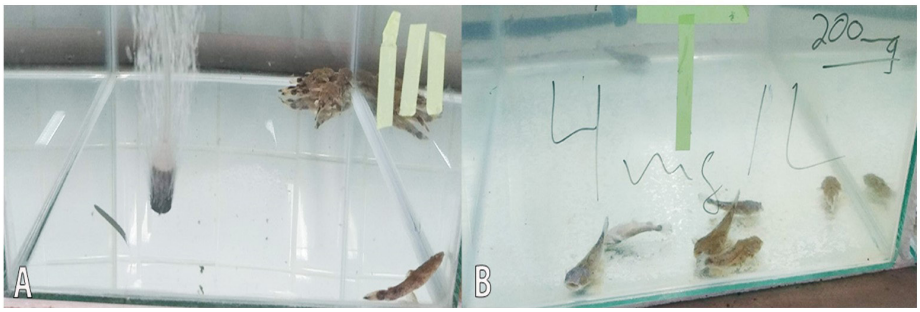

Figure 1. Social behavior of the Pacamã. (A) Fish not low concentrations of Mancozeb stay together; (B) Fish exposed to 4mg/L of Mancozeb (group T4) scattered around the aquarium.

slide); and 3 – severe (above five occurrences of alteration per slide). Using these scoring values, an average degree of alteration was calculated for each sample time, and they were classified as absent (without alterations), mild (0.1 to 1.0), moderate (1.1 to 2.0), and severe (2.1 to 3.0).

2.3. Statistical analysis

To determine the LC50%, the observed mortality data were evaluated with the Mosaic statistical software (University of Lyon, 2017), using the maximum likelihood analysis method by Bayesian inference, under the mathematical formula 1:

$$f(c) = \frac{d}{1 + \left(\frac{c}{e}\right)b} \quad (1)$$

The analysis of the difference between groups and within groups in the acute test was performed using the analysis of double variance ANOVA. For all statistical analyzes performed, a significance level (p) of 5% ($p<0.05$) was adopted.

3. Results

During both experiments, in the control group water quality remained within normal limits with an average pH of 7.4±0.1, dissolved oxygen (DO) of 7.3±0.6mg/L, and an average temperature of 24±0.4 °C. As for the groups exposed to the toxic agent, there was only a slight variation in the pH, where an average of pH 8.1±05 was observed, remaining the other measures similar to the control group. Water conductivity and ammonia levels remained within standard values (below 500 µS/cm and 0.05 mg/L, respectively) (Masood et al., 2022).

3.1. Acute toxicity test

Regarding fish behavior, a reduction in social activity and swimming patterns was observed in the three groups exposed to higher concentrations (T3, T4, and T5, respectively). In these groups, the fish were scattered throughout the aquarium, while those in the other groups (T1, T2, and control) tended to remain together, stacked one on top of the other, as is usual for the species (Figure 1).

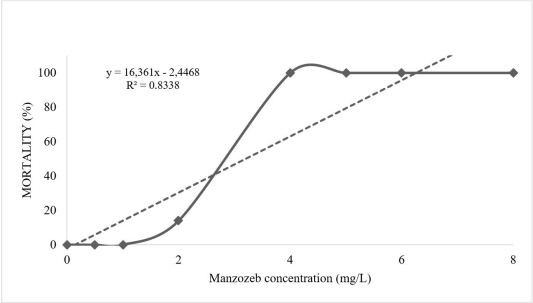

Figure 2. Mortality of Pacamã fingerlings exposed to different concentrations of Mancozeb for 96 hours.

During the test period, fish from all exposed groups showed increased mucus secretion in the gills since the first hours of exposure. Secretion was more evident in groups exposed to higher concentrations, especially hours before death. The mucus presented was colorless, smooth, and slightly sticky, and was found all over the animal's body, more prominently around the gills.

Loss of balance was not observed in the groups treated with doses of less than 2mg/L of Mancozeb (T1 and T2), but it was evident in the groups treated with higher concentrations (T4 and T5) after a few hours of exposure, becoming more evident moments before the death of individuals. In the group exposed to 2mg/L (T3), moderate loss of balance was observed after 48 hours in three of the seven fish, however not assuming the same intensity observed in the other groups until the last day of the experiment.

As for mortality, no deaths were observed in the control group during the experiment, as well as in the groups treated with doses under 2mg/L of Mancozeb (groups T1 and T2). In the exposed groups, mortality varied according to the time and dose of exposure. There was a significant difference ($p<0.05$) in the mortality of the groups exposed to concentrations above 2mg/L (groups T4 and T5) compared to the other exposed groups (Figure 2).

Variation in the mortality time was observed between groups T3, T4, and T5, where in the last two groups (T4 and T5) a complete mortality of the animals was observed at 24 hours, while in the T3 group, only one death was observed at the end of the experiment. Mortality ranged from 0% in

the control group and in the groups exposed to doses lower than 2mg/L (T1 and T2), to 14% in the group T3 exposed to moderate doses (2mg/L), and 100% in the groups treated with 4 and 8mg /L (T4 and T5, respectively) of Mancozeb.

The toxicity assessment of Mancozeb was conducted through the analysis of the lethal concentration observed at different levels, from 1 to 80, focusing on the LC50 corresponding to the lethality rate for 50% of the population, the most used measure in ecotoxicological studies (Table 1).

Mancozeb showed a dose and time-dependent toxic effect. The concentration found for the LC50 in the tested species, Pacamã, was initially 2.76mg/L at 24 hours (shortest measured exposure time), remaining relatively stable and presenting values of 2.76 and 2.77 at 48 and 72 hours, respectively, and finally showing its lowest value, 2.29mg/L, at 96 hours (longest measured exposure time).

3.2. Sublethal toxicity test

Only one death was observed throughout the sublethal experiment, in group T4, on the second day of exposure.

Histopathological alterations were found in the livers of fish exposed to Mancozeb and also in those belonging to the control group, as shown in details in Table 2. More frequently, the presence of cytoplasmic vacuolization was observed, followed by necrosis (Figure 3). Only in the exposed groups, the occurrence of inflammatory infiltrate was observed (Figure 3). The occurrence of vacuolation

Table 1. Values (mg/L) of lethal concentration at 50% of Mancozeb in Pacamãs at different times (hours).

Lethal Concentration	Exposure time			
	24 hours	**48 hours**	**72 hours**	**96 hours**
CL50	2.76mg/L [2.11;3.73]	2.76mg/L [2.12;3.72]	2.77mg/L [2.12;3.74]	2.29mg/L [2.00;3.09]

Table 2. Percentage and absolute distribution of hepatic changes according to the group and grading.

Groups	Graduation	Hepatic Vacuolization	Hepatic Necrosis	Inflammatory Infiltrate
Control	**Absence**	71.43% (5)	71.43% (5)	100% (7)
	Grade I	28.57% (2)	28.57% (2)	-
	Grade II	-	-	-
	Grade III	-	-	-
T1	**Absence**	-	-	85.71% (6)
	Grade I	85.71% (6)	100% (7)	14.28% (1)
	Grade II	14.28% (1)	-	-
	Grade III	-	-	-
T2	**Absence**	14.28% (1)	14.28% (1)	14.28% (1)
	Grade I	57.14% (4)	85.71% (6)	71.43% (5)
	Grade II	28.57% (2)	-	14.28% (1)
	Grade III	-	-	-
T3	**Absence**	-	14.28% (1)	28.57% (2)
	Grade I	42.85% (3)	57.14% (4)	57.14% (4)
	Grade II	42.85% (3)	28.57% (2)	-
	Grade III	14.28% (1)	-	14.28%
T4	**Absence**	-	-	14.28% (1)
	Grade I	28.57% (2)	100% (7)	28.57% (2)
	Grade II	57.14% (4)	-	42.85% (3)
	Grade III	14.28% (1)		-
T5	**Absence**	-	-	14.28% (1)
	Grade I	14.28% (1)	-	28.57% (2)
	Grade II	71.43% (5)	85.74% (6)	57.14% (4)
	Grade III	14.28% (1)	14.28% (1)	-

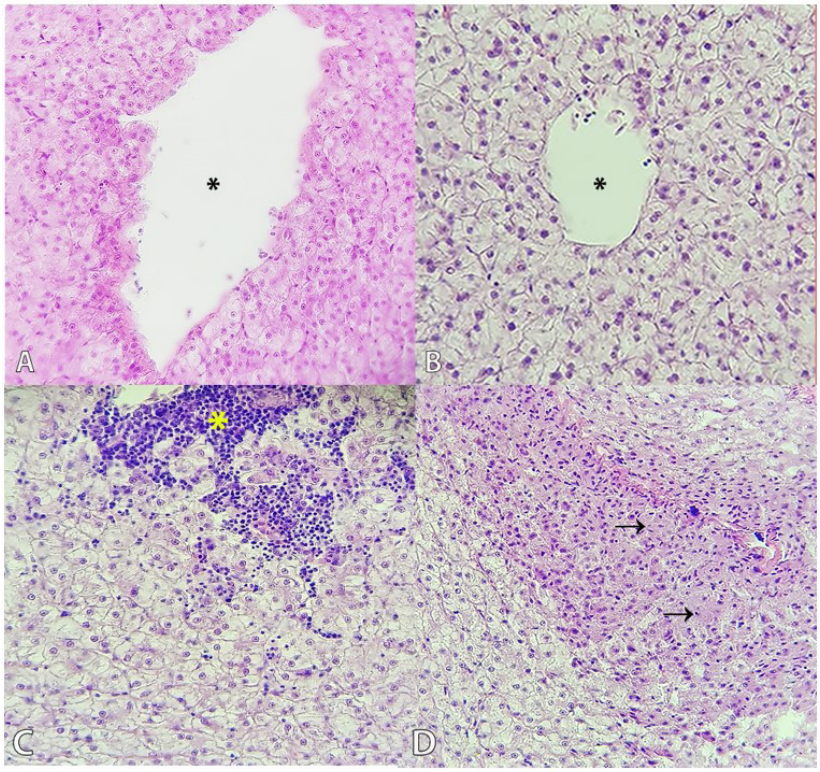

Figure 3. Histological sections of the liver tissue. (A) Fish exposed to T5 (0.8mg/L of Mancozeb), showing mild vacuolation. HE – obj. 40x; (B) Fish exposed to T5 for 15 days showing severe tissue vacuolation. HE – obj. 40x; (C) Inflammatory infiltrate (yellow asterisk) in an animal exposed to T5. HE – obj. 40x; (D) Animal exposed to T5, an extensive area of tissue necrosis is observed, with the presence of pyknotic nuclei (black arrows). HE – obj. 40x. Black asterisks (*) indicate central vein.

was observed in all groups, including the control group, at different intensities, with groups T3 and T4 presenting a greater intensity of vacuolation.

The occurrence of necrosis was observed in all groups as well as in the control group, however in the groups exposed to Mancozeb the grade of necrosis increased according to the dose of exposure; particularly at a higher dose (group T5), necrosis was more evident, being present in all animals and occupying larger areas of the tissue. Inflammatory infiltrate was observed only in the exposed groups, with greater intensity in group T4. The control group did not present inflammatory infiltrate in the liver.

4. Discussion

The water parameters observed in all groups, including the control group, during both tests, were considered within the comfort level for tropical species, which has an optimal pH range between 6 and 8, dissolved oxygen (DO) greater than 5 and less than 9, and temperatures between 20 and 28 °C (Masood et al., 2022).

4.1. Acute toxicity test

The evaluation of animal sensitivity to xenobiotics, through assays that demonstrate a correlation between the exposure dose and the animal's physiological response to a given agent, is important in ecotoxicological studies. Such sensitivity varies between species and age groups, with younger individuals being more susceptible; such factors should be taken into account when interpreting biomarkers (Sousa et al., 2019).

No previous studies were found about the toxic effect of Mancozeb on the species in question, Pacamã, however, through the results obtained here, parallels can be made with studies carried out on other species of fish.

Hejduk and Svobodová (1980) in their study with different species of fish using toxic agents based on carbamates, as well as Saha et al. (2016), who tested Mancozeb in Mozambican tilapia (*Oreochromis mossambicus*), found an increase in mucus secretion on the epidermal surface of the fish. This response corroborates what was found here and is considered something expected since the change in water quality with the addition of a potentially toxic agent generates stress on the animal and the production of mucus is one of the most common physiological responses. Changes in mucus production due to stress caused by exposure to toxic substances have been widely reported in fish (Ayoola, 2008; Dash et al., 2018). The composition and quantity produced also depend on the agent causing the stress and the producing species (Dash et al., 2018). Thus, together with the other alterations observed here, it can be assumed that Mancozeb, even in non-lethal doses, generates stress and immune response in affected animals.

The occurrence of increased movement (swimming activity) and loss of balance over the time of exposure to Mancozeb was also reported by Hejduk and Svobodová (1980) in *Cyprinus carpio, Salmo gairdneri, and Poecilia reticulata*, and by Shahi and Singh (2015) in catfish. This is probably due not only to the stress imposed on the animals but also to the action of Mancozeb on the nervous system of the fish (Srivastava and Singh, 2014), causing motor coordination disorders.

As for the variation in the value of the lethal concentration of Mancozeb for fish, the concentration varies greatly between studies. However, it is important to note that different animal species have different levels of tolerance to carbamate toxicity (Hano et al., 2017), such as Mancozeb. Therefore, the LC50 values obtained depend on the test organism and also on the age group (Sousa et al., 2019).

Goldoni and Silva (2012) in their study on adult fish of the species *Astyanax jacuhiensis*, used doses of Mancozeb varying between 0.3mg/L to 2.5mg/L (maximum dose) in a single exposure for a test lasting 120 hours, however, the authors did not observe mortality in any of the exposed groups. This differs from what was observed in this study, where the group exposed to a dose of 2mg/L had a mortality rate of 14% at 96 hours. This difference may be because the fish used in our study were younger, and therefore more susceptible to toxic effects (Sousa et al., 2019), or to a possible greater sensitivity of the species in question.

The present result corroborates with what was found by Saha et al. (2016), who studied the toxic effect of Mancozeb (pure formulation) on *Oreochromis mossambicus*, using concentrations of the agent from 8 to 18.5mg/L, observing mortality after 72 hours (8mg/L) and total mortality on the first day in animals exposed to the highest concentration (18mg/L), showing great variation between the values observed in the groups according to dose and time. Shahi and Singh (2015) in an acute toxicity study in catfish (*Clarias batracus*) exposed to doses of 30 to 40mg/L, also reported a positive correlation between the concentration of Mancozeb and the mortality rate, with total mortality at 24 hours in the group exposed to the highest dose, so that mortality increases with the increase of dose.

The LC50 found in the present experiment varied in relation to the exposure time, as previously described. This is in agreement with what was observed by Hejduk and Svobodová (1980), where there was variation in the LC50 observed in the three tested species *Cyprinus carpio, Salmo gairdneri, and Poecilia reticulata,* with variations in concentration over a period of 48 hours (maximum time of the test imposed), with a reduction over time in the concentration necessary for the occurrence of death in 50% of the exposed group.

In the literature, in all studies found, differences in the LC50 value between species were observed. In *Cyprinus carpio, Salmo gairdneri*, and *Poecilia reticulata* the values were respectively 24, 1.85, and 2.2 mg/L at 48 hours using concentrations based on a product with 80% Mancozeb (Hejduk and Svobodová, 1980). In catfish (*Clarias batrachus*), Srivastava and Singh (2014) found a CL50 of 14.36mg/L at 96 hours, while Shahi and Singh (2015) obtained divergent values in a more recent test on the same species, with concentrations from 33.43 to 37.50mg/L over the span of 96 hours. Saha et al. (2016) verified lethal concentrations between 14.4 and 11.68mg/L in Mozambican tilapia (*Oreochromis mossambicus*) exposed to Mancozeb for a period of 96 hours. Lastly, in *Punctius tictus*, a value of 12.95mg/L was observed at 96 hours (Sharma et al., 2016).

Hano et al. (2017) in their work with three species of fish exposed to dithiocarbamate fungicides, observed lethal concentrations of 22-29, 239-553, and 301-364mg/L at 96 hours in *Pagrus major*, *Verasper variegatus,* and *Pleuronectes yokohamae*, respectively. These results among the tested species indicate that species is one of the biggest determining factors for LC50 (Hano et al., 2017). The findings of Hejduk and Svobodová (1980) in *Poecilia reticulata,* with values of LC50 at 2.2mg/L at 48 hours, corroborate with the values of LC50 found in the present study, presenting values closer to those obtained in the tests with Pacamã (2.76 to 2.29mg/L) throughout the period of 96 hours.

IBAMA (2017) classifies pesticides according to their potential for environmental hazard (PEH), which assesses the toxicity of a substance for a given species through the LC50 values obtained in studies. Mancozeb is classified as moderately toxic, however, its toxic potential is considered high for some species of aquatic organisms (IBAMA, 2017). In our study, the LC50 values at 96 hours (2.29mg/L) indicated that Mancozeb can be classified as very toxic for Pacamã fingerlings, according to the current IBAMA PEH classification (2017).

This result is in line with what was observed in *Poecilia reticulata* and *Salmo gairdneri*, which also showed high sensitivity to Mancozeb, which proved to be very toxic for the species (Hejduk and Svobodová, 1980). However, species such as *Clarias batrachus* showed much greater resistance to the toxic effect of the fungicide (Shahi and Singh, 2015), with Mancozeb being only moderately toxic for this species according to the PEH classification. This difference was already expected, as different organisms have different degrees of sensitivity to toxic agents (IBAMA, 2017; Sousa et al., 2019). This difference between species is very important and must be taken into account when determining safety limits for the use of pesticides.

It is necessary to establish safety limits that are in accordance with the toxicity that the pesticide presents to the species that inhabit the place where this compound will be applied and that may come into direct or indirect contact with the agrochemical.

4.2. Sublethal toxicity test

The liver is one of the main organs evaluated in ecotoxicological studies (Wolf and Wheeler, 2018), due to the large blood supply it receives, this organ is considered a target organ in intoxications (Novaes et al., 2018; Stoyanova et al., 2019). Toxic agents pass through biological barriers and enter the bloodstream, lodging in the internal organs of fish (Yancheva et at., 2016). Several pathological changes can be observed in this organ and used as reliable biomarkers in studies on the effects of toxic substances, including pesticides (Stoyanova et al., 2019). However, as Wolf and Wolfe (2005) report, such morphological changes resulting from the cytotoxic effect

are often exacerbated forms of findings that can also be seen in normal or control fish.

The histopathological changes that occur in the liver are due to increasing concentrations of toxic substances received in the organ (Bukhari et al., 2012), such changes depend not only on the time of exposure of organisms to toxic agents but also on the type of agent and its concentration. Several substances can generate these changes, making them useful as possible non-specific biomarkers in ecotoxicological studies (Yancheva et at., 2016).

Although studies related to the action of Mancozeb in Pacamãs have not been found, alterations such as vacuolation, inflammation, and necrosis are among the most observed in similar studies carried out in other species of fish and with other toxic agents.

In a similar study, carried out in the same species, Albinati et al. (2017) studied the effects of the pesticide thiamethoxam on Pacamã fingerlings over a period of 15 days, finding alterations in liver tissue, such as cytoplasmic vacuolation, congestion, and necrosis.

In a study carried out with Mancozeb, but in a different species, Choudhury and Das (2020) observed the occurrence of vacuolization, degeneration, and tissue necrosis in the liver, as well as in the kidney of *Channa punctatus* exposed to Dithane M-45 (80% Mancozeb) at concentrations of 3 ppm over 15 and 30 days, noting that the severity of the lesions varied according to the time of exposure, with a greater degree of degeneration due to necrosis after 30 days.

Nataraj et al. (2017) and Ullah et al. (2018) in their experiments *using Labeo rohitae* exposed to organophosphate pesticides for 21 days, identified several liver lesions, among them the occurrence of necrosis, cytoplasmic vacuolation, and several types of degeneration. Similar findings were found in the livers of *Aristichthys fish nobilis Richardson*, exposed to fungicides for a period of 96 hours (Yancheva et al., 2015). Such studies are in agreement with what we found in our histopathological evaluation of the liver.

Increased hepatic vacuolization is the histopathological finding most commonly found in ecotoxicological studies in fish (Wolf et al., 2015), occurring due to increased energy reserve or as a result of a degeneration process, it is cited as an indication of toxicological response (Wolf and Wheeler, 2018). However, as Wolf and Wheeler elaborated in their study, the presence of vacuolization alone cannot be considered an indicator of liver injury.

In the liver tissue of these animals, energy reserves can be stored as glycogen or lipid within the cytoplasm of hepatocytes (Wolf et al., 2015). The occurrence of vacuolization is less important than the change in severity and frequency, which are usually linked to exposure to chemical agents (Wolf and Wheeler, 2018).

When in a situation of environmental stress, glycogen is one of the nutrients most used in biochemical adaptations (Portz and Furuya, 2012). Fish under stress, with poor diet and diseased, often exhibit a decrease in hepatic energy stores, decreasing hepatocellular vacuolation (Wolf et al., 2015). Such a drop can be a direct consequence of an intoxication process, however, exposure to toxic substances can also lead to the accumulation of glycogen or lipid in the liver, generated by the reduction in the breakdown of glycogen due to the cytotoxic effect of the agrochemical on hepatocytes (Wolf and Wolfe, 2005).

The formation of vacuoles with an accumulation of fat or glycogen may occur due to the reduction in glycogen breakdown due to hepatocellular toxicity resulting from exposure to toxic agents (Wolf and Wolfe, 2005). Hepatic vacuolization in fish is also commonly associated with stressors, such as exposure to chemical agents (Shukla and Trivedi, 2018), and even the presence of other foreign compounds, such as plastic particles, resulting from water pollution (Iheanacho and Odo, 2020).

In our work, vacuolation was observed in different groups, including the control. Therefore, the occurrence of vacuolation in our experiment may be a normal finding in fish kept in captivity. However, the increase in the frequency and intensity of vacuolation may indicate a response to the offending agent in the groups treated with Mancozeb, especially in the T3 and T4 groups, which showed a higher rate of vacuolation.

Morphological changes characteristic of inflammation are among the main tissue responses to physiological stress and cellular damage (Wolf et al., 2015). The presence of an inflammatory infiltrate in the liver may indicate responses to cellular damage, immunological stimuli, and/or physiological disturbances (Wolf and Wheeler, 2018). According to Weber et al. (2020), the occurrence of inflammatory infiltrate as well as necrosis may be related to increased oxidative stress in hepatocytes of fish exposed to toxic agents.

According to Yancheva et al. (2016), inflammatory lesions are more observed in fish collected from polluted environments when compared to fish collected in reference sites. Ayoola (2008) in a study on the toxicity of glyphosate in the liver tissue of *Clarias gariepinus*, observed the occurrence of lymphocytic infiltrate among other lesions resulting from exposure to the toxic agent. Chamarthi et al. (2014) found similar findings in their work with *Cyprinus carpio* exposed to an organophosphate insecticide. Thus, the presence of such a finding in our study can be considered an indication of a response to liver injury as a result of exposure to Mancozeb.

As for necrosis, the observation of this alteration, in a focal or diffuse form, is the second most described alteration in fish liver tissue, followed by other degenerative lesions (Wolf and Wheeler, 2018). According to Vigário and Sabóia-Morais (2014), toxic agents can lead to the occurrence of epithelial necrosis and degeneration. Hepatotoxicity can lead to cell death, via apoptosis or necrosis, depending on the action taken. The mechanisms involved in this phenomenon are linked to the type of drug or toxic agent, the degree of liver injury, and the mechanism that triggers the cell death process (Lorga et al., 2017).

In our study, necrosis proved to be an important finding, more frequently observed in the groups T4 and T5 treated with high doses of Mancozeb (0.4 and 0.8mg/L, respectively). The exposure of this group to Mancozeb at a higher dose than the other groups, for a period of 15 days, may have allowed the toxic action of the agent to affect the fish more aggressively, limiting the tissue repair process and resulting in disseminated necrosis of the fabric.

Wolf and Wolfe (2005) classify vacuolation, as well as the inflammatory process, necrosis, and various forms of cell degeneration, as important alterations to identify the cytotoxic potential of exogenous agents on hepatocytes. Yancheva et al. (2016, 2020) in extensive reviews on histopathological changes as biomarkers in fish, reiterate these findings, considering these changes as important non-specific biomarkers that can be found in fish exposed to toxic compounds. Therefore, we consider the liver alterations observed in our experiment can be used as biomarkers to help determine the toxic potential of Mancozeb in fish.

5. Conclusion

Mancozeb is toxic to fish of the Pacamã species and can lead to death. The 50% lethal concentration of Mancozeb (under the ManzateWG® form) at 96 hours for Pacamã fingerlings was 2.29mg/L (2.29ppm), classifying it as a very toxic pesticide for the species. Mancozeb was toxic to acutely exposed Pacamã fingerlings and showed evidence of a sublethal toxic potential. This fungicide can have serious effects on fish even at low doses, such as behavioral changes (increased mucus production and reduced social activity) and histological changes, such as the occurrence of inflammatory processes and hepatic necrosis; such alterations can be used as non-specific biomarkers of the toxic potential of this agent. However, more studies are needed to elucidate the mechanisms of action of this toxic agent in the species, especially those related to the behavioral changes caused by it.

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