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Original Communication

Toxicological effects of a glyphosate-based formulation on the liver of *Poecilia reticulata*

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ABSTRACT

The toxicity of the glyphosate-based herbicide formulation Roundup Transorb® was determined for *Poecilia reticulata* (guppy). The mean lethal concentration (LC_{50}) for 12, 24, 48, 72 and 96 h of R. Transorb® were 11.2, 8.5, 6.5, 6.1 and 5.6 µl/L, respectively, indicating that this species is very sensitive to this herbicide. Our recent study showed its genotoxicity to *P. reticulata* at low-level exposures of 1.41, 2.83, 4.24 and 5.65 µl/L which increased the frequencies of micronucleus and DNA damage in peripheral erythrocyte cells. Toxicity bioassays were conducted to evaluate the effects of sublethal concentrations of herbicide in the acute treatment for 24 h. Fish exposed to the herbicide showed behavioral changes such as aggressiveness and loss of sense of direction. Several different pathological alterations were observed in the liver, such as vacuolization, cytoplasmic degeneration and hyperemia. Therefore, results confirm the risks this herbicide formulation poses to aquatic organisms.

KEYWORDS: acute toxicity, liver histopathology, Roundup®, herbicides, guppy

INTRODUCTION

Roundup Transorb® is a widely used herbicide in Brazil's mid-west savannah-like biome. This herbicide is applied on leaves as desiccant on sugarcane, soybeans, coffee, maize and citrus crops. The formulation is based on isopropylamine salt of glyphosate at a concentration of 648 g/L, acid equivalent of N-(phosphomethyl) glycine (glyphosate) at a concentration of 480 g/L and with inert ingredients at a concentration of 594 g/L [1]. The surfactant polyethoxylene amine (POEA) is used in the most common glyphosate-based product Roundup® and is more toxic than pure glyphosate [2]. Thus, commercial formulations of glyphosate are more toxic than pure glyphosate [3].

Due to its high solubility in water and extensive use, the exposure of non-target aquatic organisms to this herbicide has caused great concern [3]. For aquatic organisms, the surfactant POEA in Roundup® is the main toxic component in the formulation [4]. In a previous study, POEA was found to be more toxic to fish than pure glyphosate [5]. A bioassay of acute toxicity of glyphosate on *Cyprinus carpio* showed a $LC_{50,\ 96h}$ of 620.0 mg/L [6]. However, with regard to the glyphosate formulation Roundup®, the $LC_{50,\ 96h}$ ranged from 2.0 to 55.0 mg/L, depending on the fish species, confirming the highest toxicity of glyphosate-based formulations, compared with its active ingredient [7].

Histological alterations were observed in the liver, gills and kidneys of the Nile tilapia *Oreochromis niloticus* after acute and chronic exposure to sublethal concentrations of Roundup® [7].

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The acute effects of this herbicide on metabolic and enzymatic parameters for *Leporinus obtusidens* and *Rhamdia quelen* were investigated by Glusczak *et al*. [8], in a study which observed a significant reduction in the enzyme acetyl-cholinesterase (AChE) in the fish's brain at all the studied concentrations of glyphosate. An increase in lipid peroxidation, glucose and proteins in the liver were also noted. To date, the toxicological responses of tropical fish to herbicides based on glyphosate have remained largely unclear.

Measurement and evaluation of the chemical compounds using histo-cytopathological tests have been increasingly recognized as a relevant tool in evaluating the impact of pollutants on fish [9, 10]. The gills, liver and kidney are common primary target organs for many chemical compounds, due to the processes of detoxification and ionic regulation that they perform in fish organism. The liver has been presented as the main target organ for determination of histo-cytopathological biomarkers in studies that investigate aquatic pollution as it is responsible for homeostasis maintenance, nutrient metabolism, glycogen and fats storage, and synthesis and secretion of proteins among other functions; particularly important is its metabolism of toxic agents [10, 11]. The notably dynamic nature of the liver and its regulation in various metabolic and physiological processes makes this organ an important experimental model for the study of mechanisms and processes involving activity and action of bioactive compounds [12]. Many contaminants tend to accumulate in fish liver, exposing the tissues of this organ to comparatively higher levels than those found in other organs [13]. This histopathological study of the liver of *P. reticulata* was motivated by the scarcity of data in the literature referring to the effects provoked by the glyphosate-based formulation R. Transorb®.

MATERIAL AND METHODS

Collection of fish

Thirty-six adult female fish of the species *P. reticulata* were used in each replicate (three bioassays) for the toxicity test, and 30 adult fish of both sex for the test on sublethal effects. These animals were obtained from the Agronomy School of the University of Goiás. Previously, the fish had been acclimatised in aquariums with filtered water, aeration and constant temperature (25 °C ± 2) for 15 days. The physicochemical parameters of the water, such as pH, conductivity, temperature and ammonia, were duly controlled, with constant renewal of water. The bioassays followed the recommendations of Organization for Economic Cooperation and Development (OECD) Protocol number 203, Guideline for Testing Chemicals - "Fish, Acute Toxicity test" [14]. Fish were fed *ad libitum* with supplemented fish-food from Alcon Colours®. Exposures were carried out in the fish maintained at a constant temperature of 26 °C ± 2 °C; ammonium level and hardness of the water were constantly monitored. Dissolved oxygen was kept higher than 60%, conductivity at 500 mS and pH at 7.2. The procedures were accepted and approved by the Committee on Ethics in Animal Experimentation of the Federal University of Goiás under case number 126. Glyphosate is a highly water-soluble herbicide, presenting a value of 11,600 mg/L at 25 ºC. Glyphosate is stable in water at pH 3, 5, 6 and 9, at a temperature of 35 ºC, with an aquatic half-life ranging from 2 to 14 days, and its main surfactant component, polyetoxylated tallowamine (POEA), has an aquatic half-life from 21 to 42 days. Glyphosate showed photodegradation stability at pH 5, 7 and 9 and is broken down primarily by microorganisms [15].

Determination of the average lethal concentration ($LC_{50;\ 12\text{-}96\ h}$)

A static system was used with constantly renewed aeration. Each aquarium received 6 fish weighing on average 0.4 g ± 0.2 g (24.2 ± 8.6 mm), at the following concentrations of Roundup Transorb®: 2, 4, 8, 16 and 32 µl/L plus the control group (CG). There was no periodical renovation of the herbicide in the experimental aquariums, as it does not undergo degradation or reduction in the experimental conditions to which it was submitted for 96 h. Behavioral studies: during exposure to R. Transorb® herbicide, the specimens' behavior was observed. Behavioral manifestations were recorded in the first 30 minutes and then at intervals of 2, 4, 8 and 12 h, during the day, each observation period lasting for 15 minutes. The following behavioral manifestations were analyzed: alterations in swimming, localization in the

aquarium, distribution in the aquarium, clinical manifestations, dead and stationary animals. Physicochemical parameters of the water in the aquariums during the exposures were maintained constant.

Sublethal test

To evaluate the effects of R. Transorb® the bioassays were carried out in four groups of six fish exposed plus a control non-exposed group. Sublethal exposures were based on 25, 50, 75 and 100% of the $LC_{50, 96h,}$ for 24 h. Fish were placed in glass aquariums of 7 L, each containing six fish (0.6 g ± 0.2 g; 28.5 ± 7.2 mm). The tissue fragments were dehydrated in graded concentrations of ethanol, and then embedded in historesin. After this, sections of 2.5 µm width were cut in the ultramicrotome (Leica Ultracut UCT). Histopathological and histomorphometric analyses: samples of liver were fixed in Karnovsky fixative, embedded in historesin, sectioned (2.5 µm), and stained with toluidine blue 1% for microscopic analyses. The images obtained were processed using the computer program ImageLab (version 2.3). Changes in liver tissue induced by R. Transorb® herbicide were evaluated using the Index of Histopathological Alterations (IHA), which is based on the severity of the lesions and the possibility of recovery. To calculate the IHA, hepatic alterations were classified in three progressive stages of hepatic functional insufficiency: stage I = changes that do not damage the liver tissue in a way that the organ cannot repair itself; stage II = reparable changes that are more severe, affecting the function of the associated tissues; stage III = changes that prevent the liver's functional structure from being re-established, even with a change in environment (improvement in water quality). The IHA was calculated by the sum of the number of types of lesions in each of the three stages and multiplied by the stage index, using the following mathematical equation proposed by Poleksić and Mitrovic-Tutundžić [16]: $IHA = (1\times\Sigma I) + (10\times\Sigma II) + (100\times\Sigma III)$, where I, II and III are the number of lesions in phase I, II and III, respectively. The value obtained for each IHA in the fish was used to calculate the mean index of each group exposed to the herbicide and its respective control. The IHA means were divided into five categories, 0-10: liver functioning normally; 11-20: liver function slightly to moderately damaged; 21-50: liver moderately to strongly damaged; 51-100: liver severely damaged; 100: liver irreparably damaged.

The histometric measurements were carried out in the Image Pro-Plus 6.0 program (Media Cybernetics, Silver Spring, USA/Microsoft® Windows 32-bit Systems Windows® XP, Vista). To this end, three slides were produced for each fish, each one containing 10 histological sections. The images generated were obtained by 40X magnification. Three images of different fields were thus acquired, from one section randomly selected from each histological slide, recording nine images of the liver per animal, 54 images for each group (CG and EG: 1.4, 2.8, 4.2 and 5.6 µl/L), totaling 270 images; 4,050 hepatocytes and 810 sinusoids were analyzed. A Samsung Color Digital SHC 410 NAD camera was used, coupled to an optical microscope (Leica DMLB). Quantification of the hepatocytes was obtained through the images of 15 hepatocytes chosen randomly to quantify the parameters of volume, area and diameter, plus three sinusoid vessels for each image to quantify their diameters. The measured variables were adapted from Vertemati [17] and Golalipour [18] (Table 1).

The volumetric measurements were estimated from studies carried out by Ke *et al*. [19]; these authors considered the hepatocyte as a sphere of $V = 4/3\pi R^3$, where the calculation of the radius (R) is given by the formula for area $A = \pi R^2$. All the calculations were carried out from values found by the use of tools available in the program Image Pro Plus 6.0.

Statistical analysis

The results obtained were expressed in the form of mean, standard deviation from the mean and standard error of the mean (SD). For analysis of variance, ANOVA with a level of significance of $\alpha = 0.05$ and $\alpha = 0.01$ and Tukey's Honest Significant Differences test (HSD test, parametric) were used by means of the program PDF Word Count & Frequency Statistics Software 7.0. Correlations were also analyzed by calculating the Pearson (r) correlation index, with its respective levels of significance. Guppy mortality rates in each aquarium in the periods of 12, 24, 48, 72 and

Table 1. Parameters adopted for histomorphometric analysis of the liver of *P. reticulata*.

Volume	Area	Diameter
$HV = 4/3\pi R^3$ ($R = HA*\pi^2$)	HA = mean	HD = mean-HD more + HD less
$CV = 4/3\pi R^3$ ($R = CA*\pi^2$)	CA = mean-(HA-NA)	ND = mean-ND more + ND less
$NV = 4/3\pi R^3$ ($R = An*\pi^2$)	NA = mean	SD = mean - 3 mean in 3 sinusoids
	HR = mean (CA/NA)	

HV = Hepatocyte volume; CV = Cytoplasm volume; NV = Nucleus volume; HA = Hepatocyte area; CA = Cytoplasm area; NA = Nucleus area; HR = Hepatocyte ratio; HD = Hepatocyte diameter; ND = Nucleus diameter; SD = Sinusoid diameter.

96 hours were used to estimate the LC_{50} of the herbicide for each exposure time using Graph software Prism-5 with transformation of the concentration into agonist log of the $LC_{50.}$ The values of the LC_{50} calculated by Prism-5 were confirmed with TSK-Trimmed Spearman-Karber (TSK), version 1.5 from the US Environmental Protection Agency (EPA) [20].

RESULTS

Determination of the LC_{50}

It was thus possible to determine the $LC_{50;\ 12,\ 24,\ 48,\ 72\ and\ 96h,}$ finding the mean of the three bioassays: 11.2 ± 0.13, 8.5 ± 2.6, 6.5 ± 2.1, 6.1 ± 1.4 and 5.6 ± 0.7 µl/L, respectively (Figure 1). The percentage mortality of fish exposed to the herbicide was concentration-dependent. At the highest concentrations (16 and 32 µl/L) 100% of the fish died in the first four hours of exposure, confirming the high toxicity of these concentrations. Lower exposure-levels of 2 and 4 µl/L presented a lower mortality rate during bioassays (Figure 1).

Figure 2 shows the mortality percentages of *P. reticulata*, obtained from exposures carried out with R. Transorb® to achieve the $LC_{50,\ 96h.}$ By transforming the concentrations into agonist log, it could be observed that in the three acute toxicity bioassays the mortality rate increased with an increase in the herbicide concentration. The linear regression coefficient calculated for the data obtained in 12 h ($R^2 = 0.8486$), 24 h ($R^2 = 0.9406$), 48 h ($R^2 = 0.9232$), 72 h ($R^2 = 0.9025$) and 96 h ($R^2 = 0.8776$) (Figure 2) support this trend, leading to the conclusion that the toxic effects of the herbicide appear to be concentration-dependent.

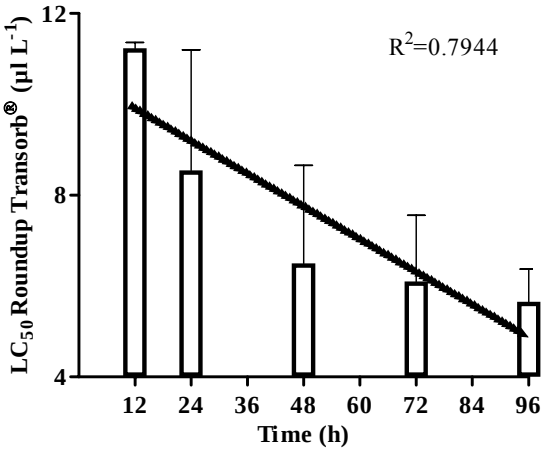

Figure 1. LC_{50} of Roundup Transorb® herbicide at 12, 24, 48, 72 and 96 h of exposure. Error bars represent 95% confidence intervals. Assays were carried out in triplicate.

The correlation between time (12-96 h) and mean lethal concentration (LC_{50}) was demonstrated to be inversely proportional ($R^2 = 0.7944$); the lower the exposure time to the herbicide, the greater the value of LC_{50}. The values of $LC_{50;12\text{-}96h}$ presented a tendency to decrease time-dependently in the three bioassays (Figure 2).

In the control group, normal behavior and coordinated group swimming were observed. In the aquariums of the exposed groups in the first and second observations, some behavioral manifestations were recorded, such as defecation, aggressiveness and irritability, which were seen at concentrations of 2 µl/L and 4 µl/L. Loss of the escape reflex, colliding with the wall of the aquarium, contortion, tremors, mydriasis, loss of sense of direction, premature laying and dead

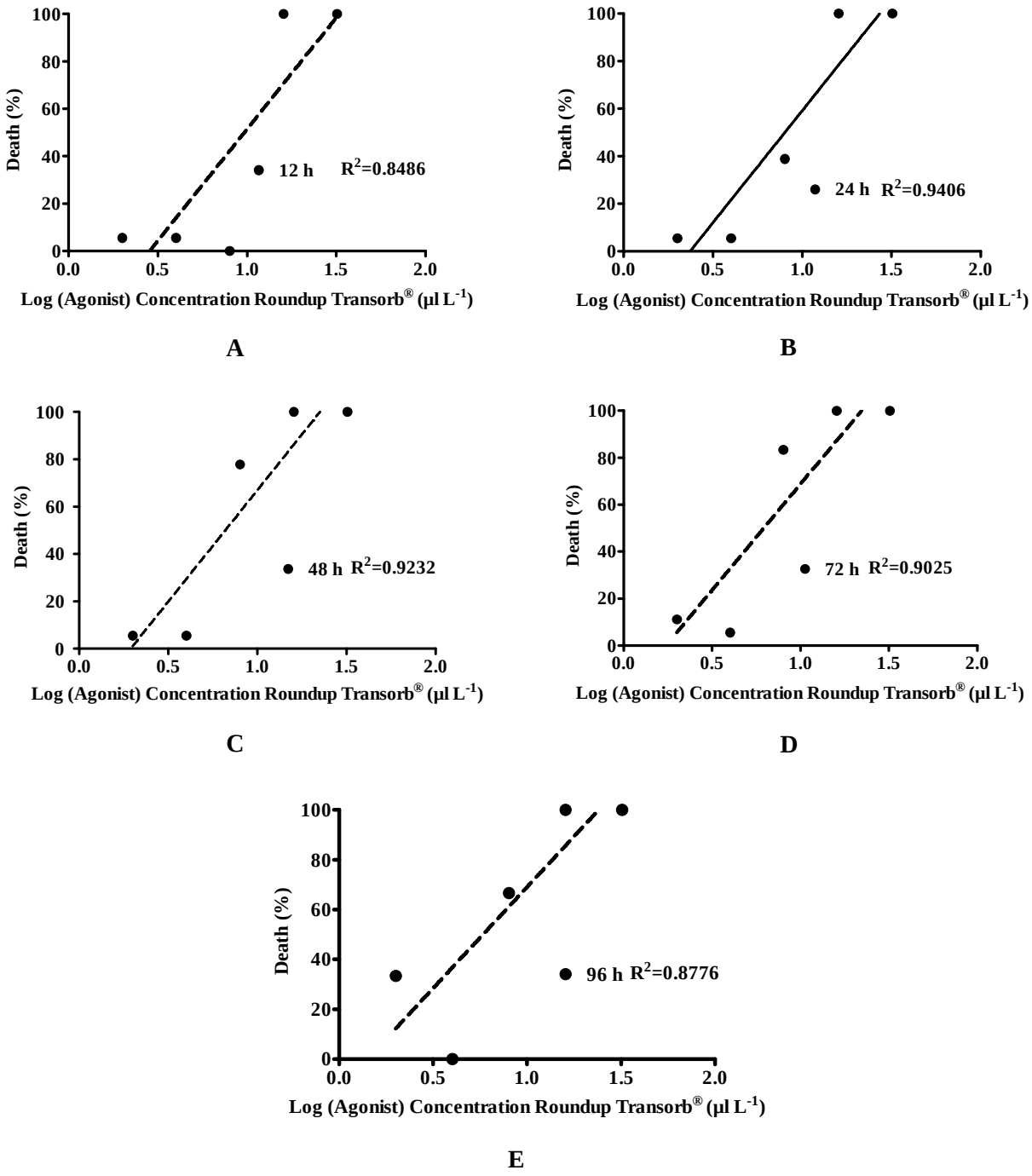

Figure 2. Mortality (%) of *P. reticulata* after exposure to five concentrations of Roundup Transorb®. Correlation between time and median lethal concentration (LC_{50}) for *P. reticulata*. **A**- 12h; **B**- 24h; **C**- 48h; **D**- 72h; **E**- 96h. Assays were carried out in triplicates.

larvae, darkening of the body surface, distribution throughout two-thirds of the aquarium, staying at the bottom of the aquarium, frequently going up to the surface, slow movements of the side and tail fins, and few movements in all the fins at the same time; these manifestations were observed at concentrations of 8 µl/L, 16 µl/L and 32 µl/L. In addition to the above behavior, the following were

also seen at concentrations of 16 µl/L and 32 µl/L: erratic swimming, swimming in circles, anesthesia, swimming in a spiral with the belly up and convulsions. For the third, fourth and fifth observations, the behavioral manifestations were the same as those described for the previous ones. In the behavioral analyses there was an increase in mortality, and at higher concentrations (16 µl/L and 32 µl/L) all the fish died after 4 h of exposure, with intensely yellow gills compared to the control group. At the other concentrations there was an increase in the number of stationary fish, not moving their fins, with wide-open opercula and gills colored a medium red. Histopathological effects and histomorphometry of the guppy liver: microscopic analyses of the liver of *P. reticulata* showed that all the exposed specimens presented a liver with alterations of varying degrees, with a deformed appearance, looking extremely fragile and, at the highest concentrations (2.8, 4.2 and 5.6 µl/L), looking more bulky and swollen. The fish exposed to all concentrations of R. Transorb® showed various pathological alterations in the liver. Of these, the most frequent were vacuolization, cytoplasmic degeneration and hyperemia, which is the substantially increased flow of blood in the liver (Figure 3-B), degeneration of the cytoplasm and nucleus (Figure 3-C), cellular hypertrophy and nuclear hypertrophy (Figure 3-E) and degeneration of the nucleus (Figure 3-F). Table 2 presents the frequency of alterations in the guppy liver. The Index of Histopathological Alterations (IHA) was determined for the guppy liver after 24 h of exposure at 1.4, 2.8, 4.2 and 5.6 µl L^{-1} of R. Transorb®, and throughout the whole exposure period to higher concentrations of this herbicide; these alterations were significantly higher than in the control (Figure 4).

An increase in the cellular and nuclear volume of hepatocytes (phase I) can be considered as a response to a stressor agent, because this indicates activation of the functions and does not compromise hepatic performance, pointing to the intensification of metabolic activity of the hepatocytes in adverse conditions. These alterations were frequently observed in fish exposed to 2.8 µl/L, 4.2 µl/L and 5.6 µl/L of R. Transorb® during the 24 h period. In contrast, the cytoplasmic and nuclear degenerations represent more serious lesions (phase II), albeit reversible, which may prevent the liver from carrying out its functions, because the area of active hepatic tissue is metabolically reduced. These alterations may correspond to direct effects caused by exposure to the herbicide, as they are found in all the exposed fish, but not in the fish of the control group (Table 3). In the control group, only vacuolizations and some less serious and less frequent pyknotic nuclei (in only two specimens) were found, probably representing part of the normal cellular activity process of the tissue. Nuclear vacuolization and pyknotic nuclei were also found in fish exposed to concentrations of 2.8, 4.2 µl/L and 5.6 µl/L of the herbicide R. Transorb® throughout the whole exposure period, discussed in Table 4. Vacuolization of the nucleus occurs in the liver of many aquatic vertebrates and invertebrates in the presence of pollutants, and this event can lead to subsequent degeneration of the nucleus, indicated by the presence of pyknotic nuclei.

According to Tukey's HSD test there was a significant difference between the control group and the treatment groups at the highest concentrations (4.2 µl/L and 5.6 µl/L), and significant differences also occurred between the treatment groups at concentrations of 1.4 µl/L and 4.2 µl/L, 4.2 µl/L and 5.6 µl/L ($p > 0.05$), 1.4 µl/L and 5.6 µl/L and 2.8 µl/L and 5.6 µl/L ($p > 0.01$). These differences indicate a concentration-effect relationship caused by the herbicide exposures, compromising the proper functioning of the liver (Figure 4 and Table 3).

Using quantitative evaluation obtained by histomorphometry, it was confirmed that animals submitted to acute treatment with R. Transorb® herbicide presented significant alterations in the hepatocytes, nuclei and sinusoids. The main alteration observed was cellular increase in the hepatocyte, together with the nucleus, in terms of area, diameter and volume, and hyperemia of the sinusoid vessels (Table 3 and 4). Hyperemia indicates an acute inflammatory process in the liver promoted by the presence of the herbicide, which under chronic exposure can evolve into necrosis, hemorrhage or even hepatic fibrosis.

From histomorphometrical analysis of the hepatic tissue the mean and standard deviation were

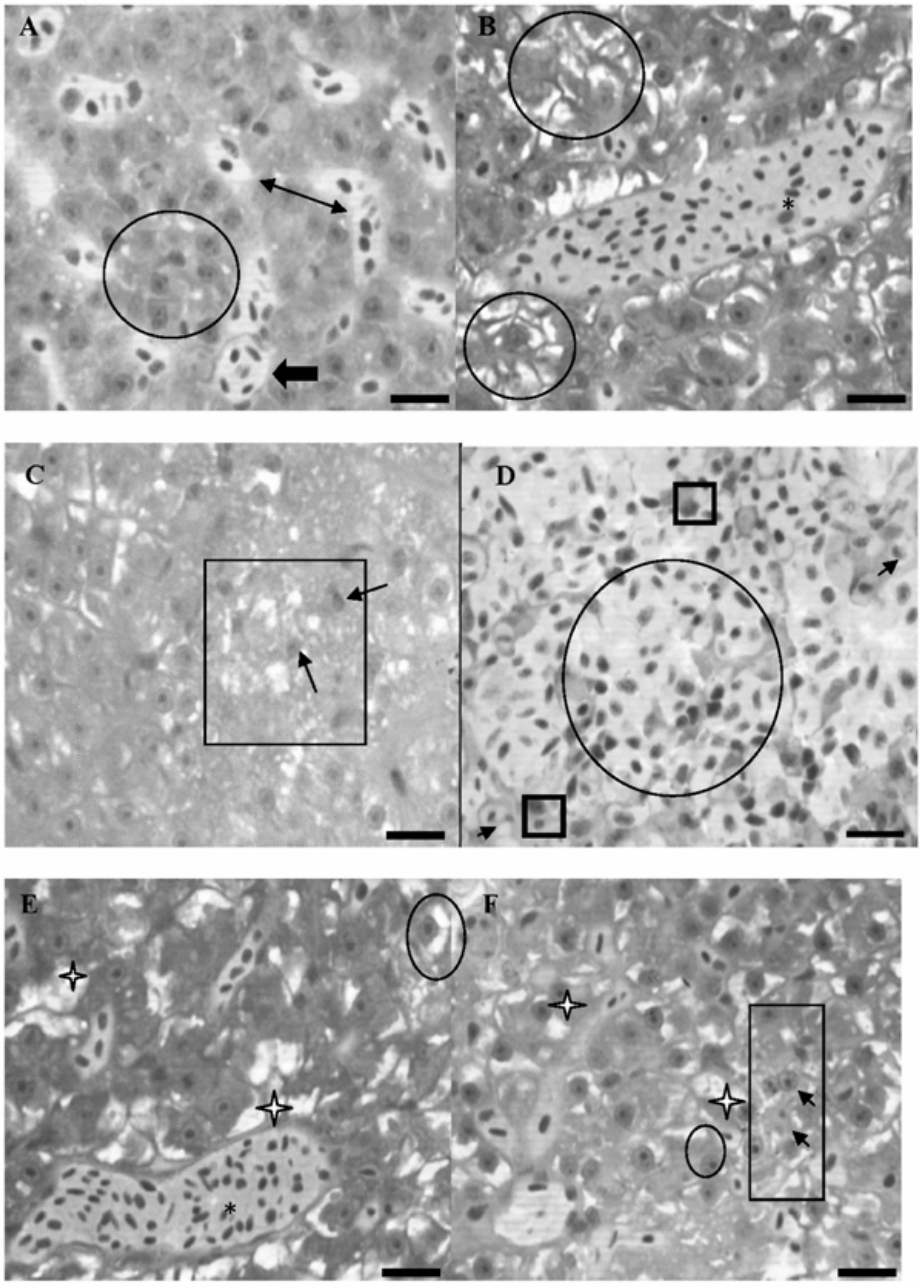

Figure 3. Photomicrograph of the hepatic tissue of *P. reticulata*. (A) Non-exposed fish, normal hepatic tissue, hepatocytes (circle), sinusoids (two-headed arrow) and interhepatic pancreas (wide arrow). (B) Fish exposed to 1.4 µl/L of herbicide, vacuolization of the cytoplasm (circles) and hyperemia (asterisk). (C) Fish exposed to 2.8 µl/L, degeneration of the cytoplasm (rectangle) and degeneration of the nucleus (arrows). (D) Fish exposed to 5.6 µl/L, pyknotic nucleus (squares), hemorrhage (circle) and vacuole in the nucleus (arrows). (E) Fish exposed to 5.6µl/L, hyperemia (asterisk), cellular hypertrophy (stars) and nuclear hypertrophy (circle). (F) Fish exposed to 4.2 µl/L, cellular hypertrophy (stars), degeneration of the cytoplasm (rectangle), degeneration of the nucleus (arrows) and nuclear hypertrophy (circle). Scale bar of 10 µm. Fish exposed to R. Transorb® showed hepatic histological alterations (Figure 3). Quantitative analysis of these hepatic alterations showed that the liver of the guppy was affected after exposure to the herbicide with mean values for IHA of 14.8 in 1.4 µl/L, 26.8 in 2.8 µl/L, 56 in 4.2 µl/L and 94.2 in 5.6 µl/L, indicating that the occurrence of these alterations significantly interferes with normal functioning of this organ, compared with the control group's IHA of 2.5 (Figure 4).

Table 2. Frequency of histological alterations in the liver of *P. reticulata* under acute exposure (24 h) to the herbicide Roundup Transorb®.

Alterations	Stage	0 µl/L	1.4 µl/L	2.8 µl/L	4.2 µl/L	5.6 µl/L
Cellular hypertrophy	I	0	++	+++	++++	++++
Nuclear hypertrophy	I	0	++	+++	+++	+++
Irregular cells	I	0	+++	+++	++++	++++
Irregular nucleus	I	0	++	++	+++	++++
Peripheral nucleus	I	0	++	+++	+++	+++
Vacuole cytoplasm	I	+	+++	++++	++++	++++
Nucleus vacuole	II	0	0	+++	++++	++++
Cytoplasm degeneration	II	0	+++	+++	++++	++++
Nucleus degeneration	II	0	+++	+++	++++	++++
Hyperemia	II	0	++	++++	++++	++++
Pyknotic nucleus	II	+	++	+++	+++	++++
Bleed	II	0	0	0	0	++

Legend: 0 = absence; + = low frequency; ++ = frequent; +++ = very frequent; ++++ = high frequency.

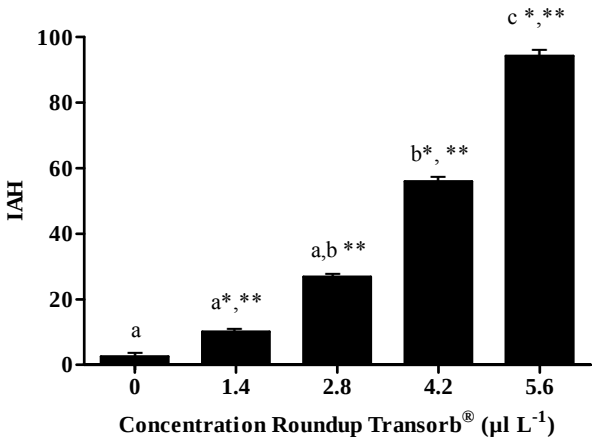

Figure 4. Mean values of the IHA for hepatic tissue of *P. reticulata* exposed to graded concentrations of Roundup Transorb® for a period of 24 h. The signs * and ** indicate significant differences from 0 µl/L, $p < 0.05$ and $p < 0.01$, respectively (Anova and Tukey's HSD test). Different letters 'a', 'b' and 'c' correspond to the statistical difference among groups. Error bars represent 95% confidence intervals.

obtained. Statistical analysis between treatment and control groups presented a significant difference ($p < 0.05$ and $p < 0.01$). The parameters used to analyze histomorphological alterations presented a difference, at all concentrations, when compared to the control (Tables 1 and 3-4). Differences were also noted between the treatment groups using Tukey's HSD test, with differences being more evident between measurements found at the highest concentrations (4.2 and 5.6 µl/L) (Table 3 and 4), indicating that the concentrations were sufficiently spaced for the increase in histological alterations to be observed at the qualitative and quantitative levels. These were promoted by the toxic action of the herbicide on the hepatocytes, and grew as concentrations increased.

Total analysis of the area, diameter and volume of the hepatocytes and their nuclei demonstrated that the treatment groups (1.4, 2.8, 4.2 and 5.6 µl/L of herbicide) presented significantly greater areas than those found in the control group (Tables 3 and 4). These results indicate that there was significant cellular hypertrophy of the studied hepatocytes under acute exposure to R. Transorb® herbicide, which promoted a concentration-effect response, confirmed by analyses of the histopathological alterations that took place in exposed groups.

DISCUSSION

It can be suggested that in short-term exposures, higher amounts of the herbicide are necessary to

Table 3. Measurements of area, diameter, radius and volume of the hepatocytes, and volume and area of the cytoplasm in treatments with Roundup Transorb®.

Exposure ($\mu l\ L^{-1}$)	HA (μm^2)	HD (μm)	HV (μm^3)	CA (μm^2)	CV (μm^3)	HR CA/NA (μm^2)
0	66.3 ± 15.4	8.8 ± 1.1	414.5 ± 139.1	58.2 ± 15.5	343.1 ± 132.9	7.1 ± 2.3
1.4	78 ± 21.0**	10.1 ± 1.3**	533.6 ± 220.0**	67.8 ± 19.7*	432.9 ± 193.9*	6.5 ± 2.8
2.8	76.4 ± 12.3*	10.0 ± 1.0**	509.6 ± 147.4*	66.7 ± 14.6*	417.6 ± 132.1	6.9 ± 1.4
4.2	89.1 ± 12.4**	10.9 ± 0.7**	637.2 ± 132.4**	77.1 ± 11.4**	513.5 ± 114.2**	6.4 ± 1.6
5.6	88.8 ± 19.0**	11 ± 1.1**	640.4 ± 203.3**	77.7 ± 18.4**	526.2 ± 187.6**	7.0 ± 1.8

HA = hepatocyte area; HD = hepatocyte diameter; HV = hepatocyte volume; CA = cytoplasm area; CV = cytoplasm volume; HR = hepatocyte ratio; NA = nuclear area.
* and ** indicate significant differences from 0 μlL^{-1}, $p < 0.05$ and $p < 0.01$, respectively (Anova and Tukey's HSD test), 4,050 hepatocytes were analysed.

Table 4. Evaluated measurements of the hepatocyte nuclei and diameter of the sinusoid in treatments with Roundup Transorb®.

Exposures $\mu l\ L^{-1}$	NA μm^2	ND μm	NV μm^3	SD μm
0	8.1 ± 2.3	3.22 ± 0.2	17.9 ± 7.0	2.8 ± 0.5
1.4	10.4 ± 2.8**	3.64 ± 0.5**	25.8 ± 11.9**	11.8 ± 2.7**
2.8	9.6 ± 1.4**	3.62 ± 0.2**	22.6 ± 5.2*	7.3 ± 3.2**
4.2	11.9 ± 1.6**	4.02 ± 0.2**	31.5 ± 6.5**	15.6 ± 4.1**
5.6	11.1 ± 1.8**	3.96 ± 0.2**	28.1 ± 7.4**	13.2 ± 3.4**

NA = nucleus area; ND = nucleus diameter; NV = nucleus volume; SD = sinusoid diameter.
* and ** indicate significant differences from 0 µl/L, $p < 0.05$ and $p < 0.01$, respectively (Anova and Tukey's HSD test). 4,050 nuclei and 810 sinusoids were analysed.

cause mortality in 50% of fish, which was confirmed by the $LC_{50;\ 12\ and\ 24h}$. It appears likely that this resistance occurred in function of the quick changes in metabolic activity in various enzymes after exposures, demonstrating a high capability of responses during this initial period (24 h) of exposure (Figure 2). Toxicity depends on the susceptibility of the organisms to the toxic agent. Different species have different susceptibility, depending on their metabolic apparatus, their feeding habits, their behavior, development phase and other aspects. Young or immature individuals are generally more susceptible to chemical agents than are adults, probably due to the differences in the development of their detoxification mechanisms [21].

In this study, *P. reticulata* showed behavioral abnormalities, being more resistant to R. Transorb® herbicide in the first 24 h of exposure. However, continuous exposures of the aquatic environment to this herbicide or chronic exposure may cause higher mortality in this species, even at low exposure-concentrations, such as those determined in the $LC_{50,\ 96h}$ (5.6 ± 0.7 µl/L). The investigation of behavioral manifestations is based on the analysis of individual symptoms, such as respiratory frequency, motor coordination and balance, which provides a series of strategies to guarantee their

survival in natural surroundings, often leading to a greater adaptive capacity to deal with unexpected situations and survive. These observed behavioral alterations are typical of the action of herbicide on the nervous system. This herbicide causes inhibition of the enzyme acetylcholinesterase (AChE), which plays important roles in central and peripheral cholinergic neurotransmission [22]. It hydrolyzes cholinesterases and helps to detoxify substances that present a toxic threat to the organism. Cholinesterases are widely distributed in both vertebrates and invertebrates [23]. This herbicide is an inhibitor of the enzyme AchE and consequently causes an accumulation of acetylcholine in the central cholinergic synapse and neuromuscular junctions, leading to overstimulation of the target cells. In consequence, these disturbances can affect the locomotion and balance of exposed organisms [24, 25]. Disturbances observed in swimming behavior were similar to those already described in other species of fish exposed to the herbicides Atrazine [24] and Diuron [24, 26]. Exposure of fish to other herbicides is also related to alterations in the activity of cholinesterases [27, 28].

Cellular and nuclear degradation were the most frequent histological alterations observed, besides an increase in nuclear volume, cytoplasmic and nuclear vacuolization, the presence of pyknotic nuclei, cellular and nuclear hypertrophy, and hyperemia (Table 4). These alterations were observed in all fish exposed to the herbicide, corroborating the data of Jiraungkoorskul *et al.* [7] and Langiano and Martinez [29], in which the occurrence of the same hepatic lesions was observed in *Oreochromis niloticus* exposed to 36 mg/L of glyphosate-based herbicide (Roundup Original®) and in *Prochilodus lineatus* exposed to 7.5 and 10 mg/L of the same herbicide [7, 29]. Szarek *et al.* [30] studied the hepatic ultrastructure of *Cyprinus carpio* exposed to Roundup® and also confirmed the occurrence of various liver areas with vacuoles in the hepatocytes, as well as degeneration of mitochondria and an increase in the Golgi complex, confirming that Roundup® can cause significant damage to the fish hepatocytes.

Our previous study showed the genotoxicity of R. Transorb to *P. reticulata* at very low exposure-levels, increasing the frequencies of micronucleus and DNA damage through comet assay in gill erythrocyte cells [31].

CONCLUSION

In conclusion, the glyphosate-based herbicide (R. Transorb®) caused behavioral changes in all exposed groups, with a clear action on the nervous system and liver. The $LC_{50;\ 96h}$ of 3.6 µg/ml determined in this study indicated that this fish species is more sensitive than those quoted in other studies. Histopathological evaluation of *P. reticulata* liver showed that R. Transorb® caused many hepatic damages at cellular level. Due to its use on genetically tolerant plants, this herbicide is the most widely applied around the world. That is the reason why more accurate studies must be done for safety evaluation of the risks to the aquatic organisms.

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CONFLICT OF INTEREST STATEMENT

Authors declare no conflict of interests.

REFERENCES

1. De Oliveira-Filho, E. C. and Lima, J. E. F. W. 2002, Potencial de impacto da agricultura sobre os recursos hídricos na região do cerrado, Embrapa Cerrados, Ministério da Agricultura, Pecuária e Abastecimento, Brasil, 49.
2. Rank, J., Jensen, A.-G., Skov, B., Pedersen, L. H. and Jense, K. 1993, Mut. Res. Gen. Tox., 300(1), 29-36.
3. De Amarante Junior, O. P. and Santos, T. C. R. 2002, Quim. Nova, 25(4), 589-593.
4. Tsui, M. T. and Chu, L. 2003, Chemosphere, 52(7), 1189-1197.
5. Williams, G. M., Kroes, R. and Munro, I. C. 2000, Reg. Tox. and Pharm., 31(2), 117-165.
6. Nešković, N. K., Poleksić, V., Elezovíc, I., Karan, V. and Budimir, M. 1996, Bull. of Envir. Cont. and Tox., 56(2), 295-302.

7. Jiraungkoorskul, W., Upatham, S. E., Kruatrachue, M., Sahaphong, S., Vichasri-Grams, S. and Pokethitiyook, P. 2002, Science Asia, 28, 121-127.
8. Glusczak, L., Miron, D. S., Crestani, M., Fonseca, M. B., Pedron, F. A., Duarte, M. F. and Vieira, V. L. P. 2006, Ecotox. and Envir. Safety, 65(2), 237-241.
9. Bernet, D., Schmidt, H., Meier, W., Burkhardt-Holm, P. and Wahli, T. 1999, J. of Fish Dise., 22(1), 25-34.
10. Au, D. W. T. 2004, Mar. Pollu. Bull., 48(9), 817-834.
11. Braunbeck, T. 1998, Ecotox. Springer, 61-140.
12. Segner, H. 1998, Comp. Biochem. and Phys. Part A: Mol. & Int. Phys., 120(1), 71-81.
13. Teh, S. J., Adams, S. M. and Hinton, D. E. 1997, Aquat. Tox., 37(1), 51-70.
14. Organization for Economic Cooperation and Development (OECD), 1998, Guidelines for Testing of Chemicals, OECD Publishing.
15. Kollman, W. and Segawa R. 1995, Interim report of the pesticide chemistry database, Environmental Hazards Assessment Program, EH95-04, State of California, Environmental Protection Agency, Department of Pesticide Regulation, Environmental Monitoring and Pest Management Branch, Sacramento.
16. Poleksic, V. and Mitrovic-Tutundzic, V. 1994, Fish gills as a monitor of sublethal and chronic effects of pollution, 339-352, R. Müller and R. Lloyd (Ed.), Sublethal and Chronic effects of pollutants on freshwater fish, Oxford, Fishing News Books.
17. Vertemati, M., Vizzotto, L., Moscheni, C., Dhillon, A. and Quaglia, A. 2008, Micro. Res. and Tech., 71(8), 606-613.
18. Golalipour, M. J., Ghafari, S. and Farsi, M. M. 2009, Int. J. Morphol., 27(4), 1339-1344.
19. Ke, W. M., Xie, S. B., Yu, L. N., Liu, T., Lai, J., He, D. Q., Li, X. H., Gao, Z. L., Ke, Y. and Chen, P. J. 2008, Intervirology, 51(4), 235-240.
20. Hamilton, M. A., Russo, R. C. and Thurston, R. V. 1977, Env. Sci. & Tech., 11(7), 714-719.
21. dos Santos, J. R. and da Silva, J. M. 2007, Oecologia Brasiliensis, 11(4), 565-573.
22. Ferrando, S. and Santos, T. C. R. 2006, J. of Zoo., 73(01), 7-14.
23. De la Torre, F., Ferrari, L. and Salibián, A. 2002, Comp. Biochem. and Phys. Part C: Tox. and Pharm., 131(3), 271-280.
24. Saglio, P., Olsén, K. H. and Bretaud, S. 2001, Arch. of Env. Cont. and Tox., 41(2), 192-200.
25. Saglio, P. and Trijasse S. 1998, Arch. of Env. Cont. and Tox., 35(3), 484-491.
26. Bretaud, S., Toutant, J.-P. and Saglio P. 2000, Ecotox. and Env. Saf., 47(2), 117-124.
27. Sancho, E., Fernandez-Vega, C., Sanchez, M., Ferrando, M. D. and Andreu-Moliner, E. 2000, Ecotox. and Env. Saf., 46(1), 57-63.
28. Dutta, H. M. and Arends, D. A. 2003, Env. Res., 91(3), 157-162.
29. Langiano, V. C. and Martinez, C. B. 2008, Comp. Biochem. and Phys. Part C: Tox. and Pharm., 147(2), 222-231.
30. Szarek, J., Siwicki, A., Andrzejewska, A., Terech-Majewska, E. and Banaszkiewicz, T. 2000, Mar. Env. Res., 50(1), 263-266.
31. De Souza Filho, J., Sousa, C. C. N., Da Silva, C. C., De Sabóia-Morais, S. M. T. and Grisolia, C. K. 2013, Bul. of Env. Cont. and Tox., 91(5), 583-587.