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Experimental acute lead toxicity in zebrafish (*Danio rerio*) with emphasis on clinicopathological findings[1]

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ABSTRACT.- Marcelino SAC, Lacerda MSC, Tavares GC, Pereira CLM, Hoyos DC, Nascente CC, Santana GC, Pierezan F. **Experimental acute lead toxicity in zebrafish (*Danio rerio*) with emphasis on clinicopathological findings.** *Pesquisa Veterinária Brasileira 46:e07776, 2026.* Departamento de Clínica e Cirurgia Veterinária, Universidade Federal de Minas Gerais, Campus Pampulha, Av. Pres. Antônio Carlos 6627, São Luiz, Belo Horizonte, MG 31270-901, Brazil. E-mail: fpierezan@gmail.com

Recent ecological disasters following the rupture of mining tailings dams in Brazil, which contaminated the nearby river basin, highlight the urgent need for a better understanding of the effects of short- and long-term exposure of fish to heavy metals. In the present study, acute lead (Pb) toxicity was assessed in zebrafish (*Danio rerio*) with a focus on clinical and pathological findings. Dilutions of lead acetate, in distilled water, were used in the following concentrations: 0, 0.21, 0.46, 1.02, 2.24, and 4.92 mg/L. The experiment was designed as a semi-static acute toxicity assay. For each concentration, two aquariums with six fish were used, totaling 72 fish (12 fish per group). Evaluations of clinical signs, mortality, and anatomopathological findings were performed. The clinical signs noted included hypoactivity, abnormal surface distribution, under-reactivity, abnormal bottom distribution, loss of shoaling behavior, and changes in ventilatory functions. The 96 h mortalities indices were 33.3% in the 0.46 mg/L group, 41.7% in the 1.02 mg/L group, 75% in the 2.24 mg/L group, and 91.7% in the 4.92 mg/L group. There were no deaths in the control group and in the 0.21 mg/L group. The 96 h LC50 was 1.1 mg/L Pb. No macroscopic lesions were observed, but histological lesions were noted in the gills of fish exposed to 0.46 mg/L and higher concentrations. Given the presence of autolytic changes in fish that died spontaneously during the experiment, only lesions in survivors were considered in this study. Gill lesions include hyperplasia of epithelial cells, with lamellae fusion and the formation of cavities, which were occasionally filled with cell debris (necrotic material). High levels of Pb were identified in the gills of fish from all challenged groups by inductively coupled plasma mass spectrometry (ICP-MS). Pb was toxic to zebrafish at the evaluated doses and caused mortalities that were dose-dependent. The main organ affected was the gill because of its direct contact with dissolved Pb and its role in the absorption of this metal in freshwater fish. The gills had proliferative and necrotic lesions that compromised the fish's respiratory capacity and triggered clinical signs related to swimming behavior and to the ventilatory function. As the lesions found are nonspecific

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for Pb toxicity, histopathology in combination with toxicological analyses of water or fish tissues is recommended to confirm the diagnosis in spontaneous cases.

INDEX TERMS: Icthyiopathology, heavy metals, aquatic toxicology, metal poisoning.

RESUMO.- [**Avaliação da toxicidade de chumbo em peixe-zebra (*Danio rerio*) com ênfase em achados clínico-patológicos.**] Desastres ambientais recentes, decorrentes da ruptura de barragens de rejeitos de mineração e da contaminação da bacia hidrográfica periférica, demonstraram a necessidade de um melhor entendimento dos efeitos a curto e a longo prazo da exposição de peixes a metais pesados. No presente estudo, a toxicidade aguda do chumbo (Pb) foi avaliada em peixe-zebra (*Danio rerio*), com ênfase nos achados clínicos e patológicos. Foram utilizadas diluições de acetato de chumbo em água destilada nas concentrações de 0, 0.21, 0.46, 1.02, 2.24 e 4.92 mg/L. Os experimentos foram delineados como ensaios de toxicidade aguda semiestáticos. Para cada concentração, foram utilizados dois aquários com seis peixes, totalizando 72 peixes (12 por grupo). Foram realizadas avaliações de sinais clínicos, de mortalidade e de achados anatomopatológicos. Os sinais clínicos presentes foram hipoatividade, distribuição anormal na superfície, subreatividade, distribuição anormal no fundo, perda do comportamento de cardume e alterações das funções ventilatórias. Os índices de mortalidades em 96 h foram 33.3% no grupo 0.46 mg/L, 41.7% no grupo 1.02 mg/L, 75% no grupo 2.24 mg/L e 91.7% no grupo 4.92 mg/L. Não houve mortes no grupo controle nem no grupo 0.21 mg/L. A CL50 96 h foi de 1.1 mg/L de Pb. Não foram observadas lesões macroscópicas, mas houve lesões histológicas nas brânquias dos peixes expostos a concentrações a partir de 0.46 mg/L. Dada a presença de alterações autolíticas em peixes que morreram espontaneamente durante o experimento, apenas lesões em sobreviventes foram consideradas neste estudo. As lesões branquiais incluem hiperplasia de células epiteliais, com fusão de lamelas e formação de cavidades, que ocasionalmente eram preenchidas com restos celulares (material necrótico). Altos níveis de Pb foram identificados nas brânquias de peixes de todos os grupos desafiados por espectrometria de massa com plasma indutivamente acoplado (ICP-MS). O Pb foi tóxico para o peixe-zebra nas doses avaliadas e provocou mortalidade dose-dependente. O principal órgão acometido foi a brânquia, por ter contato direto com o Pb dissolvido e por ser a principal responsável pela absorção do metal em peixes de água doce. As brânquias apresentaram lesões necróticas e proliferativas agudas que comprometeram a capacidade respiratória dos peixes e desencadearam sinais clínicos de alterações no comportamento de natação e na função ventilatória. Como as lesões encontradas não são específicas para a toxicidade pelo Pb, recomenda-se a histopatologia associada a análises toxicológicas da água ou dos tecidos do peixe para a definição do diagnóstico.

TERMOS DE INDEXAÇÃO: Ictiopatologia, metais pesados, toxicologia aquática, intoxicação por metais.

INTRODUCTION

Anthropogenic activities, such as battery and paint manufacturing, agriculture, and, particularly, mining operations, are associated with increased levels of metals in river basins (Kim & Kang 2015, Lee et al. 2019). The storage of waste from mining operations also poses a risk of river contamination due to potential leaks or the collapse of storage dams. This risk was evident in the ecological disasters that occurred in Mariana and Brumadinho, Minas Gerais, Brazil, in 2015 and 2019, respectively (Freitas et al. 2019). Following these two incidents, the highest recorded levels of lead (Pb) in the water of the affected rivers were 0.9 mg/L in Mariana (SEMAD 2016) and 0.147 mg/L in Brumadinho (IGAM 2020, Ramos et al. 2020). In general, expected levels of Pb in water are low: 0.0002 mg/L in saltwater and 0.002 mg/L in freshwater (Labrot et al. 1999).

Pb can be absorbed in fish through the gills or gastrointestinal tract. The metal is absorbed, carried by the bloodstream, metabolized in the liver, deposited in different organs, and excreted by the kidneys and gills. The particularities of these pathways may affect different target organs and aspects of bioaccumulation. The gills play a primary role when dissolved Pb levels in the water are high. This pathway is especially important in freshwater fish, as the constant ionic exchange required to maintain homeostasis between the body (hypertonic) and freshwater (hypotonic) makes the gills the primary organ for bioaccumulation. On the other hand, the gastrointestinal route is associated with sediments or diets with high Pb levels. This pathway is particularly important in marine fish, as these animals need to ingest greater amounts of water to maintain body homeostasis (hypotonic) in a hypertonic environment (salt water), making the intestines the principal organs of bioaccumulation (Kim & Kang 2015, Lee et al. 2019).

Several studies have investigated Pb toxicity in various species of fish, demonstrating both the acute (Rogers et al. 2003, Rabitto et al. 2005, Paul et al. 2014, Roy et al. 2015, Liu et al. 2019, Macirella et al. 2019) or the chronic forms (Dai et al. 2009, Al-Balawi et al. 2013, Łuszczek-Trojnar et al. 2013, Kim & Kang 2015, Hwang et al. 2016). However, the reported anatomopathological findings differ across studies (Rabitto et al. 2005, Dai et al. 2009, Al-Balawi et al. 2013, Macirella et al. 2019). Lesions described in the gills include secondary lamellae congestion, epithelial cell hypertrophy, epithelial detachment, secondary lamellae degeneration, aneurysms, chloride cell hypertrophy, and hyperplasia (Al-Balawi et al. 2013, Macirella et al. 2019). In the liver, described lesions include vacuolar degeneration and individual hepatocyte necrosis (Rabitto et al. 2005, Dai et al. 2009, Al-Balawi et al. 2013). In the kidneys, described lesions include multifocal areas of tubular necrosis and neutrophilic and histiocytic infiltrates in the cranial kidney, in addition to glomerular expansion (Rabitto et al. 2005, Al-Balawi et al. 2013). Some lesions attributed to Pb toxicity have also been identified as artifacts of tissue fixation and processing or as species-specific characteristics, including hyperplasia of the final portion of the primary lamellae, congestion, lamellar telangiectasias (lamellar aneurysms), detachment of lamellar epithelium, granulocytic infiltrates, vacuolation of hepatocytes and vacuolation of renal proximal convoluted tubule epithelium

(Wolf et al. 2015, Wolf & Wheeler 2018). Thus, there is a need for additional research using histopathological methods in toxicity trials or in instances of river contamination. Although numerous studies have shown that Pb can be toxic to fish and that consuming contaminated fish poses a risk to human health (Kim & Kang 2015, Lee et al. 2019, Liu et al. 2019), more in-depth evaluations of the effects of lead on animal models are essential for a comprehensive understanding of the short- and long-term impacts of such environmental disasters on fish populations. The present study focused on the acute toxicity of lead in zebrafish (*Danio rerio*), highlighting both clinical and pathological findings. Identifying these findings in zebrafish aims to establish parameters for interpreting lesions and clinical signs and for diagnosing cases of lead intoxication in fish.

MATERIALS AND METHODS

Ethical approval. All experiments were conducted in accordance with the standards of good experimental practice recommended by the "Conselho Nacional de Controle de Experimentação Animal" (National Council for the Control of Animal Experimentation – CONCEA) and the "Colégio Brasileiro de Experimentação Animal" (Brazilian College of Animal Experimentation – COBEA). The experiment project was evaluated and approved by the Ethics in Animal Experimentation Committee (CEUA) of UFMG (Protocol 61/2020). The methodology was prepared in accordance with the "Brazilian Standard for Aquatic Ecotoxicology – Acute Toxicity – Test Method with Fish (Cyprinidae)" (ABNT NBR 15088) (ABNT 2016) and the international guide "Test Guideline No. 203 Fish – Acute Toxicity Testing" (OECD 2025).

Location. The experiments were conducted at the "Laboratório de Terapêutica Veterinária" (Laboratory of Veterinary Therapeutics) at the "Universidade Federal de Minas Gerais" (UFMG).

Obtaining and keeping fish. The adult zebrafish, male and female, with an average weight of 0.35 g, were kindly donated by the Laboratory of Ornamental Fish of the "Laboratório de Aquacultura" (Aquaculture Laboratory – LAQUA) at UFMG. The fish were kept in the vivarium of the "Laboratório de Terapêutica Veterinária" at UFMG. Acclimatization lasted two weeks. The fish were randomly distributed in 25-liter (L) glass aquariums (ratio of body mass to water volume of 1 g/L). Each aquarium received dechlorinated water. During the acclimatization period, water quality was monitored daily and temperature, dissolved oxygen (DO), and pH were measured. The physicochemical parameters of solid particles, total organic carbon, non-ionized ammonia, nitrate, residual chlorine, organophosphates, organochlorines, aluminum, arsenic, chromium, cobalt, copper, iron, lead, nickel, zinc, cadmium, mercury, silver and the chemical oxygen demand (COD) were in accordance with the "Test Guideline" No. 203 (OECD 2025). The temperature and preserved oxygen were monitored with an Instrutherm MO-900 multiparameter meter, and the pH with a Tecnopon mPA-210 benchtop pH meter. Approximately 25% of the total water volume was replaced every five days. Oxygen level was maintained at ≥ 5.0 milligrams (mg)/L (> 60% of the air saturation value) and pH between 6.0 and 8.5 (OECD 2025). The fish selected for the trials were free of any apparent malformations and showed no signs of stress, bleeding along the body, excessive mucus or atypical swimming (ABNT 2016). No lesions (that could indicate any infectious agent or previous degenerative disease) were identified in the 10 fish submitted for gross and microscopic examination before the trial.

Experimental design. The experiment was designed as a semi-static acute toxicity test. The fish were exposed for 96 h, and the test solution was renewed after 48 h. Five lead concentrations in water were evaluated: 0, 0.21, 0.46, 1.02, 2.24, and 4.92 mg/L. Seventy-two fish were weighed (to measure the average weight of each experimental group) and randomly distributed in 12 round 2 L glass aquariums. For each tested concentration, two aquariums with six fish were used (12 fish per concentration and 12 in the control group). The protocol used complies with the "Test Guideline" No. 203 (OECD 2025) for testing metal concentrations, which specifies a minimum of seven fish per concentration and does not require replication.

The stock solution was prepared with lead acetate (Pb $(C_2H_3O_2)2$, $3H_2O$ in analytical grade), in the form of a powder, diluted in distilled water (concentration of 981.24 mg/L of Pb). Calculated amounts of the stock solution were diluted in 2 L of distilled water to obtain the following concentrations: 0.21, 0.46, 1.02, 2.24, 4.92 mg/L of total lead (dilution factor 2.2). The pH was adjusted when necessary. Immediately after dilution, the solutions were sent to the "Departamento de Química" (Department of Chemistry) at UFMG for the measurement of metal levels through inductively coupled plasma mass spectrometry. Subsequently, an aeration system connected to Pasteur pipettes was installed in each container and remained active for 12 hours to supply oxygen and stabilize pH. After 48 hours of fish allocation, the solutions were renewed (preparation followed the same methodology). During the experiment, pH, temperature, and dissolved oxygen (DO) were measured twice a day. The concentrations used in the experiment were chosen based on pilot tests with Pb concentrations of 1.02 and 4.92 mg/L and on descriptions of toxicity tests, in which mortalities were associated with lead concentrations ranging from 1 to 31.5 mg/L (Noga 2010).

An inductively coupled plasma mass spectrometer (ICP-MS) from Agilent Technologies (model 7700 Series) was used to determine the Pb concentration in water samples collected from aquariums. These samples were diluted and acidified (with ultrapure water and HNO_3 2% v/v, obtained from a purification system), then centrifuged at 12,000 rpm for 5 min to avoid clogging of the nebulizer. One multi-element stock solution (10 µg mL -1 in 2% HNO_3) containing Pb was diluted in ultrapure HNO_3 1% v/v to construct an analytical curve ranging from 0 to 100 µg L -1.

Analysis procedures for changes in behavior and mortality. Mortalities and behavioral changes were recorded at 4-hour intervals. The fish were physically monitored, and video recordings of the aquaria were obtained to assist in the evaluation of behavioral changes and the documentation of clinical signs. At the end of the test, the mortality percentage was calculated at different concentrations across all experimental groups. The results were considered valid because, at the end of the experimental period, the dead fish rate in the control group was < 10%, as established by NBR 15088 (ABNT 2016). Lethal concentrations (LCs) (10% mortality (LC10); 50% mortality (LC50); 90% mortality (LC9096h); and 99% mortality (LC99) were estimated by probit-log (dose) regression models (95% confidence level) established by Lei & Sun (2018).

Fish were considered dead when there was no visible opercular movement and when touching the caudal peduncle did not produce any reaction. Statistical analysis was performed using GraphPad Prism version 11.0.0; *p*-values < 0.05 were considered significant. Probability of survival was estimated by Kaplan-Meier curves, and the log-rank test of Mantel-Cox was used to compare the curves.

Behavioral changes indicative of toxicity were classified as: 1) loss of balance; 2) swimming behavior; 3) respiratory function; 4) appearance; and 5) other visible abnormalities of appearance and behavior. The examples, criteria, and definitions of the evaluated clinical

signs were adapted from the "Test Guideline" No. 203 (OECD 2025) (Table S1). Dead fish were removed from the aquariums during the observation interval. Euthanasia criteria to avoid extreme suffering from acute intoxication were adopted during trials.

After the experimental period, the animals that did not die were euthanized with an overdose of eugenol associated with rapid cooling (CONCEA 2018). Spontaneously dead animals and those euthanized were sent in equal portions for toxicological evaluation and for histopathological evaluation.

Collection processing and histopathological evaluation. Necropsies and histological analysis were conducted at the "Laboratório de Patologia" (Laboratory of Veterinary Pathology) at UFMG. Immediately after death/euthanasia, the fish were kept in 10% buffered formalin for 48 hours.

Later, the fish were decalcified with 24% formic acid for 48 hours. Sections with five micrometers (μm) were routinely stained with hematoxylin and eosin (HE). An adaptation of the recommendations of the "Histopathology Guidance Document for the Medaka Extended One-Generation Reproduction Test" (OECD 2023) was carried out, so that slides were produced at three different depths for each fish. The tissues of six fish from each experimental group were histologically evaluated. These fish were identified numerically, and the lesions were correlated with the time of death.

Collection and procedures for toxicology. Fish submitted for toxicological analysis were sent to the "Departmento de Química" at UFMG. The selected samples were the gills, viscera (liver, kidney, heart, spleen, pancreas and intestines) and musculature (epaxial and hypoaxial) in addition to bones and skin. Composite samples were collected (six fish per experimental dose) because the amount of available material was small.

For the toxicological analysis, the gills, coelomic viscera, and the carcass (muscle and bones) were separated. As the amount of available material was small, analyses were carried out on pools containing samples of five fish. The tissue samples were crushed for homogenization, weighed, and digested in an oxidizing medium (HNO_3 65% m/m and H_2O_2 30% m/m). The digests were voluminous and analyzed by inductively coupled plasma mass spectrometry (ICP-MS). The method was based on the methodology of Renieri et al. (2017). These tests were carried out in Laboratory 157 of the "Departamento de Química" at UFMG.

RESULTS

Mortality and clinical signs

After 96 hours, mortality rates were 0% in the 0.21 mg/L group, 33.3% (4/12) in the 0.46 mg/L group, 41.7% (5/12) in the 1.02 mg/L group, 75% (9/12) in the 2.24 mg/L group, and 91.7% (11/12) in the 4.92 mg/L group (Fig. 1). There were no deaths in the control group. Mortality in the Pb 0.46 mg/L and Pb 1.02 mg/L groups began in the first 5 hours, whereas in the Pb 2.24 mg/L and Pb 4.92 mg/L groups it began in the first 3 hours. Most deaths occurred within 24 hours of experimentation, and then, there was a mortality plateau, even though, in the Pb 4.92 mg/L group, the last death occurred during the interval between 68 and 72 hours. Survival probability differed significantly among groups ($p < 0.0001$). The 96 h LC50 for Pb was 1.1 mg/L.

The most commonly observed clinical sign category was abnormal swimming behavior. The main sign in this category was hypoactivity. The second most prominent clinical sign was the abnormal bottom distribution. In many cases, concomitantly with these clinical signs, under-reactivity and loss of shoaling behavior were observed. The least frequent abnormal swimming behavior was the abnormal surface distribution, which was also one of the last clinical signs observed before death and was usually accompanied by abnormalities in the ventilatory function.

The ventilatory abnormalities observed were hyperventilation, irregular ventilation, and fish gulping. These signs were usually observed in the final hours before the fish died. In one fish, bleeding from the gills was visible (Fig. 2-3).

A pattern of progression of clinical signs was noted. Initially, from the first 2 hours of exposure, hypoactivity was stressed and was generally associated with under-reactivity and abnormal bottom distribution. This set of clinical signs affected a large proportion of the fish and persisted throughout the experiment. Later, the fish exhibiting the previous clinical signs showed an abnormal surface distribution, in addition to hyperventilation, irregular ventilation, and gulping. After a few hours with these clinical signs, the animals died.

In addition to the progression of the clinical signs, a small number of fish remained in an abnormal bottom distribution, with intense hypoactivity, until the end of the experiment. Some of these fish progressed to unresponsiveness to visual and vibratory stimuli, hyperventilation, irregular ventilation, and finally, death. One fish from the Pb 1.02 mg/L group showed abnormal surface distribution with mild gill bleeding between 4 and 8 hours. Some fish showing abnormal bottom or surface distribution, occasionally followed by hyperventilation, recovered and survived until the end of the experiment. There were no clinical signs in the control group.

Pathological findings

Except for one fish in the 1.02 mg/L group, which showed gill bleeding, no significant macroscopic lesions were found in fish from the challenged and control groups.

The microscopic lesions in the challenged groups were concentrated in the gills. Lesions found in fish that survived 96 h and were submitted to euthanasia were characterized by marked thickening of the secondary lamellae, with slight to moderate lamellar disorganization due to hyperplasia of the pavement epithelial cells with moderate fusion of secondary lamellae (Fig. 4-5) and accumulation in variable amounts of granular to amorphous, eosinophilic material (cell debris) in well-defined spaces. In the control group, no histological changes were observed in the gills. No other significant findings were observed in the remaining organs.

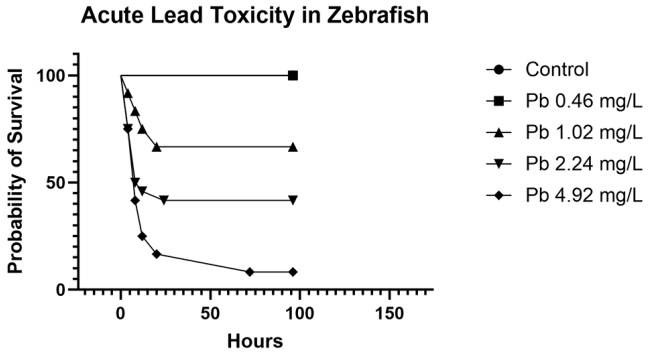

Fig. 1. Experimental acute lead toxicity in zebrafish (*Danio rerio*). Probability of survival of fish exposed to dilutions of lead acetate at the concentrations of 0 (control), 0.21, 0.46, 1.02, 2.24, and 4.92 mg/L for 96 hours.

The relationships among types of death, time of death, injury pattern, and injury intensity are shown in Table 1.

In the control group, no significant histopathological alterations were observed. The gills of these animals showed adequate morphological conformation of the primary and secondary lamellae.

Toxicological findings

High levels of Pb were identified in the gills of fish from all challenged groups, with groups 2.24 and 4.92 mg/L showing the highest levels (710.06 mg/kg wet base and 1207.86 mg/kg wet base, respectively). The levels of PB were relatively slower and varied between the groups in samples from the carcass (ranging from 3.60 to 9.85 mg/kg wet base) and from coelomic viscera (ranging from 6.47 to 52.50 mg/kg wet base) (Table 2). No statistical analysis was conducted to compare the levels of Pb in the different groups and organs, due to the reduced number of tested samples.

Water parameters by group

The main water parameters analyzed were: temperature, pH and dissolved oxygen (DO). These parameters were measured twice daily and generally conformed to the OECD (2025). The average of the water parameters is shown in Table S2.

Table 1. Intensity of the histological findings in zebrafish (*Danio rerio*) exposed to concentrations of lead (Pb)

Concentration	Fish	Type of death (time to death)	Intensity
Pb 0.46 mg/L	3.3	Euthanasia (96 h)	Marked
	3.5	Euthanasia (96 h)	Mild
	4.4	Euthanasia (96 h)	Marked
	4.6	Euthanasia (96 h)	Moderate
Pb 1.02 mg/L	5.3	Euthanasia (96 h)	Marked
	5.5	Euthanasia (96 h)	Moderate
	6.5	Euthanasia (96 h)	Mild
Pb 2.24 mg/L	7.5	Euthanasia (96 h)	Marked
Pb 4.92 mg/L	9.6	Euthanasia (96 h)	Mild

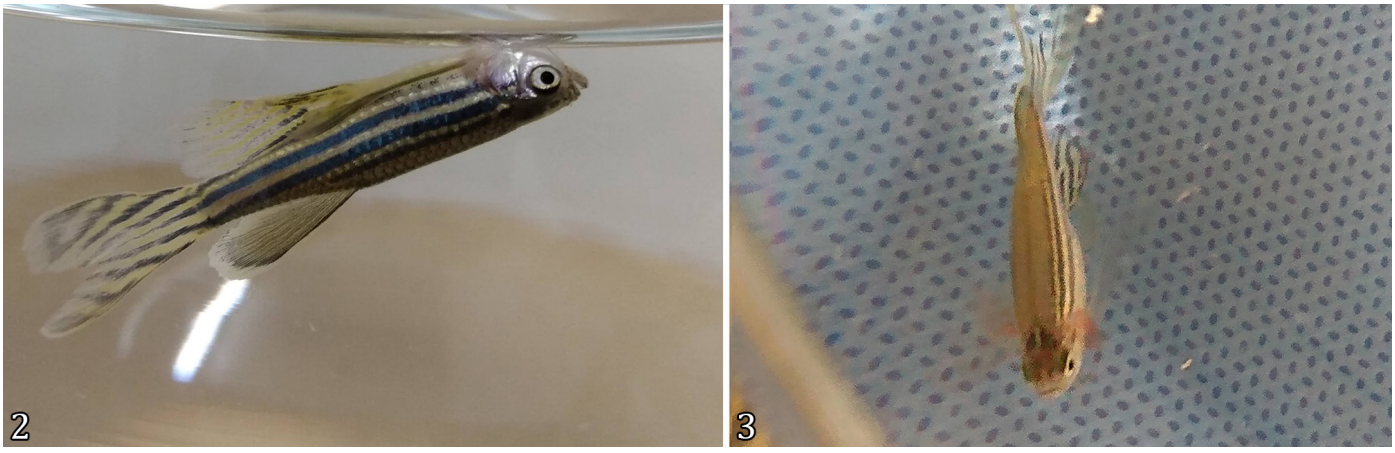

Fig. 2-3. Experimental acute lead toxicity in zebrafish (*Danio rerio*). (**2**) Dead fish from the group 4.92 mg/L. (**3**) Fish exposed to 1.02 mg/L of Pb presented gill hemorrhage, evidenced by red-tinged fluid oozing from the opercular cavity.

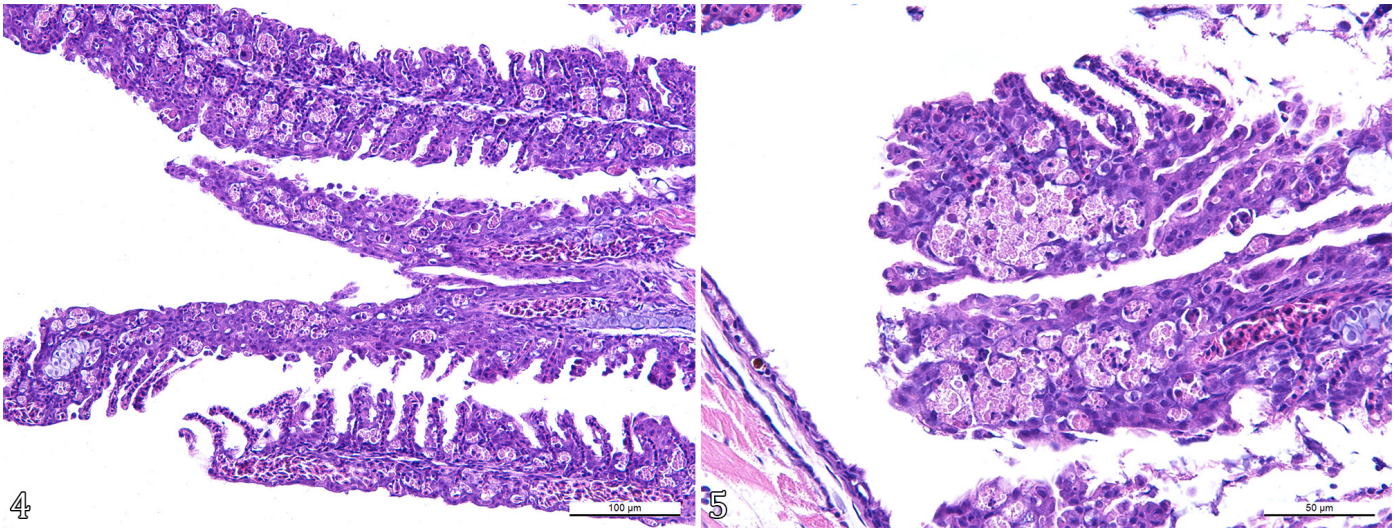

Fig. 4-5. Experimental acute lead toxicity in zebrafish (*Danio rerio*). Fish were exposed to 2.24 mg/L of Pb. Note the marked pavement cell hyperplasia and accumulation of eosinophilic granular material (cellular debris) in cavities formed by the fusion of the lamellae. (**4**) HE, obj. 20x. (**5**) HE, obj. 40x.

DISCUSSION

Lead dilutions of 0.46, 1.02, 2.24, and 4.92 mg/L caused clinical signs, significant microscopic lesions, and mortality in zebrafish (*Danio rerio*). When compared to concentrations used in other studies, regarding the value and ability to induce injuries or deaths, differences were observed and are likely related to the wide variation of the other tested concentrations, the different challenged fish species, and the focus of each study. In general, concentrations reported in other studies ranged from 0.4 to 925 mg/L, and the described clinical signs, microscopic lesions, and mortality rates were variable. Still, they were generally observed at concentrations similar to or slightly higher than those used in this study. Factors such as different fish species, age, eating habits, gender, composition of salt mixed with the metal, and experimental conditions can influence the relationship between dose and mortality in studies, even when the same fish species is challenged (Noga 2010, Singh & Ansari 2017).

The concentrations evaluated in the present study were closer to those used in studies that assessed the toxicity of this metal in tilapia (*Oreochromis* spp.) (Doaa & Hanan 2013) and in marine fish (Macirella et al. 2019). The study on the toxicity of the metal in tilapia, focusing on histopathological analysis, used lead acetate at 0.4 and 0.7 mg/L for three weeks and found no mortality. In that case, the concentrations were considered sub-lethal, although lesions in the gills, ovary, and liver were described. A positive correlation between injuries and concentration at the times tested was observed (Doaa & Hanan 2013). In the study with a marine species ornate wrasse fish (*Thalassoma pavo* L.), with emphasis on the morpho-functional and structural analysis of the gills, dilutions of lead nitrate were used at concentrations of 0.8, 2.0, and 12.0 mg/L, for up to 196 hours. The study also showed no mortality at any of the tested doses; however, lesions were observed at the highest concentration, and their severity depended on the duration of exposure (Macirella et al. 2019).

Higher doses than those evaluated in the present study have been described in tests in zebrafish (*Danio rerio*) (Singh & Ansari 2017), African catfish (*Clarias gariepinus*) (Al-Balawi et al. 2013), in Spotted snakehead (*Channa punctata*) (Paul et al. 2014) and in curimbatá (*Prochilodus lineatus*) (Martinez et al. 2004). In the study of lead and cobalt toxicity in adult zebrafish, lead acetate was diluted in distilled water to obtain concentrations of 10, 15, 20, 25, and 30 mg/L. Only mortality was assessed in this study, and the LC50 of 41.27 mg/L at 24 hours and 21.63 mg/L at 96 hours was determined (Singh & Ansari 2017). In a study with a focus on histological lesions and bioaccumulation in African catfish exposed to dilutions of lead acetate, an LC50 of 122 mg/L was determined, as well as sub-lethal levels of the metal of 6.1 (5% LC50), 12.2 (10% LC50), and 24.4 mg/L (20% LC50), for six weeks. Sublethal doses induced histological changes in the gills, liver, and kidneys, and bioaccumulation was mainly observed in the gills and liver (Al-Balawi et al. 2013). The immunotoxic potential of lead on spotted snakehead was tested with lead acetate dilutions based on an LC50 of 925 mg/L. In that study, the level of 9.43 mg/L (1.02% LC50) was tested for 96 hours, and the results supported impairment of the phagocytosis capacity of intestinal macrophages and injuries to the intestinal mucosa associated with the metal (Paul et al. 2014). Tests evaluating injuries to the gills and blood alterations in curimbatá used diluted lead acetate based on an LC50 of 95 mg/L. Sub-lethal levels of 24 and 71 mg/L for 96 hours were used, and histological changes in the gills and hyperglycemia were observed (Martinez et al. 2004).

The levels evaluated in the present study were higher than those described by the normative deliberation DN COPAM/CERH No. 001/2008 for class II waters (0.01 mg/L) in Brazil (SEMAD 2016). This deliberation distinguishes the water that can be destined for human consumption after simplified treatment from the water used for the protection of aquatic communities, primary contact recreation, and the irrigation of vegetables consumed raw and fruits that develop close to the ground. However, the levels evaluated in this study were similar to or higher than the maximum levels of lead found in river water after dam failures in Mariana/MG (0.9 mg/L recorded in the Rio Doce after the Fundão dam rupture) (SEMAD 2016) and Brumadinho/MG (0.147 mg/L recorded in the Paraopeba River after the rupture of the Córrego do Feijão dam) (IGAM 2020). It is important to note that the tested Pb concentrations used in this study were set to induce histological lesions in the fish.

Compared with other studies of Pb toxicity in fish, similarities in clinical signs and behavioral changes with those observed in the present study are evident. Changes in erratic swimming, loss of balance, and fish clustered in one corner and resting on the bottom of the aquarium were recorded in a study evaluating the toxicity of lead and cobalt in zebrafish. The authors comment that the fish frequently remained on the surface, with progression of clinical signs to a ventilatory disorder characterized by difficult breathing, stronger opercular movement, and excessive mucus secretion from the body surface (Singh & Ansari 2017). Another study that tested lead acetate in tilapia reported decreased swimming activity and increased mucus production (Doaa & Hanan 2013).

Table 2. Lead (Pb) levels in the water and tissues from the challenged and control groups

Expected dilutions (mg/L)	Samples			
	Water (Time 0) (mg/L) (SD)	Organs (mg/Kg – wet base)		
		Gills	Carcass	Coelomic viscera
0	0.000 (0.00)	3.58	0.35	0.29
0.46	0.494 (0.042)	124.2	3.91	6.47
1.02	0.889 (0.030)	180.67	9.85	52.50
2.24	2.331 (0.085)	710.06	3.98	7.49
4.92	4.643 (0.181)	1207.86	5.40	38.87

SD = standard deviation.

Rajeshkumar et al. (2017) reported a variety of similar signs, including increased opercular movements and coordination disorders during swimming, especially at the beginning of exposure to the metal, with a return to normal behavior after prolonged contact. Other works point to a decrease in the response to the stimulus, reduction of the escape response, change in swimming pattern and speed (Lee et al. 2019, Roy et al. 2015). Some clinical signs reported in the literature were not observed in the present study, such as food refusal and neurological disorders (Lee et al. 2019, Roy et al. 2015, Rajeshkumar et al. 2017).

Some studies attribute this set of clinical signs to the theory of "coagulation film anoxia". This theory discusses the coagulation capacity of the mucus present in the body and, mainly, in the gills of fish, when these fish are exposed to water with toxic amounts of lead or other metals such as zinc, iron, copper, cadmium, mercury, manganese, cobalt, nickel, silver, gold and aluminum. This precipitated mucus over the gills would hinder breathing, promoting respiratory distress and death by suffocation (Aronson 1971).

The results of the present study demonstrate that the gill is the target organ for injury by Pb at doses of 0.46, 1.02, 2.24, and 4.96 mg/L, in a 96-hour test. Lesions on the gills are clearly proliferative and necrotic, correlating with the clinical signs and justifying them. These lesions were characterized by thickening of the secondary lamellae, with lamellar disorganization due to hyperplasia of the lining epithelial cells, moderate fusion of secondary lamellae, and accumulation of cell debris in well-defined spaces between the epithelial cells of the lamellar coating. Although this set of injuries is not directly related to the death of the animals, it proves the substantial impairment of the respiratory capacity of the exposed fish; after all, all these fish were hypoactive, under-reactive, and abnormally distributed on the bottom or on the surface of the aquarium.

When compared with other studies, the convergence of these results with the literature is evident, particularly in the demonstration of the gill as a target organ. However, the discernment and interpretation of lesions in the present study differed from those in other studies. After all, several studies cite other findings in the gills, such as epithelial detachment, lamellar aneurysms (telangiectasias), hyperplasia and hypertrophy of chlorine cells and mucus-producing cells, and changes in chondrocytes in the afferent portions of the filaments (Martinez et al. 2004, Simonato et al. 2008, Al-Balawi et al. 2013, Macirella et al. 2019). No hepatic, renal, ovarian, or intestinal lesions, as reported in other studies (Simonato et al. 2008, Dai et al. 2009, Al-Balawi et al. 2013, Doaa & Hanan 2013, Rajeshkumar et al. 2017, Xia et al. 2018) were observed in the present study.

The gill was the most affected organ, probably because it is an important pathway for lead absorption and bioaccumulation, especially in freshwater species, particularly when the metal is diluted in water (Kim & Kang 2015, Lee et al. 2019). Even though no statistical analysis was performed on the data from the toxicological analysis, our results support the higher bioaccumulation of Pb in the gills. In addition, the gill is possibly impacted by free radicals produced by lead, being exposed to cellular ion regulation damage, protein damage, and membrane damage from oxidative stress (Rogers et al. 2003, Verstraeten et al. 2008, Lee et al. 2019). It is suggested that no lesions were found in other organs due to the fast occurrence of mortalities and the short exposure time (96 hours) of the fish to the metal. Although the study was conducted in zebrafish, we believe the findings may be extrapolated to other species, particularly freshwater fish exposed to elevated concentrations of lead in the aquatic environment. Therefore, the present study contributes to the anatomopathological characterization of lead toxicity in fish. Further studies are required to evaluate interspecific susceptibility under varying exposure concentrations and durations of the metal.

CONCLUSION

Lead (Pb) was toxic to zebrafish at the evaluated doses and caused dose-dependent mortalities. The main organ affected was the gill, as it has direct contact with dissolved lead and is responsible for absorbing the metal in freshwater fish. The gills had proliferative and necrotic lesions that compromised the fish's respiratory capacity and led to clinical signs of altered swimming behavior and ventilatory dysfunction. Injuries vary according to exposure time and individual susceptibility. As the lesions found are not specific for lead, histopathology, in combination with toxicological analyses of water or fish tissues, is recommended to establish the diagnosis in suspected natural cases of toxicity.

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Data availability statement.- The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Table S1. Examples, definitions, and criteria of clinical signs, adapted from the "Test Guideline" No. 203

CLINICAL SIGNS	
LOSS OF BALANCE	
1. Abnormal horizontal orientation	Loss of balance with abnormal horizontal orientation in relation to the water column.
2. Abnormal vertical orientation	Head up or down posture.
3. Loss of buoyancy control	Floating on the surface or sinking to the bottom.
ABNORMAL SWIMMING BEHAVIOR	
1. Hypoactivity	Decreased spontaneous activity, lethargy and apathy.
2. Hyperactivity	Increased spontaneous activity, agitation and erratic swimming.
3. Corkscrew swimming	Rotation around the long axis, erratic movements, often in bursts, rolling, spiraling, spiral swimming, falling or circular movements.
4. Seizures	Involuntary and uncontrolled abnormal contraction of muscles (spasms).
5. Tetany	Rigid body muscles (intermittent or permanent) paralysis.
6. Skin irritation	Flashing, scraping, and rubbing against the side of the aquarium.
7. Abnormal surface distribution	Abnormal depth selection, near water/air interface, jumping, stays at the surface or just below the surface/top.
8. Abnormal bottom distribution	Abnormal depth behavior, near the base of the tank, prone diving, static at the bottom of the aquarium or just above the bottom.
9. Overreactive to stimuli	Flight (startle) or avoidance response to: visual (hand passing over the tank, luminous (light beam), tactile (touch), or vibration stimulus (tank tapped lightly).
10. Underreactive to stimuli	Decreased avoidance response to visual, tactile, or vibration stimuli
11. Dropping behavior	Individual fish show loss of aggregation and social interactions.
12. Dense schooling behavior	Increased association with excessive crowding of fish.
ABNORMAL VENTILATORY (RESPIRATORY) FUNCTION	
1. Hyperventilation	Increased frequency of opercular ventilatory movements, with possible open mouth and open opercula.
2. Hypoventilation	Decreased frequency of opercular movements, shallow ventilatory movements.
3. Irregular ventilation	Irregular opercular movements.
4. Coughing	Rapid expansion of the reflex of the mouth and opercula not into the water or surface - assumed to clear the ventilatory channels.
5. Swallowing	Movements of the mouth (and opercular membranes) on the surface of the water result in the entry of water and air.
6. Head shaking	Rapid lateral movements of the head.

ABNORMAL SKIN PIGMENTATION	
1. Darkened	Changed/increased/darkened color.
2. Pallor	Pale/altered/weak pigmentation.
3. Discolored spots	Discolored spots.

OTHER VISIBLE ABNORMALITIES (APPEARANCE AND BEHAVIOR)	
1. Exophthalmia	Swelling within the orbital cavity(s), which results in bulging of one or both eyes.
2. Edema	Abdominal edema due to fluid accumulation. It can cause raised scales and/or fissures in the abdominal wall.
3. Hemorrhage	Petechiae (pinhead-sized spots) and/or hematoma (blood collection area) due to intradermal or submucosal bleeding.
4. Excess mucus production	Excess mucus production.
5. Fecal (anal) cast	String of feces hanging from the anus or the bottom of the tank.
6. Aggressiveness	Aggression, direct attack, domination of tank selection locations.
7. Cannibalism	Attacking and ingesting parts of other fish or eating the bodies of dead fish.

Reference: OECD (2019).

Table S2. Water parameters for the acute lead (Pb) toxicity test

Water Parameters			
	Group	Average	Standard deviation
Temperature	Pb 0.21 mg/L	25	0.54
	Pb 0.46 mg/L	25.6	0.62
	Pb 1.02 mg/L	25.6	0.57
	Pb 2.24 mg/L	25.6	0.65
	Pb 4.92 mg/L	25.8	0.65
	Control	25.6	0.62
pH	Pb 0.21 mg/L	6.5	0.23
	Pb 0.46 mg/L	6.8	0.35
	Pb 1.02 mg/L	6.6	0.27
	Pb 2.24 mg/L	6.8	0.2
	Pb 4.92 mg/L	6.5	0.26
	Control	6.8	0.37
Dissolved oxygen	Pb 0.21 mg/L	4.90	1.3
	Pb 0.46 mg/L	4.92	1.6
	Pb 1.02 mg/L	4.70	1.35
	Pb 2.24 mg/L	4.67	1.12
	Pb 4.92 mg/L	4.69	1.13
	Control	4.86	1.61