TRABAJO ORIGINAL

Hepatotoxic effects of low-concentration exposure to the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) in adult male zebrafish (*Danio rerio*)

Efectos hepatotóxicos de la exposición a bajas concentraciones del herbicida ácido 2,4-diclorofenoxiacético (2,4-D) en peces cebra machos adultos (*Danio rerio*)

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Abstract. 2,4-Dichlorophenoxyacetic acid (2,4-D) is an herbicide widely used around the world. It has been detected in water samples, with a half-life ranging from 15 to 300 days depending on environmental conditions. This study aimed to investigate the effects of a commercial formulation containing 2,4-D on oxidative stress markers, as well as on liver histopathological and histochemical parameters, in adult male *Danio rerio* for 7 days. The results revealed structural and vascular lesions in the livers of zebrafish across all groups exposed to 2,4-D (at concentrations of 0.03, 0.3 and 3.0 mg/L). Analysis of the Histopathological Alteration Index suggests severe (3.0 mg/L) or moderate (0.03 and 0.3 mg/L) liver impairment in zebrafish exposed to 2,4-D. Exposure to the herbicide also led to a reduction in acid polysaccharides (0.03 and 3.0 mg/L) and glutathione (GSH) levels (at concentrations of 0.03 and 3.0 mg/L), and increased levels of the oxidized glutathione (GSSG) (at concentrations of 0.03 and 0.3 mg/L). No significant changes in lipid peroxidation levels were observed. These findings suggest that as little as 7 days of exposure to permissible concentrations of 2,4-D (0.03 mg/L) or higher (0.3 and 3.0 mg/L) can negatively affect biochemical, histochemical, and histopathological parameters, as well as the integrated biomarker response index in the liver of adult zebrafish. This study provides the first evidence of the hepatotoxic effects of the herbicide 2,4-D in adult male *Danio rerio*.

Keywords: Ecotoxicology; Pollution; Pesticide; Liver; Histopathological; Aquatic environment.

Resumen. Ácido 2,4-Diclorofenoxiacético (2,4-D) es un herbicida ampliamente utilizado en todo el mundo. Se ha detectado en muestras de agua, con una vida media que varía entre 15 y 300 días dependiendo de las condiciones ambientales. Este estudio tuvo como objetivo investigar los efectos de una formulación comercial que contiene 2,4-D sobre los marcadores de estrés oxidativo, así como sobre los parámetros histopatológicos e histoquímicos del hígado en machos adultos de *Danio rerio* durante 7 días. Los resultados revelaron lesiones estructurales y vasculares en los hígados de los peces cebra en todos los grupos expuestos al 2,4-D (a concentraciones de 0,03, 0,3 y 3,0 mg/L). El análisis del Índice de Alteración Histopatológica sugiere un deterioro hepático severo (3,0 mg/L) o moderado (0,03 y 0,3 mg/L) en los peces expuestos al 2,4-D. La exposición al herbicida también condujo a una reducción en los polisacáridos ácidos (0,03 y 3,0 mg/L) y en los niveles de glutatión reducido (GSH) (a concentraciones de 0,03 y 3,0 mg/L), así como a un aumento en los niveles de glutatión oxidado (GSSG) (a concentraciones de 0,03 y 0,3 mg/L). No se observaron cambios significativos en los niveles de peroxidación lipídica. Estos hallazgos sugieren que tan solo 7 días de exposición a concentraciones permitidas de 2,4-D (0,03 mg/L) o superiores (0,3 y 3,0 mg/L) pueden afectar negativamente los parámetros bioquímicos, histoquímicos e histopatológicos, así como el índice de respuesta de biomarcadores integrados en el hígado de peces cebra adultos. Este estudio proporciona la primera evidencia de los efectos hepatotóxicos del herbicida 2,4-D en machos adultos de *Danio rerio*.

Palabras clave: Ecotoxicología; Contaminación; Pesticida; Hígado; Histopatología; Medio acuático.

INTRODUCTION

Large-scale agricultural production has historically relied on pesticide application to address the challenges associated with pest control and soil degradation, both of which threaten crop productivity (Moraes 2019). In recent decades, pesticide sales have significantly increased, with global consumption reaching 3.7 million tons of active ingredients annually (FAO 2022). Only in Brazil, over 800 thousand tons of pesticides were sold in 2022 (IBAMA 2023). Due to their intensive use, pesticides can negatively impact health and the environment through soil and water contamination, harming non-target plants and animals. This reduces biodiversity and affects living organisms, including humans (FAO 2022). Among the classes of pesticides, herbicides are the most widely used and are intended to control weeds (Gupta 2019).

2,4-Dichlorophenoxyacetic acid (2,4-D) is a widely used herbicide globally and ranks as the second most sold pesticide in Brazil (IBAMA 2023). This compound belongs to the class of phenoxyacetic acids and serves as the primary active ingredient in over 1,500 chemical formulations (Islam *et al.* 2018). Known for its high selectivity in targeting dicotyledonous plants, 2,4-D is applied as a pre- and post-emergent systemic herbicide across various crops. Acting as a growth regulator, it mimics the plant hormone auxin, disrupting normal growth processes and ultimately leading to plant death (Martins *et al.* 2024). Despite being one of the earliest herbicides developed and a former component of Agent Orange, a defoliant widely used during the Vietnam War and subsequently banned due to its toxic effects, its high efficacy and low cost continue to render it a commercially viable option (Islam *et al.* 2018).

Due to its extensive and widespread use, 2,4-D concentrations have been detected in both surface and drinking water in several countries (Hernández *et al.* 2011; Rodil *et al.* 2012; Ensminger *et al.* 2013; Yamini and Saleh 2013; Tsaboula *et al.* 2016; Martins *et al.* 2024). In aquatic environments, the half-life of 2,4-D can range from 15 to 300 days, depending on environmental conditions (Islam *et al.* 2018), raising increasing concerns about the contamination of water bodies and the exposure of non-target organisms to this environmental pollutant. Brazilian regulatory agencies have been detecting the occurrence of 2,4-D in water samples from surface and groundwater sources, as well as in drinking water (CETESB 2018; PEVASPEA 2023), raising concerns about its presence and potential toxic effects on non-target organisms.

Research on the toxicity of 2,4-D has historically concentrated on several key areas, including herbicide resistance or tolerance, occupational risk, neurotoxicity, and the effects on non-target species (Zuanazzi *et al.* 2020). Among non-target organisms, fish are excellent bioindicators of environmental contamination, as they are sensitive to different types of pollutants and are frequently used in xenobiotic-induced toxicity studies. Some studies have shown that different fish species are susceptible to 2,4-D toxicity, whose documented effects include alterations in biochemical, morphophysiological and behavioral parameters (DeQuattro and Karasov 2016; Ruiz de Arcaute *et al.* 2016; Dehnert *et al.* 2019). Among fish, the zebrafish *Danio rerio* stands out as a widely used model in areas such as toxicology, pharmacology and neuroscience. Studies using early stages of embryonic and larval development of zebrafish, in short periods of exposure, showed toxic effects induced by 2,4-D during development, which include decreases survival of zebrafish embryos and larvae, changes in hatching rates, different types of malformations, such as pericardial and yolk sac edema, as well as neurological and behavioral changes (Dehnert *et al.* 2019; Gaaied *et al.* 2020; Martins *et al.* 2021).

In addition to its impact on normal development, the liver is recognized as one of the organs affected by the herbicide 2,4-D. However, most studies investigating the hepatotoxic effects of this herbicide have focused on rodent models or on different fish species exposed to high concentrations of 2,4-D, and in many cases, the sex of the fish used was not specified (Neskovic *et al.* 1994; Cattaneo *et al.* 2008; Matviishyn *et al.* 2014). Because liver detoxification systems in fish are largely driven by hormonal regulation, liver metabolism may differ between males and females, making it important to discriminate the hepatotoxic effects of 2,4-D by considering the sex of the animal. For example, estrogen in females can up- or down-regulate certain enzymes in the liver, leading to differences in how toxins are metabolized (Nebert and Dalton 2006). Moreover, studies on 2,4-D-induced hepatoxicity in zebrafish are scarce, limited to one study conducted at early stages of development in this model (Martins *et al.* 2021). During these early periods, protective systems are not fully developed, making embryos and larvae particularly susceptible to toxic effects that may be specific to a particular developmental stage (Phelps *et al.* 2020). This increased sensitivity complicates the extrapolation of findings to later developmental stages or other species. Therefore, there is a gap in knowledge regarding the hepatotoxic potential of 2,4-D in adult fish, especially in the zebrafish model.

Given the high commercialization volumes of 2,4-D in Brazil and worldwide, its persistence in the environment, and its detection in rivers and lakes, it is evident that concerns persist regarding the contamination of non-target organisms through water bodies, particularly the potential for liver damage (Martins *et al.* 2024),

which may lead to the development of various diseases, including hepatitis, fibrosis, steatosis, cirrhosis, and hepatocellular carcinoma (Elufioye and Habtemariam 2019). The aim of the study was to investigate the effects of a commercial formulation containing 2,4-D on liver histopathological and histochemical parameters, oxidative stress markers, and the integrated biomarker response (IBR) index in adult male *D. rerio*.

MATERIALS AND METHODS

Animals

Adult male fish of the species *Danio rerio* were obtained from aquarium stores in Santa Catarina. They were acclimated for two weeks in the vivarium of the Federal University of Santa Catarina (UFSC). The fish were housed at a stoking density of 1 gram (g) of fish per liter (L) under standardized laboratory conditions. These conditions included dechlorinated and filtered water, a controlled temperature (27 ± 1 ºC), a light-dark cycle of 14-10 h. They were fed twice a day with commercial fish food (composition: 46% protein, 6% fat, 1.8% fiber and 9.5% inorganic matter - Ca, F and Mg). Water parameters were monitored daily and maintained within the following ranges: pH 7.0 (± 0.5), 0 ~ 0.25 ppm of toxic ammonia (NH_3), and 0.001 ~ 0.01 ppm of nitrite (NO_2). The conditions for housing the animals, as described above, were in accordance with the Ethics Committee on Animal Use – UFSC (protocol approved by CEUA / UFSC No. 6778251119).

Herbicide

The commercial formulation U46 BR (Nufarm Chemical and Pharmaceutical Industry S/A), containing 806.0 g/L of 2,4-D, was utilized in the experiments. The dilution was prepared using dechlorinated and filtered water, taking into account the concentration of 2,4-D in the formulation.

Experimental Procedures

After the acclimatization period, the animals were randomly divided into 4 groups (n = 35/group): a control group (0.0) and groups exposed to 2,4-D at concentrations of 0.03, 0.3, and 3.0 mg/L. The designated exposure duration was 7 days, due to a lack of literature on the effects of 2,4-D after this duration of exposure. On the 4th day of exposure, the water was completely replaced by a new solution containing the same concentrations of 2,4-D corresponding to each experimental group, ensuring continued exposure to the herbicide. At the end of the 7th day of exposure, the male specimens were euthanized for liver collection. Euthanasia was conducted by immersing the specimens in eugenol at a concentration of 75 mg/L of water. All procedures were executed in accordance with Normative Resolution No. 37 of the National Council for the Control of Animal Experimentation (CONCEA).

Biochemical Analysis

Liver samples (consisting of 5 livers/sample) were thawed (n=6/group), homogenized in phosphate buffer (0.3 M, pH 7.4), and subsequently centrifuged (10 min, 10,000 rpm, 4 °C). The resulting supernatant was used to determine protein concentration, reduced glutathione (GSH) levels, oxidized glutathione (GSSG) levels, lipid peroxidation (LPO) levels, and NADPH oxidase activity. Protein concentration (mg/mL) was determined by the Bradford method (Kruger 1994), with bovine serum albumin as standard, in an Infinite M200 TECAN microplate reader, measuring absorbance at 595 nm. GSH and GSSG levels were measured as previously described (Rahman *et al.* 2007). Briefly, GSH reacts with dithionitrobenzoic acid (DTNB) to form a conjugate (GSH-TNB), which was measured by spectrophotometry at 412 nm, using a GSH concentration curve as the standard. The GSSG measurement was based on its reduction to GSH by glutathione reductase, which reduces all GSSG to GSH. Subsequently, free GSH levels were measured using a reaction with DTNB. To calculate GSSG, the GSH measurement was subtracted by GSH measurement after glutathione reductase, this difference was the GSSG concentration (Huber *et al.* 2008). The end product of lipid peroxidation, malondialdehyde (MDA), was measured in tissue homogenates through its reaction with thiobarbituric acid (TBA), forming a pink-colored fluorescent complex. The MDA produced was determined by the fluorescence of the MDA-TBA complex, with excitation at 515 nm and emission at 553 nm, using MDA as the standard (Ohkawa *et al.* 1979). To evaluate NADPH oxidase enzyme activity, 1.25 µL of 10 mM lucigenin were inserted per well, together with NADPH buffer (50 mM PBS, 0.01 mM EDTA, pH 7.40). An initial reading was taken to determine the background signal. Then, 25 µL of 10 mM NADPH were added per well, and continuous readings were taken for 15 min at 37 °C to evaluate luminescence (Janiszewski *et al.* 2002).

Histological Analysis

For using light microscopy analyzes, liver samples were dissected, fixed in alcoholic Bouin solution for 24 h and subsequently preserved in 70% ethanol (n = 3/group). Following this, the samples were dehydrated in an ascending ethanolic series 70% - 100%, clearing in xylene and embedding in paraffin. Tissue sections of 6 µm thickness were obtained using a Leica RM2255 rotary microtome at the "Laboratório Multiusuário de Estudos em Biologia" (LAMEB). The resulting slides were then subjected for staining techniques with Hematoxy-

lin and Eosin (HE), and Toluidine Blue (TBO).
For morphological analyses of the liver samples, 10 photomicrographs were analyzed per animal, totaling 30 photos per group. Evaluations were performed by calculating the Histological Alteration Index (HAI), which considers the frequency and severity of each histological change (lesion). Lesions were categorized into progressive stages based on tissue function impairment, following the criteria outlined by Poleksic and Mitrovic-Tutundzic (1994), using the formula:
$HAI = 10^0 . \Sigma^I + 10^1 . \Sigma^{II} + 10^2 . \Sigma^{III}$
Σ^I, Σ^{II}, and Σ^{III} represent the total number of changes according to their respective stage, while 10^0, 10^1, and 10^2 are the factors used to calculate the HAI, based on the severity of the injury. HAI values ranging from 0 to 10 indicate normal organ function; 11 to 20 suggest slight damage; 21 to 50 indicate moderate damage; 50 to 100 indicate severe damage, and values exceeding 100 indicate irreversible tissue damage, as adapted from Poleksic and Mitrovic-Tutundzic (1994). The stained sections were photographed using an Olympus BX41 upright microscope, and images were captured with the Q-imaging 3.3-megapixel color digital camera and the Q-imaging Q-capture Pro 5.1 image capture software.
Slides stained with TBO were used to examine the histochemical profile of acidic polysaccharides, employing integrated density analysis with ImageJ software (Schneider *et al.* 2012; Hartig 2013). In this analysis, the photomicrograph is converted to an 8-bit grayscale, and squares of defined area were employed for all measurements. These squares were randomly positioned within the image, and six areas of 4588.23 μm² each were measured in each section. The data were then extracted and plotted using GraphPad Prism statistical software for further analysis.

Integrated Biomarkers Response (IBR)
The Integrated Biomarker Response (IBR) is a technique used in ecotoxicological studies to analyze and integrate the responses of different biomarkers in organisms exposed to environmental stressors, and it was determined according to Beliaeff and Burgeot 2002. Based on the data collected for the different biomarkers analyzed in this study [Reduced glutathione levels (GSH), Oxidized glutathione levels (GSSG), NADPH oxidase activity (NADPH), Thiobarbituric Acid Reactive Substances (TBARS), Integrated density of acidic polysaccharides by toluidine blue-stained sections, and Histopathological Alteration Index (HAI)], the values of each biomarker in liver of fish exposed to 2,4-D (0.03, 0.3, and 3.0 mg/L) were compared to the mean of the control group. Subsequently, the logarithm of each ratio [Log(Yi+1)] was calculated, followed by the determination of the mean (μ) and standard deviation (σ). The logarithmized values (Yi) were standardized to compare the responses of the different biomarkers using the formula [$Zi = (Yi-\mu)/\sigma$]. Then, the difference between the standardized values of the exposed groups (Zi) and the control group (Zo) was calculated for each biomarker ($A = Zi - Zo$). The resulting difference value (A) was used to represent the deviation of each biomarker in the different experimental groups subjected to 2,4-D relative to the control group, using a radar graph. The IBR index was then obtained by summing all the absolute values of A for the different biomarkers for each experimental group ($IBR = \sum|Ai|$).

Statistical Analysis
Results are presented as mean ± standard error. The data were subjected to the Kolmogorov-Smirnov normality test, and outliers were identified using the ROUT method. Identified outliers resulted in the removal of the respective animal from the analysis. Statistical analyses were performed using one-way Analysis of Variance (ANOVA), followed by Tukey's post-test, in GraphPad Prism version 8.0.2 software. Differences were considered significant at $p<0.05$. Study images were captured using Adobe Photoshop CS6 software.

RESULTS

Effects induced by 2,4-D on markers of oxidative stress in the liver
Exposure to the herbicide 2,4-D induced changes in the levels of the GSH in the liver of zebrafish [$F(3,19) = 8.247$, $p<0.001$], resulting in decreased GSH levels in the 0.03 and 3.0 mg/L groups of 2,4-D ($p<0.01$). The levels of the GSSG were also affected by 2,4-D [$F(3,18) = 4.404$, $p<0.05$], with significant increases observed in the groups exposed to 0.03 and 0.3 mg/L of 2,4-D ($p<0.05$). While evaluating the activity of the NADPH oxidase enzyme in the liver, no significant differences were found compared to the control group, but rather between the groups exposed to 2,4-D [$F(3,20) = 3.124$, $p<0.05$], where the 0.3 mg/L group exhibited higher activity compared to the 0.03 mg/L group of 2,4-D ($p<0.05$). Regarding lipid peroxidation levels (expressed as MDA), no changes were observed in the liver of zebrafish after exposure to the 2,4-D herbicide (*Figure 1*).

Histopathological and histochemical changes in the liver after exposure to 2,4-D
The histopathological analysis of the liver of adult male zebrafish *D. rerio* is shown in *Figure 2*. The normal liver structure primarily comprises hepatocytes, blood vessels, and blood cells traversing these vessels (*Figure 2A*). In zebrafish exposed to concentrations of 0.03, 0.3 and 3.0 mg/L of 2,4-D, structural and vascular le-

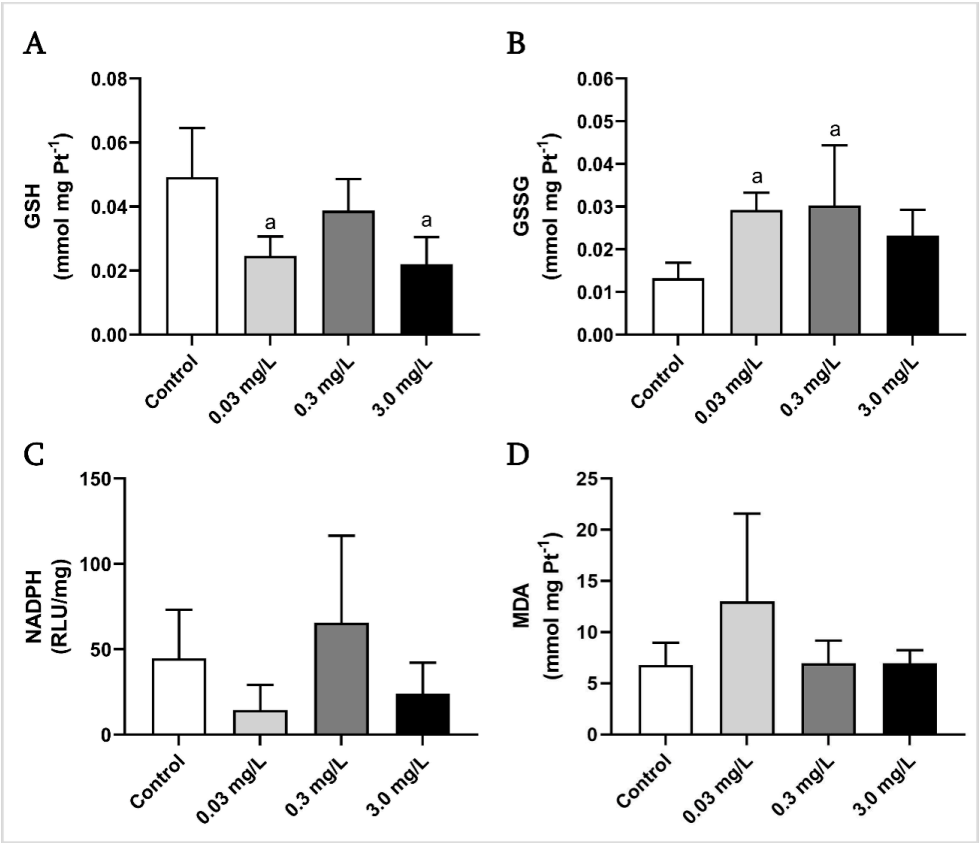

Figure 1. Oxidative stress markers in the liver of adult male zebrafish (*Danio rerio*), control and exposed to 2,4-D (0.03; 0.3; 3.0 mg/L) for 7 days. A) GSH (reduced glutathione) (mmol mg Pt^{-1}); B) GSSG (oxidized glutathione) (mmol mg Pt^{-1}); C) NADPH oxidase (RLU/mg); D) MDA (malondialdehyde) (mmol mg Pt^{-1}). (a)Significantly different ($p < 0.05$) from control evaluated by ANOVA followed by Tukey post-test.

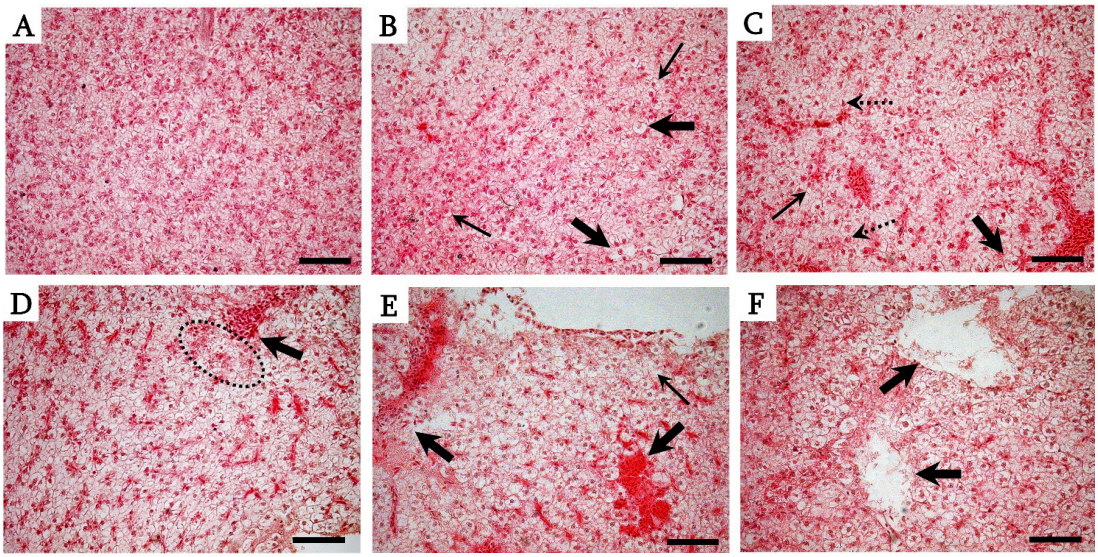

Figure 2. Histopathological changes in liver tissue of adult male zebrafish (*Danio rerio*), control and exposed to 2,4-D (0.03; 0.3; 3.0 mg/L) for 7 days. (A) Control; (B) Exposed to concentrations of 0.03 mg/L of 2,4-D with the presence of cytoplasmic vacuoles (thin arrow) and cellular hypertrophy (thick arrow); (C and D) Fish exposed to a concentration of 0.3 mg/L of 2,4-D. In (C) there is the presence of vacuoles (thin arrow), deformation in the morphology of the nucleus (dashed arrow) and cellular hypertrophy (thick arrow) and in (D) we observe hyperemia (thick arrow) and tissue disarray (dashed circle); (E and F) Fish exposed to 3.0 mg/L 2,4-D. In (E) hemorrhage (thick arrow) and loss of cell boundaries (thin arrow) are observed, and in (F) points of necrosis are observed in the tissue (thick arrows). Color: HE. Bar scale: 50 µm.

Table 1. Frequency table of histopathological lesions according to their stage in the liver of adult male zebrafish ( ), control and after exposure to the herbicide 2,4-D (0.03; 0.3; 3.0 mg/L) for 7 days. - = Absent or rarely frequent; + = Infrequent; ++ = Moderately frequent; +++ = Very frequent.

Histological changes	Stage	Groups (mg/L)			
		Control	0.03	0.3	3.0
Vacuolation	I	+	++	+++	+++
Tissue disarray	I	+	++	++	+++
Cytoplasmic hypertrophy	I	+	+	++	+++
Nuclear hypertrophy	I	-	+	+	+
Nuclear deformation	I	-	++	++	++
Cell membrane rupture	II	-	+	+	++
Sinusoidal expansion	I	+	++	+	+
Hyperemia (vascular congestion)	I	+	+	++	++
Bleeding	II	-	+	++	++
Necrosis	III	-	-	-	++

sions were observed in the liver tissue. The predominant lesions included vacuolization, cellular and nuclear hypertrophy, nucleus deformation, tissue disarray, sinusoid dilation, hyperemia and in some instances, hemorrhages and necrosis (*Figures 2B-F*). No deaths of zebrafish were found in any of the groups evaluated in the study.

Histopathological analyses of zebrafish livers exposed to 2,4-D demonstrated an increase incidence of tissue lesions compared to the control group (*Table 1*). Calculation of the Histopathological Alteration Index (HAI) revealed a significant difference [$F(3,8) = 7.60$; $p<0.01$] of the 3.0 mg/L 2,4-D group compared to the control group ($p<0.01$) and also compared to the other exposed groups ($p<0.5$). The HAI of the control group indicates healthy liver function (range of 0 to 10), the 0.03 and 0.3 mg/L groups exhibited higher values within the range of 20 to 50, indicating moderate liver tissue damage. Notably, the group exposed to 3.0 mg/L demonstrated an HAI value exceeding 100, indicating that the organ presents irreparable tissue damage and suggesting a serious impairment of the organ (*Figure 3*).

The analysis of acidic polysaccharides through TBO labeling demonstrated that exposure to 2,4-D can affect the levels of these molecules in the liver of zebrafish [$F(3,716) = 47.27$, $p<0.001$]. It was observed that the groups exposed to 0.03 and 3.0 mg/L of 2,4-D showed a decrease in relation to the control group ($p<0.001$). Additionally, the 3.0 mg/L of 2,4-D group exhibited lower levels of acidic polysaccharides compared to the other groups exposed to 2,4-D ($p<0.001$, for both groups) (*Figure 4*).

Integrated Biomarker Response (IBR) analysis

The IBR results shown in the radar graph indicate that all exposed groups (0.03, 0.3, and 3.0 mg/L of 2,4-D) exhibited changes in the biomarkers analyzed in the liver of adult male zebrafish after 7 days. The deviations in

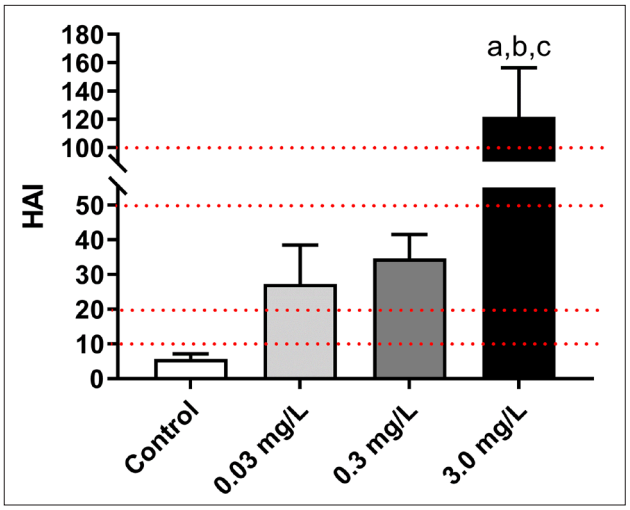

Figure 3. Histopathological Alteration Index (HAI) of the liver of adult male zebrafish (*Danio rerio*), control and exposed to the herbicide 2,4-D (0.03; 0.3; 3.0 mg/L) for 7 days. The red dotted lines delimit each range (0 to 10 - healthy; 11 to 20 - mild damage; 21 to 50 - moderate damage; 50 to 100 - severe damage and above 100 - irreparable damage). Data are presented as means ± SEM. Significantly different ($p < 0.05$) from control (a), (b) 0.03 mg/L 2,4-D, and (c) 0.3 mg/L 2,4-D evaluated by ANOVA followed by Tukey's post-test.

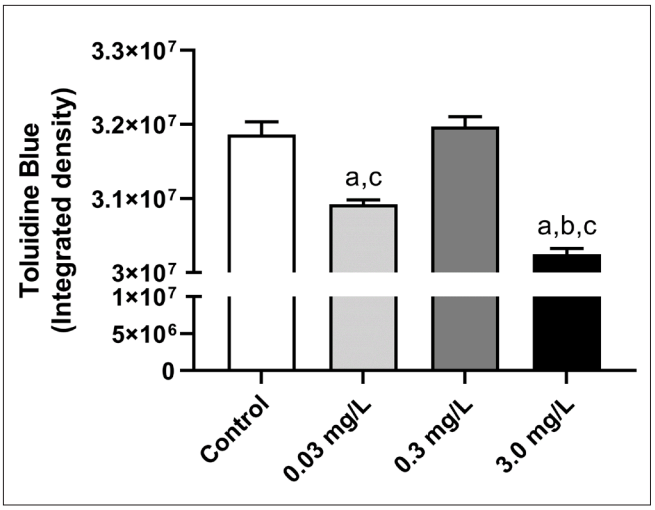

Figure 4. Integrated density graph of acidic polysaccharides labeled with toluidine blue in hepatocytes of adult male zebrafish (*Danio rerio*), control and exposed to the herbicide 2,4-D (control, 0.03, 0.3, and 3.0 mg/L) for 7 days. Data are presented as means ± SEM. Significant differences ($p < 0.05$) from the control are indicated by (a), from 0.03 mg/L 2,4-D by (b), and from 0.3 mg/L 2,4-D by (c), as determined by ANOVA followed by Tukey's post-test.

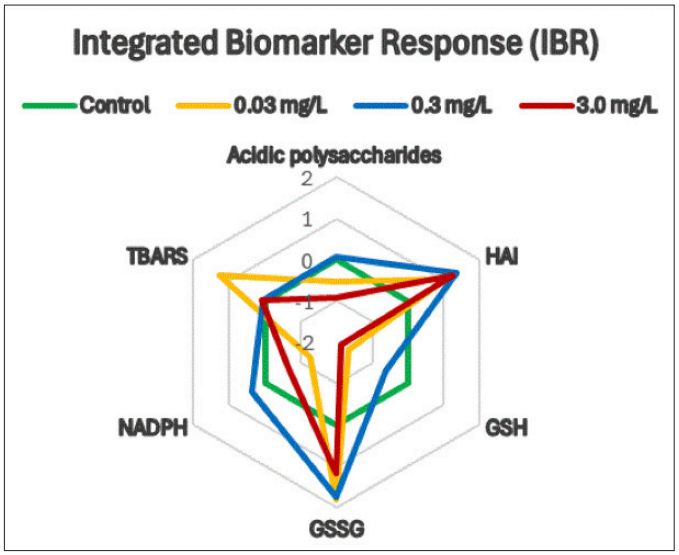

Figure 5. Integrated Biomarker Response (IBR) values for each biomarker evaluated in the liver of adult male zebrafish (*Danio rerio*), in control and groups exposed to the herbicide 2,4-D (0.03, 0.3, and 3.0 mg/L) for 7 days. Biomarkers: Reduced glutathione levels (GSH), Oxidized glutathione levels (GSSG), NADPH oxidase activity (NADPH), Thiobarbituric Acid Reactive Substances (TBARS), Integrated density of acidic polysaccharides in toluidine blue-stained sections, and Histopathological Alteration Index (HAI). For the control group, the IBR value = 0.

biomarker levels, both positive and negative, were more pronounced for the oxidative stress biomarkers GSH and GSSG, suggesting that they are the most sensitive to 2,4-D exposure and indicative of increased oxidative stress. The IBR index values were 7.54, 4.23, and 5.96 for the groups exposed to 0.03, 0.3, and 3.0 mg/L of 2,4-D, respectively. These findings suggest that the highest IBR index, reflecting the greatest modulation of biomarkers, was observed in the group exposed to the lowest concentration tested (0.03 mg/L), followed by the highest concentration (3.0 mg/L) of 2,4-D (*Figure 5*).

DISCUSSION

In this study, we demonstrated for the first time that the herbicide 2,4-D significantly impacts the liver of adult male *D. rerio*, affecting its biochemical, histochemical, and histopathological parameters.

In the present study, oxidative stress markers, histopathological and histochemical parameters, as well as IBR index were used to assess the impact of short-term exposure to low concentrations of a commercial 2,4-D formulation on the liver of adult male *D. rerio*. Hepatotoxic compounds can disrupt cellular processes by causing reducing antioxidant levels, and oxidative stress, promoting cell and liver tissue damage, which can lead to the development of liver disease (Elufioye and Habte-

mariam 2019). The safety of 2,4-D is a topic of ongoing research and debate. Regulatory agencies like the Environmental Protection Agency (EPA) and World Health Organization (WHO) have determined that, when used according to approved guidelines, 2,4-D poses minimal risk to human health. However, its safety can depend on various factors, including exposure levels and individual susceptibility. Studies suggest that long-term or high-level exposure to 2,4-D may lead to health risks, including histopathological damage to different organs and disruption of the endocrine system (Wisconsin Department of Health Services 2023).

In the liver tissue of adult male zebrafish, our results showed that 2,4-D exposure led to a decrease in GSH levels in the groups exposed to 0.03 and 3.0 mg/L of 2,4-D, along with an increase in GSSG levels in the groups exposed to 0.03 and 0.3 mg/L of 2,4-D. These findings suggest that 2,4-D induces the generation of oxidative molecules, causing the liver to utilize GSH to neutralize and mitigate oxidative damage. Simultaneously, the increase in GSSG reflects the oxidation of GSH during this protective process. The GSH molecule, vital for xenobiotics metabolism and cellular defense against oxidative stress (Huber *et al.* 2008), executes its protective function by promoting the reduction of reactive oxygen species (ROS), such as hydrogen peroxide and superoxide anion. GSH undergoes oxidation and is converted into GSSG. Subsequently, GSSG is regenerated back into GSH through the catalytic cycle (Huber *et al.* 2008). These findings are consistent with other studies on various fish species that examined the effects of commercial formulations of the herbicide 2,4-D. These studies linked 2,4-D exposure to increased oxidation or the production of oxidative by-products, leading to elevated ROS levels and alterations in antioxidant enzymes and molecules across different fish organs (Oruç and Üner 2000; Oruc *et al.* 2004).

Another important aspect is that, in addition to acting against oxidative stress, GSH also plays a key role in the metabolism of xenobiotics. This occurs through its conjugation with xenobiotics via the enzyme glutathione-S-transferase (GST), which renders these compounds less toxic and more water-soluble, thereby facilitating their elimination (Huber *et al.* 2008; Chowdhury and Saikia 2020). Exposure to 3.0 mg/L of 2,4-D caused a decrease in GSH but did not change the levels of GSSG and NADPH, suggesting that this decrease may be due to the formation of GSH/xenobiotic conjugates and their elimination.

Liver tissue serves several vital metabolic functions, including carbohydrate metabolism, lipid storage, synthesis and oxidation of fatty acids, glycogen storage, plasma protein synthesis, hormone metabolism and clearance, and detoxification (Hinton *et al.* 2001; Ferguson 2006; Yao *et al.* 2012; Heath 2018). Those roles render liver cells particularly susceptible to oxidative stress induced by toxic agents (Elufioye and Habtemariam 2019). Decreased antioxidant response and induction of oxidative stress led to cellular and tissue damage, as evidenced by increased MDA levels (Martins *et al.* 2024). MDA, a product of lipid peroxidation, especially of polyunsaturated fatty acids, serves as a common marker of oxidative stress and damage to lipids and cell membranes. Although the evaluation of MDA levels in the liver of zebrafish did not show significant changes at any concentration tested in this study, the modifications in antioxidant defenses induced by 2,4-D appear to have been regulated in a way that prevents increases in lipid peroxidation, likely due to resistance to oxidative stress through antioxidant mechanisms. A similar result was observed in the fish *Oreochromis niloticus* when exposed to a high concentration of the commercial formulation of 2,4-D (27 ppm) for a shorter period of time (96 h) (Oruç and Üner 2000).

While lipid peroxidation was not detected in the liver, histopathological analysis indicated liver toxicity induced by exposure to a commercial formulation of 2,4-D. Therefore, it is reasonable to postulate that other mechanisms contribute to this toxicity, such as protein and enzyme oxidation or the covalent binding of 2,4-D and its metabolite (2,4-dichlorophenoxyacetyl-S-acyl-CoA) to liver proteins, potentially compromising their function and inducing degradation, as observed in previous studies (Di Paolo *et al.* 2001; Li *et al.* 2003; Matviishyn *et al.* 2014; Tichati *et al.* 2020). This study did not assess whether protein oxidation or the formation of protein/2,4-D conjugates are involved in the mechanisms underlying liver toxicity in zebrafish, leaving this question open for future investigation. It is important to note that, despite 2,4-D being a very old pesticide with a long history of use, its full impact on non-target organisms, such as fish, remains incompletely understood (Mahmood *et al.* 2016; Marcato *et al.* 2017).

In our study, just one week of exposure to the commercial formulation of the herbicide 2,4-D induced several histopathological changes in the liver of zebrafish, including increased vacuolization, tissue disarray, cellular and nuclear hypertrophy, nuclear deformation, cell membrane rupture, and necrosis— the latter occurring at the highest concentration tested (3.0 mg/L of 2,4-D). Additionally, vascular alterations such as sinusoidal dilation, hyperemia characterized by vascular congestion, and hemorrhages were observed. Similar histopathological changes have been reported in previous studies following exposure to 2,4-D. Cattaneo *et al.* (2008) observed that *Rhamdia quelen* fish presented abnormal arrangement of hepatic cords, cell membrane rupture, and vacuolation of hepatocytes after exposure

to 700 mg/L of 2,4-D (commercial formulation) for 96 h. In guppies (*Viviparous Poecilia*), acute exposure (96 h) to 20 μL/L of the commercial formulation of 2,4-D resulted in an increase in vacuolation and cytoplasmic damage, while a concentration of 40 μL/L also caused vascular damage such as sinusoid vasodilation and vascular congestion (Vigário and Sabóia-Morais 2014).

The most frequent lesions identified in our study were vacuolization, cytoplasmic hypertrophy, and tissue disarray. Vacuolization, characterized by the formation of vacuoles in the cell cytoplasm, may indicate stored energy in the form of glycogen or lipids, or it may represent a pathological change involving the disruption of organelles such as the rough endoplasmic reticulum and Golgi apparatus, and/or the accumulation of fluid in the cytoplasm (Braunbeck 1998). Although the exact mechanisms behind vacuolization in our study are not fully understood, the alterations in organelles and cytoplasmic fluid accumulation observed in fish exposed to 2,4-D suggest disruptions in energy metabolism-related processes (Cattaneo *et al.* 2008). Therefore, it is reasonable to consider that this increase in vacuolization is due to changes in organelle structure and/or fluid accumulation in the cytoplasm, induced by exposure to 2,4-D. Further studies are needed to clearly understand the cellular events triggered by 2,4-D that contribute to this phenomenon.

In our study, cytoplasmic hypertrophy was moderately frequent at 0.3 mg/L and very frequent at 3.0 mg/L of 2,4-D, consistent with its progressive nature, which indicates that higher concentrations may lead to greater accumulation of changes in hepatocytes (Bernet *et al.* 1999). Tissue disruption and the consequent alteration of liver tissue architecture may be associated with 2,4-D-induced cytoskeletal changes. Structural reorganization and redistribution of microtubules and microfilaments, which disrupt organelle distribution, increase intracellular space, and impair hepatocyte interactions, have been previously described as effects induced by 2,4-D (Zhao *et al.* 1987). Similar changes in cytoarchitecture were observed in previous studies in silver catfish (*Rhamdia quelen*) exposed to a high concentration of commercial 2,4-D formulation (700 mg/L) (Cattaneo *et al.* 2008).

Vascular changes observed in zebrafish exposed to 2,4-D included sinusoidal dilation, hyperemia, and hemorrhage in liver tissue. These alterations may reflect an adaptive response aimed at increasing blood flow to facilitate the transport of defense cells and enhance tissue oxygenation (Santos *et al.* 2018). Given the liver's key role in the metabolism and circulation of xenobiotics, it is recognized as a primary target organ for chemical-induced tissue damage. In fish exposed to toxic agents, elevated hepatic blood flow can trigger vascular dilation and hyperemia, supporting hepatocyte catabolism and detoxification while promoting tissue oxygenation (Hinton *et al.* 2001). These processes may lead to increased hepatic vascular pressure, potentially resulting in vascular endothelial rupture. Moreover, pure 2,4-D has been shown to reduce the expression and levels of tight junction proteins in endothelial cell membranes, which can further contribute to endothelial rupture and subsequent bleeding (Pasandi *et al.* 2017).

Histopathological biomarkers are widely used to assess the toxic effects of xenobiotics in fish, serving as sensitive tools to diagnose both direct and indirect toxic effects on organisms. In this study, we used TBO, a dye with clinical utility (Sridharan and Shankar 2012), to label acidic polysaccharides in the zebrafish liver following 2,4-D exposure. Acidic polysaccharides containing uronic acid, such as glycosaminoglycans (GAGs), are commonly found in animal tissues, primarily in the extracellular matrix and mucous secretions (Cao *et al.* 2015). Among the GAGs are chondroitin sulfate, hyaluronic acid, and heparin, with heparin being particularly abundant in the liver of fish (Song *et al.* 2017). Studies have shown that GAGs like heparin and hyaluronic acid can undergo degradation as a result of oxidative stress (Sies 1987; Duan and Kasper 2011; Chowdhury and Saikia 2020). Given the ability of 2,4-D to induce ROS, the observed reduction in acidic polysaccharides might be linked to ROS-mediated degradation, specifically impacting heparin and other GAGs present in the liver (Tayeb *et al.* 2012; Pasandi *et al.* 2017).

The impact of 2,4-D on the liver of male zebrafish was assessed by considering both the severity and frequency of each lesion using the Histopathological Alteration Index (HAI) (Poleksic and Mitrovic-Tutundzic 1994). The significantly elevated HAI value in the 3.0 mg/L group was primarily driven by tissue necrosis, a severe form of damage that carries substantial weight due to its critical implications. In contrast, the 0.03 and 0.3 mg/L groups exhibited HAI values indicative of moderate tissue damage. This finding is particularly alarming from a biological and environmental perspective, as 0.03 mg/L of 2,4-D is the maximum concentration permitted for human consumption and for aquatic environments in Brazil (Brazil, Ordinance GM/MS No. 88, of May 4, 2021: Brazil, resolution No. 357, of March 17, 2005). Even more concerning is the fact that concentrations higher than 0.3 mg/L have been detected in aquatic ecosystems near plantations in Brazil (CETESB 2018).

The integrated biomarker response index (IBR) is a tool that synthesizes the responses of biological parameters to contaminants, aiding in the interpretation of biomarker results. Initially proposed by Beliaeff and Burgeot (2002), the IBR has been adopted in biomonitoring

and ecotoxicological bioassays. More recently, it has been used to assess the effects of contaminants on oxidative status and to simplify the interpretation of relationships between multiple biomarker responses and contamination levels (Caliani *et al.* 2021; Boudjema *et al.* 2023). A higher IBR value indicates a more intense response to exposure, suggesting a greater impact of the environmental stressor on the organism. In our study, the highest IBR index was observed in the group exposed to a concentration of 0.03 mg/L of 2,4-D, a level considered environmentally safe. According to the World Health Organization (WHO 2017), the maximum recommended concentration of 2,4-D in drinking water is 0.03 mg/L (or 30 μg/L). This limit is theoretically established based on prolonged exposure, with the assumption that it would not cause adverse health effects over a person's lifetime.

Finally, we acknowledge as a limitation of this study the absence of control groups using the vehicles present in the commercial formulation. This prevents a precise distinction between the toxic effects of the active ingredient (2,4-D) and those potentially caused by the solvents or emulsifiers included in the formulation. Additionally, the product label and package insert did not clearly disclose the identity or concentration of these other components. Notably, it is well recognized that so-called "inert" ingredients can sometimes be more harmful than the active compound itself (Roddam *et al.* 2025). Therefore, the results of this study should be interpreted with caution in light of this methodological limitation.

CONCLUSION

The data obtained in this study indicate that the herbicide 2,4-D significantly impacts the liver of adult male *D. rerio*, affecting its biochemical, histochemical, and histopathological parameters. Notably, even at the permitted concentration of 0.03 mg/L, 2,4-D was sufficient to trigger oxidative stress and cause moderate liver damage after only 7 days of exposure. At higher concentrations (3.0 mg/L), severe necrotic damage was evident. While antioxidant defenses in the zebrafish liver seemed to mitigate lipid peroxidation, the overall tissue damage suggests that 2,4-D poses a clear hepatotoxic threat. Importantly, this is the first study to demonstrate 2,4-D-induced hepatotoxicity in adult male zebrafish. These findings raise serious concerns about the intensive use of 2,4-D and its increasing presence in aquatic ecosystems, underscoring the urgent need for further research to fully understand its toxic effects on non-target organisms, as well as the need to review the maximum permitted concentrations for human consumption and aquatic environments.

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CONFLICT OF INTEREST

The authors have no relevant financial or non-financial interests to disclose.

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