Potential mutagenic effects of the glyphosate herbicide on *Gymnotus inaequilabiatus* (Valenciennes, 1839)

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ABSTRACT

This study analyzed the occurrence of nuclear damage in the red blood cells of Gymnotus inaequilabiatus (Valenciennes, 1839), exposed in vivo to the herbicide glyphosate. The fish were distributed in four groups, namely: control (without addition of herbicide) and contaminated groups with application of 65 µg/L (concentration allowed by CONAMA Resolution), 1 mg/L (maximum limit recommended by the World Health Organization - WHO and Food and Agriculture Organization - FAO) and 5 mg/L (overdose, five times higher than that by WHO and FAO). For the analysis of cell damage, the method of Nuclear Erythrocytic Abnormalities (NEA) was used, containing 1000 cells for duplicate elimination. An increase in cell damage was observed for 144 hours (6-days) of exposure in all controls. Even under a small concentration, there was a clear effect on segmented, renal, lobed formation and mainly on the formation of micronuclei. The high damage was caused in the first 48 hours and reduced after 144 hours, where the fish can have the herbicidal effect blocked. Studies that analyze the mechanisms of action of glyphosate-based herbicides are essential to determine the risks caused by biota, since there is a great divergence on the maximum tolerable limits in water, which affect quality and integrity of these ecosystems.

Keywords: cell damage, fish, Roundup®.

Potenciais efeitos mutagênicos do herbicida glifosato em *Gymnotus inaequilabiatus* (Valenciennes, 1839)

RESUMO

O objetivo desse estudo foi analisar a ocorrência de danos nucleares nas hemácias de *Gymnotus inaequilabiatus*, expostos *in vivo* ao herbicida glifosato. Os peixes foram distribuídos em quatro grupos, sendo eles: controle (sem a adição do herbicida) e os grupos contaminados nas concentrações de 65 µg/L (concentração permitida pela Resolução do CONAMA), 1 mg/L (limite máximo recomendado pela Organização Mundial de Saúde – OMS e Food and Agriculture Organization – FAO) e de 5 mg/L (superdose, cinco vezes maior que a estabelecida pela OMS e FAO). Para análise de danos celulares foi utilizado o método de Anormalidades Nucleares Eritrocíticas (ANE) contando 1000 células por lâmina em duplicatas. Foi observado

um aumento nos danos celulares durante as 144 horas de exposição (6 dias) em todos os tratamentos. Mesmo sob uma pequena concentração, houve um claro efeito sobre a formação segmentada, renal, lobada e principalmente na formação de micronúcleos. Os elevados danos foram causados nas primeiras 48 horas sendo reduzidos após 144 horas, onde o peixe pode ter bloqueado o efeito do herbicida. Estudos que analisam os mecanismos de ação de herbicidas à base de glifosato, são fundamentais para a determinação dos riscos causados à biota, já que há grande divergência sobre os limites máximos toleráveis na água, podendo causar impactos na qualidade e integridade desses ecossistemas.

Palavras-chave: dano celular, peixe, Roundup®.

1. INTRODUCTION

Glyphosate is an organophosphate compound absorbed by the chlorophyll region of plants belonging to the group of "glycine", its molecule is not perceived as a potential aggressor and may inhibit the synthesis of essential amino acids present only in plants (Galli and Montezuma, 2005). For this reason, this compound can be considered as of lower risk for humans and wild fauna since it basically acts on physiological reactions existing only in plants (Galli and Montezuma, 2005; Mesnage *et al.*, 2015).

However, its commercial formulation is composed of 41% glyphosate in the form of isopropylamine salt (Modesto and Martinez, 2010) and 59% inert ingredients. The inert ingredients act as solvents, preservatives or surfactants such as polyoxymethylene (POEA), whose function is to increase the contact of the product with the leaf to aid its adhesion (Folmar *et al.*, 1979). More than the effect of pure glyphosate, aggregates are reported to be 20 to 70 times more toxic to fish and aquatic invertebrates (Modesto and Martinez, 2010).

In the aquatic environment (the main receptor for these substances), the deposition of these contaminants can have short (acute), medium (sub chronic) and long-term (chronic) consequences, which may compromise behavior, growth, development, tissue structure, reproduction or even cause mass lethality in aquatic populations (Rand and Petrocelli, 1985; Bogoni *et al.*, 2014; Mesnage *et al.*, 2015).

In fish, for example, there is an accumulation of high concentrations of this pollutant, because they can accumulate higher concentrations of these substances present in the water, since these compounds can bind to the organic matter that is ingested by them (Nimmo, 1985).

Due to their relative sensitivity to changes in the environment, fish are frequently used in studies of toxicity of pesticides, and biochemical, physiological and histological changes can be observed (Glusczak *et al.*, 2011; Harayashiki *et al.*, 2013, Armiliato *et al.*, 2014; Fiorino *et al.*, 2018). In these non-target organisms, the stress caused by the accumulation of these contaminants promotes the development of cellular abnormalities or may even cause chromosome breakdown in the DNA molecules (Al-Sabti and Metcalfe, 1995), making the pollutant a potential genotoxic mechanism (Lushchak *et al.*, 2009).

Even if glyphosate is reported to have a lifespan of up to 15 days (Galli and Montezuma, 2005), it is believed that the time of continuous exposure to this substance can increase damage to cell nuclei. This study therefore analyzed the nuclear damage in the red blood cells of *Gymnotus inaequilabiatus* (Valenciennes, 1839) exposed to the herbicide glyphosate. To conduct this research, we hypothesized that: 1) Fish exposure to the herbicide will cause damage to the nuclear cells of the fish, mainly after the first 48 hours; 2) Damage to the blood cells will appear even under low concentrations of the herbicide.

2. MATERIAL AND METHODS

2.1. Fish breeding and maintenance

For the development of this experiment, the species adults *G. inaequilabiatus* (n = 80; 16.5 cm to 26.4 cm) was obtained in stores selling fish products. This species was chosen due to its frequent use as live bait in the Pantanal and for also being easy to maintain in confined environments for *in vivo* experimentation.

The individuals were transported to the Ecotoxicology Laboratory, linked to the Limnology, Biodiversity and Ethnobiology Research Center of the Pantanal (Celbe) of the University of the State of Mato Grosso on the university campus of Cáceres.

In the laboratory, in order to reduce stress and the proliferation of fungi and bacteria, the fish underwent an acclimatization period of four hours contained in four 200-liter polyethylene reservoirs. Soon after, the individuals were bathed in a solution of potassium permanganate (1 gram/20 liters of water).

The individuals were not fed during the entire experiment so that there was no interference with the treatments. It is important to note that there was no significant decrease between the average weights (grams) of the individuals between the beginning and the end of the experiment. Individuals varied between 16.5 cm to 26.4 cm in length, and their weight varied between 17.63 and 52.46 grams.

The study was approved by the Animal Use Ethics Committee of the State University of Mato Grosso - MT, under Opinion Number: 003/2019.

2.2. Experimental design and data collection

The fish were distributed in four mesocosms of 200 liters, setting a density of 1 fish for every 4 liters of water. Each mesocosm was supplied with dechlorinated water. The parameters analyzed in the water were: temperature (ºC), dissolved oxygen (mg/L), electrical conductivity (µS/cm), and pH. These parameters were monitored daily with the aid of a multiparametric probe (Horiba U-55).

Each mesocosm represented a treatment with different concentrations of the herbicide Roundup® (Monsanto do Brazil *Ltda*). The following concentrations were used: **1)** control treatment without any concentration of the herbicide; **2)** 65 µg/L (maximum concentration allowed in freshwater, determined by CONAMA Resolution No. 357/2005); **3)** 1 mg/L (maximum concentration recommended in waters by the World Health Organization - WHO and Food and Agriculture Organization - FAO); and **4)** 5 mg/L (overdose, five times greater than that recommended by WHO and FAO) (Araujo *et al.*, 2016).

For fish analysis, 5 specimens and 33.33 liters of water were removed from each aquarium (to maintain the same density in the different treatments) in the periods of 48 h, 96 h, 144 h and 192 h, corresponding to the 8 days on display.

The fish were anesthetized with benzocaine (100 mg/L) (Albinati *et al.*, 2007), biometric measurements (total length and total weight) were measured, and blood samples were collected from the gills with heparinized syringes to avoid blood clotting.

2.3. NEA methodology (nuclear erythrocytic abnormalities) and micronucleus test

To determine nuclear anomalies, the method of Al-Sabti and Metcalfe (1995) was used by means of blood smears, stained with the Rosenfeld solution for 15 minutes, then adding the same amount of distilled water for another 10 minutes.

1000 cells per slide were counted in duplicates, with the aid of an optical microscope, analyzing the cells (Figure 1) with normal nuclei and the following anomalies: formation of micronuclei, kidney-shaped nuclei, lobed nuclei and segmented nuclei. The frequency of damage was considered as the sum of the damages, disregarding the number of normal cells.
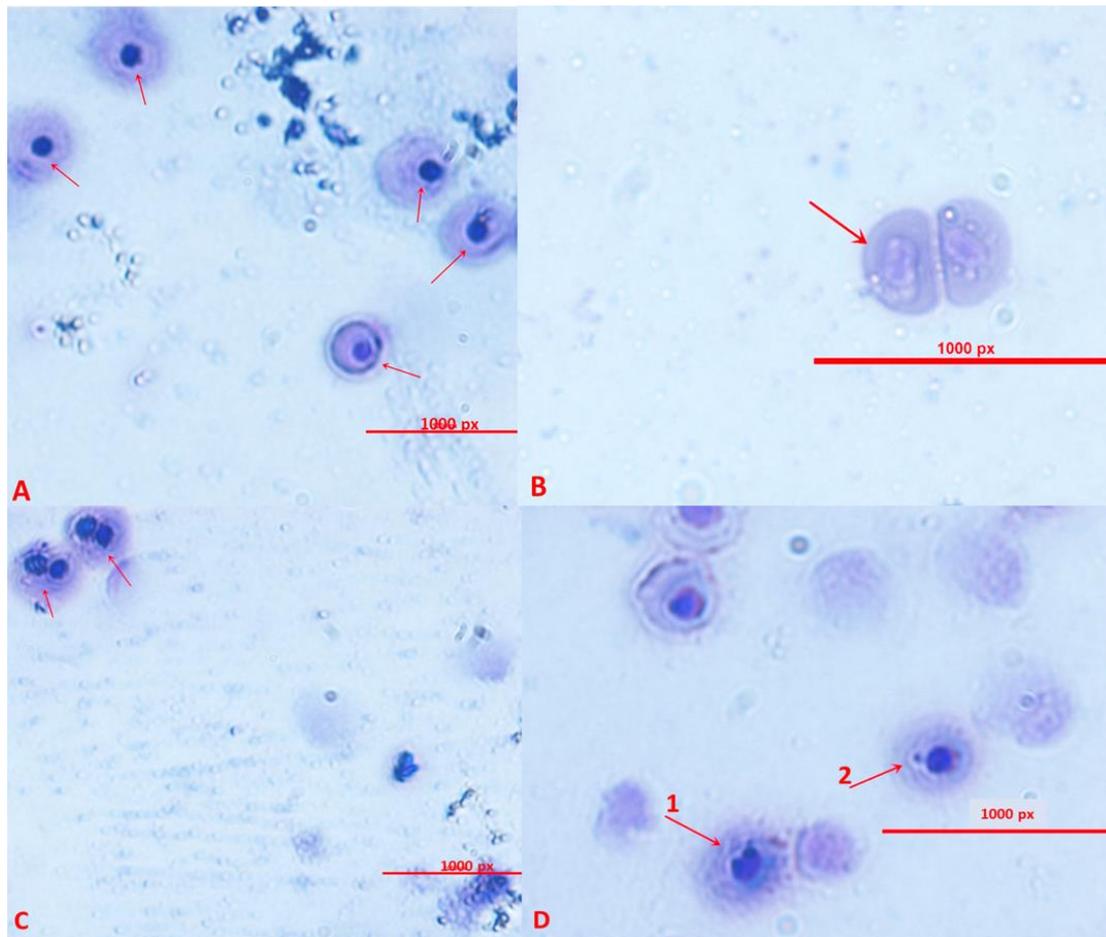
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Figure 1. Erythrocytes analyzed using the NEA method and micronucleus test (px1000). Figure A. Normal cells. Figure B. Cell showing lobed nucleus. Figure C. Cells showing segmented nuclei. Figure D1. Kidney-shaped cells. Figure D2. Cell containing micronucleus. **Source:** author.

2.4. Statistical analysis

The Kruskal-Wallis Test and Dunn's a posteriori method (1964) was used for the analysis between treatments according to the time of exposure and nuclear damage in the red blood cells (NEA).

3. RESULTS AND DISCUSSION

When comparing treatments according to the time of acute exposure with glyphosate herbicide (Figure 2), we can see that immediately after the exposure of fish to concentrations of 1 mg/L and 5 mg/L with the highest total nuclear damage were found: χ^2 (2.3) = 17.58, p = 0.000536 for 48 hours; χ^2 (2.3) = 17.58, p = 0.0005362 for 96 hours and χ^2 (2.3) = 14.31, p = 0.002512 for 144 hours; $p < 0.05$ for all comparisons. After 192 hours of exposure, only the treatment with a concentration of 5 mg/l showed higher total cell damage (χ^2 (2.3) = 12.26, p = 0.006538) for 192 hours.

According to these results, all concentrations tested in this study caused the formation of some erythrocytic nuclear anomaly compared to the control, even at the lower concentration, which is the one allowed by the Brazilian resolution (CONAMA) and suggested by WHO and FAO. The mutagenic effects caused by exposure to the herbicide glyphosate can affect the interpretation and transmission of genetic information, which can cause the observed anomalies (Srinivas *et al.*, 2019).
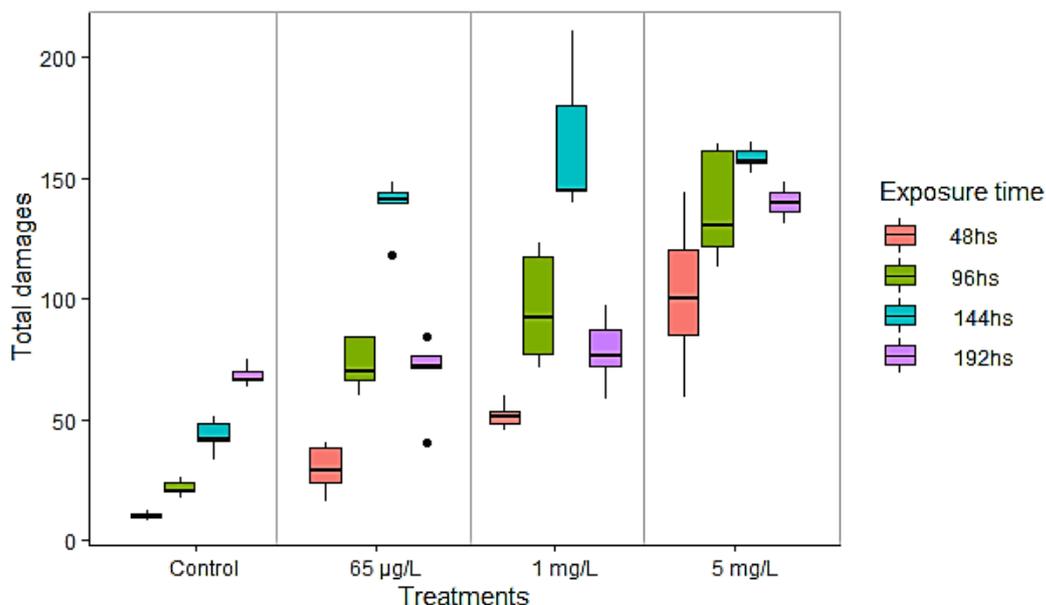
Figure 2. Boxplot showing the total cell damage within the different treatments (control and mesocosms contaminated with the glyphosate herbicide) and the time of exposure. Lines within boxes denote median fluxes. Boxes represent the 25th and 75th percentiles. Whiskers represent variability outside the upper and lower quartiles. Dots represent values outside of the range of the whiskers, but not outliers.

The oxidative stress caused by glyphosate through the accumulation of acetylcholine in the synaptic clefts generates free radicals and changes in the antioxidant system. Among the consequences caused by the primary deleterious effects of oxidative stress is the damage to the ADN molecules in which these changes occur, the potential genotoxicity mechanism (Wang *et al.*, 1998; Watt *et al.*, 2007; Lushchak *et al.*, 2009).

The occurrence of micronuclei showed that there was no significant difference in damage between the periods of exposure (192 hours) (Kruskal-Wallis test; χ^2 (2.3) = 1.49, p = 0.68 (Figure 3)).
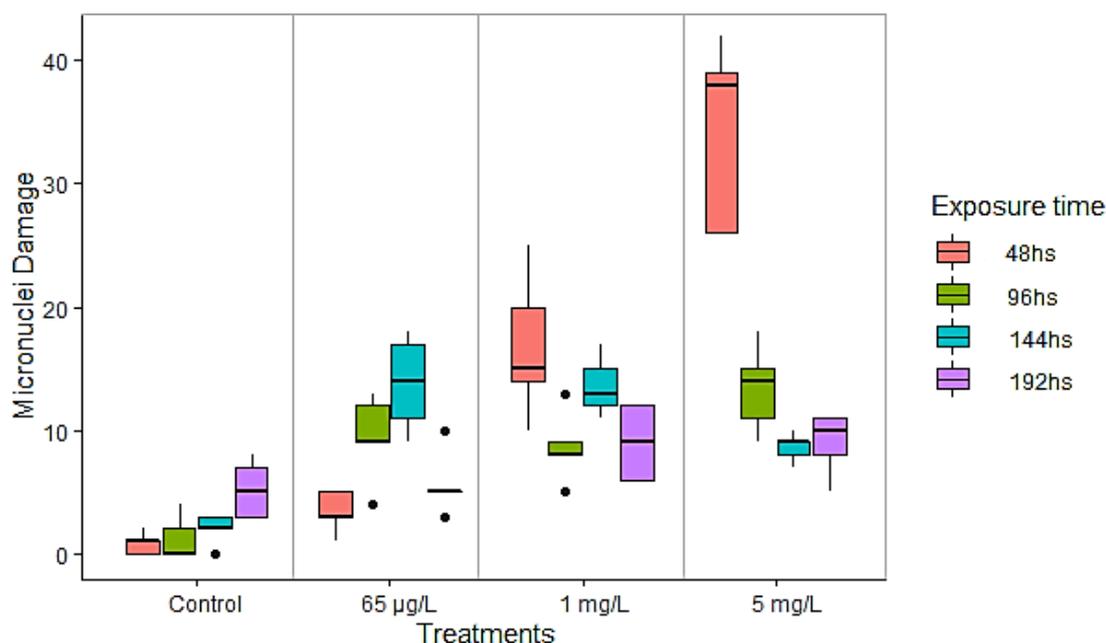

Figure 3. Analysis between the different treatments (control and mesocosms contaminated with the glyphosate herbicide) and cell damage with micronuclei formation.

However, there was a significant increase between contaminated treatments compared to the control in the formation of nuclei with lobed damage and kidney shape (χ^2 (2.3) = 28.07, p < 0.001 and χ^2 (2.3) = 30.10, p < 0.001, respectively) (Figures 4 and 5, respectively); and, in the occurrence of segmented nuclear damage (χ^2 (2.3) = 10.62, p = 0.01) (Figure 6).
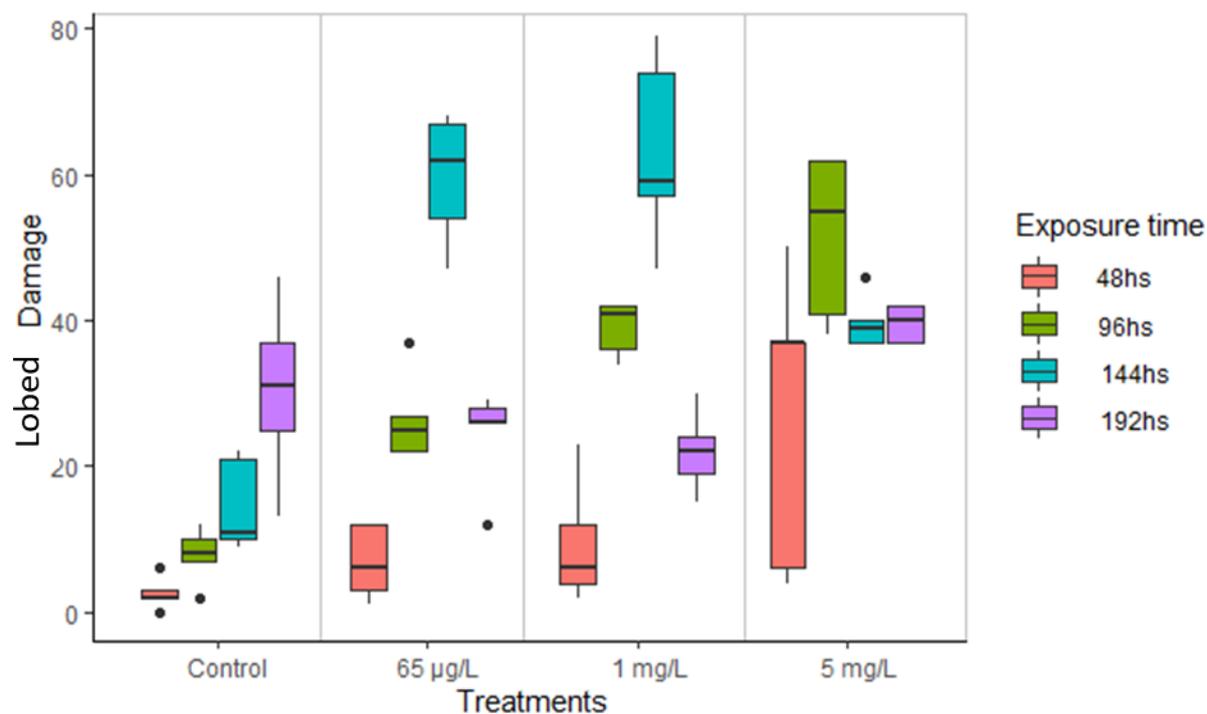

Figure 4. Analysis between the different treatments (control and mesocosms contaminated with the glyphosate herbicide) and cell damage with lobed nuclei formation.
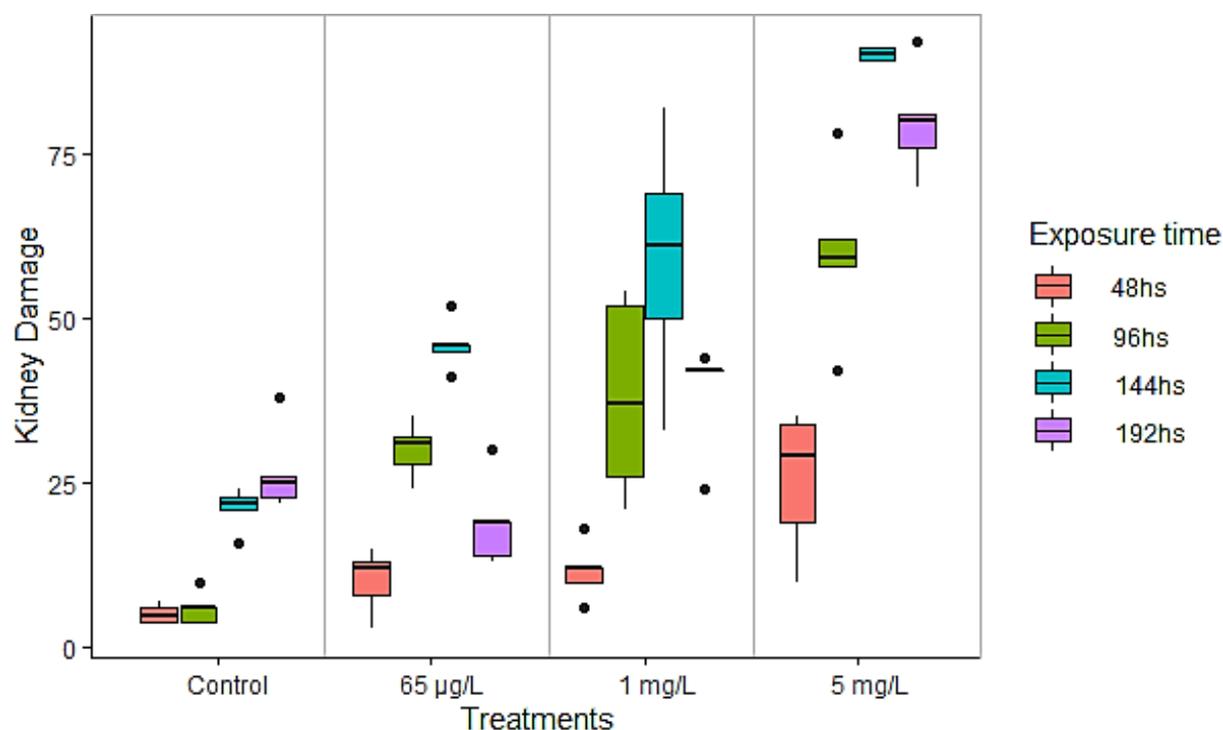

Figure 5. Analysis between the different treatments (control and mesocosms contaminated with the glyphosate herbicide) and cell damage with kidney-shaped nuclei formation.
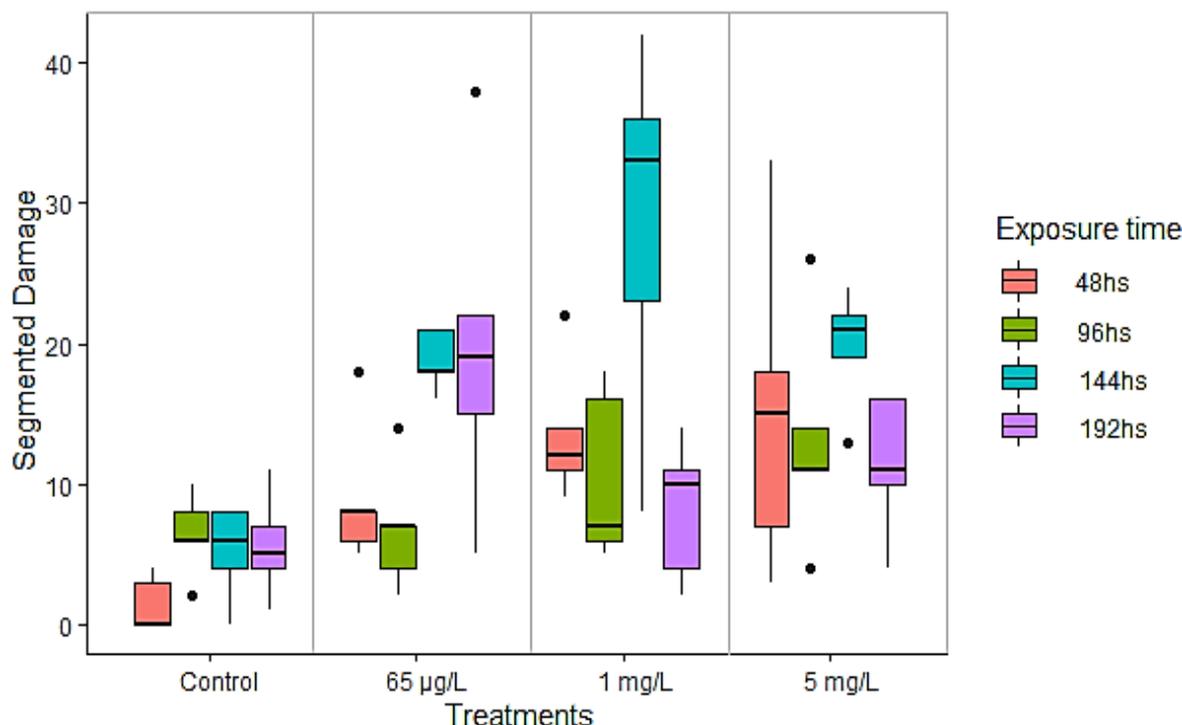
Figure 6. Analysis between the different treatments (control and mesocosms contaminated with the glyphosate herbicide) and cell damage with segmented nuclei formation.

The formation of micronuclei is the result of chromosome fragmentation, which during the cell division process (anaphase), does not migrate to the cell poles, and can also be formed by apoptosis or inactivation of the spindle formation due to chemical or physical agents (Al-Sabti and Metcalfe, 1995).

The ability of several glyphosate-based products to induce increased micronucleus formation has been found in other studies (Bolognesi *et al.*, 1997; Clements *et al.*, 1997; Williams *et al.*, 2000; Cavas, 2011; Cavalcante *et al.*, 2008; Mañas *et al.*, 2009; Poletta *et al.*, 2011). In the present study, there was no significant increase in micronucleus formation in the observed treatments. According to Bonifacio and Hued (2019), the non-significant increase in micronuclei can be explained by the fact that concentrations and exposure periods differ.

Formations of cells with lobed nuclei (Figure 4), on the other hand, may be associated with an attempt to increase the oxygen transport capacity in waters contaminated with chemical substances, when a direct cell division called "amitosis" occurs, also generating bilobed or even anucleate nuclei (Barni *et al.*, 1995). In our study, for all the treatments including the control, after 48 and 96 hours there was an increase in lobed cells. After 144 hours the damages increased only for the 65 µg/L and 1 mg/L, but not in the 5 mg/L. A similar pattern was observed for the kidney-shaped nuclei formation (Figure 5).

The morphological changes of the nuclei can be induced through the influence of cytotoxic compounds on the integrity of the nuclear lamina responsible for the stability and the regular oval shape of the nucleus (Alberts *et al.*, 2002). However, another possible explanation would be the possibility due to the body's attempt to eliminate genetic damage in situations of stress or exposure to chemical contaminants, in which when detecting the genetic damage, the process of recovery or elimination of excess chromatin begins to be moved to outside the nucleus and eliminated by exocytosis presenting imperfections before being completed (Carrasco *et al.*, 1990; Fenech *et al.*, 2011).

This process of eliminating damage by exocytosis may be a possible explanation for the reduction of damage formation between the exposure periods of 144 and 192 hours only for the concentration of overdose, with a temporary blockage by the organism to the poisoning.

We also accept that in the control treatments there were similar patterns of damages, where the total damages showed a linear increase, for instance (Figure 2). However, the contaminated treatments presented 768,11% times more damage than the control.

Greater survival of specimens at higher doses was observed, corroborating other research with pesticides (Storrs and Kiesecker, 2004; McMahon *et al.*, 2013). The occurrence of these results in a non-linear way between concentrations and mortality can be related to chemicals with endocrine-disrupting action. Even if these animals survive in high concentrations of contaminants, this period of exposure can cause other negative consequences, some irreversible (Relyea, 2005; Beausoleil *et al.*, 2013; Lagarde *et al.*, 2015).

According to Turek et al. (2018) more and more studies show that concentrations even below the limits allowed by legislation are harmful to organisms as prolonged exposure, and in this context studies like the present reinforce the need to analyze non-lethal concentrations of pesticides, whose silent effects can affect organisms on several levels.

However, the values determined as maximum limits allowed in freshwater established by Brazilian Government Resolution (CONAMA resolution n.357/2005) were defined for the active principle, disregarding the complex formulation sold commercially with surfactants, solvents and preservatives which, when associated with glyphosate, makes it more toxic (Kier and Kirkland, 2013; De Castilhos Ghisi *et al.*, 2016).

In fact, scientific studies demonstrate that glyphosate acts causing hepatic, renal and reproductive toxicity, neurotoxicity, teratogenicity, tumorigenicity, carcinogenicity and endocrine disruption (Antoniou *et al.*, 2012; Cattani *et al.*, 2014; Greim *et al.*, 2015); Mesnage *et al.*, 2015; Kegley *et al.*, 2016; Mesnage *et al.*, 2017), which caused the World Health Organization's in 2017 to alert the world about its use and effect (Cook, 2019).

4. CONCLUSION

It has been observed that the formation of cell damage increases with the period of exposure to glyphosate. We have hereby shown that the initial exposure of glyphosate increased blood-cell damage in 768, or 11%, of the exposed fish. This once more shows the effects of this herbicide used worldwide. This accretion of blood cells damage is seen for 144 hours (6-days), during which the organisms may have adapted to (blocked) the contaminant. Considering the Brazilian Government Resolution and the world organizations, we argue that even the lower concentration of the herbicide in the water causes an effect on the blood cells of fish and must be recognized as a toxic substance for the water.

Studies recognizing the mechanism of action of the herbicide, mainly for the characterization of its acute, sub chronic and chronic effects, are essential for the risk assessment of the use of these contaminants. There is a considerable divergence regarding the consensus for the maximum acceptable limits of glyphosate in the water, but even the lower concentration changes the quality and integrity of ecosystems at risk.

5. ACKNOWLEDGEMENTS

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