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ENVIRONMENTALLY TOLERATED CONCENTRATION OF GLYPHOSATE INDUCES BEHAVIORAL ALTERATIONS IN DANIO RERIO

CONCENTRAÇÃO AMBIENTALMENTE TOLERADA DE GLIFOSATO PRODUZ ALTERAÇÕES COMPORTAMENTAIS EM DANIO RERIO

LA CONCENTRACIÓN AMBIENTALMENTE TOLERADA DE GLIFOSATO PRODUCE ALTERACIONES CONDUCTUALES EN DANIO RERIO

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ABSTRACT

Over the past decades, Brazil has been increasing the use of various classes of pesticides in different types of monoculture crops, resulting in considerable contamination of water resources. This contamination represents a growing threat to public health and the entire aquatic biota. Given the public health issues and the damage to aquatic life caused by the excessive use of pesticides, the present study aimed to evaluate the behavioral alterations of the fish Danio rerio exposed to a concentration of 65 µg/L of the herbicide glyphosate, corresponding to the maximum concentration allowed in drinking water (Class 1 waters), as established by CONAMA Resolution of 2005. Our results showed that D.rerio specimens exposed to glyphosate needed to be closer to their prey before moving toward it and tended to remain longer in the light compartment, escaping more slowly from a simulated predator attack. These alterations indicate signs of increased vulnerability to predation, compromising

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the exploratory behavior of D. rerio. Our findings support the hypothesis that lethality tests commonly used in bioassays may be insufficient to assess the real impact of aquatic pollutants. Therefore, glyphosate concentrations considered acceptable under current legislation may still pose ecological and public health risks, reinforcing the need to reassess the established limits.

Keywords: Ecotoxicology. Herbicide. Zebra Fish. Brazil. Publichealth.

RESUMO
Nas últimas décadas, o Brasil, em décadas vem aumentando o uso de diversas classes de agrotóxicos em plantios de monoculturas, culminando em uma considerável contaminação dos recursos hídricos. Essa contaminação representa uma ameaça crescente à saúde pública e à biota aquática. Diante disso, o presente estudo teve como objetivo avaliar as alterações comportamentais do peixe D. rerio, exposto à concentração de 65 µg/L do herbicida glifosato concentração máxima permitida em águas de classe 1, Resolução CONAMA de 2005. Os resultados demonstraram que D. rerio expostos ao glifosato, necessitaram estar mais próximos à presa para moverem-se em sua direção, e apresentaram uma tendência a permanecerem por mais tempo no compartimento claro, fugindo mais lentamente de um ataque predador simulado. Essas alterações indicam sinais de vulnerabilidade à predação, comprometendo o comportamento exploratório do D. rerio. Estes resultados reforçam a hipótese de que ensaios baseados exclusivamente em testes de letalidade podem ser insuficientes para avaliar o impacto real de poluentes aquáticos. Portanto, concentrações de glifosato toleradas pela legislação vigente, podem representar risco ecológico e de saúde pública, reforçando a necessidade de reavaliação dos limites estabelecidos.

Palavras-chave: Ecotoxicologia. Herbicida. Zebra-Fish. Brasil. Saúde Pública.

RESUMEN
En las últimas décadas, Brasil ha incrementado el uso de diversas clases de pesticidas en distintos tipos de cultivos de monocultivo, lo que ha resultado en una considerable contaminación de los recursos hídricos. Esta contaminación representa una amenaza creciente para la salud pública y toda la biota acuática. Ante los problemas de salud pública y los daños a la biota acuática causados por el uso excesivo de pesticidas, el presente estudio tuvo como objetivo evaluar las alteraciones conductuales del pez Danio rerio expuesto a una concentración de 65 µg/L del herbicida glifosato, correspondiente a la concentración máxima permitida en agua potable (aguas de clase 1), según lo establecido por la Resolución CONAMA de 2005. Nuestros resultados mostraron que los ejemplares de D. rerio expuestos al glifosato necesitaban acercarse más a su presa antes de moverse hacia ella y tendían a permanecer más tiempo en el compartimento iluminado, escapando más lentamente de un ataque simulado de depredador. Estas alteraciones indican signos de mayor vulnerabilidad a la depredación, comprometiendo el comportamiento exploratorio de D. rerio. Nuestros hallazgos refuerzan la hipótesis de que las pruebas de letalidad comúnmente utilizadas en bioensayos pueden ser insuficientes para evaluar el impacto real de los contaminantes acuáticos. Por lo tanto, las concentraciones de glifosato consideradas aceptables según la legislación vigente aún pueden representar riesgos ecológicos y para la salud pública, lo que subraya la necesidad de reevaluar los límites establecidos.

Palabras clave: Ecotoxicología. Herbicida. Pez Cebra. Brasil. Salud Pública.

1 INTRODUCTION

Global pesticide consumption has increased sharply, placing Brazil among the leading markets worldwide. According to data from Confex Stat, the official system of the Brazilian Ministry of Development, Industry, Commerce, and Services (MDIC), between January and September 2025, the country imported approximately USD 4.1 billion worth of insecticides, herbicides, fungicides, and their derivatives, totaling around 750.1 thousand tons (Secex/MDIC, 2025). According to the Food and Agriculture Organization of the United Nations (FAO, 2022), Brazil leads global imports in this sector, spending over seven billion dollars in 2022 alone. This economic magnitude is reflected in the massive application of more than 300 thousand tons of active ingredients nationwide (EMBRAPA, 2025), corresponding to a per capita consumption of approximately 7.3 liters per inhabitant per year (CREA-AL, 2019). This intensity of use is supported by a more permissive regulatory framework compared to other major agricultural powers, such as the European Union, allowing the application of doses hundreds or even thousands of times higher, as well as the registration of substances already banned in other regions (Rodrigues *et al*, 2024). Between 2010 and 2018, the amount of pesticides sold in Brazil more than doubled relative to the growth of cultivated land in the country.

Pesticide consumption is directly associated with the increasing levels of aquatic contamination in Brazil, which, according to reports from the Brazilian Institute of the Environment (Environmental Bulletin 2021–2022), reached the highest level since 2008. In 2022, the country recorded a historic high of 562 pesticides registered for use and commercialization (Brazil, Ministry of Agriculture and Livestock, 2022). Although water resources are essential for the development of human activities, knowledge about their contamination remains limited. This gap is particularly concerning given the health impacts of pesticide exposure described by Rodrigues *et al*. (2024), especially for children and agricultural workers. Allergic diseases, neoplasms, endocrine and neurological disorders, as well as metabolic and cardiovascular alterations, have been directly linked to pesticide exposure. Poisonings are frequent in rural settings, where the excessive use of these products poses serious health risks to workers and their families, whether through multiple or single exposures during handling and equipment cleaning (Elias; Siegloch; Agostinetto, 2022). Contamination can manifest in various ways, ranging from mild symptoms such as nausea, vomiting, and mucosal irritation to more severe cases, which may even result in death (Oliveira; Zambrone, 2006).

The intensive application of these compounds results in their environmental persistence and dispersion into aquatic ecosystems through processes such as leaching and surface runoff, especially following rainfall events (Veiga *et al*, 2006). The impacts of this contamination are extensive. In the context of public health, a recent study conducted by the National Initiative for the Conservation of the Brazilian Tapir (INCAB) detected residues of pesticides and heavy metals, including glyphosate, in 38% of residents evaluated in Mato Grosso do Sul, suggesting that exposure extends beyond rural areas and also affects urban populations (Meneses; Piva, 2025). The increasing use of these products has led to changes in terrestrial and aquatic environments, promoting the accumulation of harmful substances at concentrations capable of causing ecotoxicological effects (Soares-da-Silva *et al*, 2025). After these compounds are absorbed by aquatic biota, the phenomenon of biomagnification occurs, characterized by the progressive increase in contaminant concentration along trophic levels (Begon; Townsend; Harper, 2007). Aquatic organisms, such as fish and crustaceans, often exhibit sublethal morphological, physiological, and behavioral alterations, which serve as early biomarkers of contamination (Nascimento; Naval, 2019). However, the full extent of these impacts remains partially unknown, requiring ongoing investigation (Oliveira; Carneiro; Wastowski, 2022). According to Américo *et al*. (2015), the effects on aquatic biota include abnormal behaviors such as suffocation and movement impairments, which hinder predator avoidance and food acquisition, leading to population changes and shifts in aquatic community structure and diversity. Furthermore, antipredatory behavioral changes in fish such as altered escape responses and reduced avoidance of detection (light/dark preference) may occur following contaminant exposure. Exposed individuals tend to escape more slowly from predators and spend more time in illuminated areas, which deviates from the species' natural behavior and highlights the impact of contaminants on ecological fitness (Sandoval-Herrera *et al*, 2019).

Such behavioral alterations allow for the assessment of aquatic environment quality and help predict the potential ecotoxicological effects of pesticides on the ecosystem as a whole (Ribeiro; Américo-Pinheiro, 2018). In light of the above, this study aimed to evaluate the behavioral alterations observed in the fish species *Danio rerio* exposed to a concentration of 65 µg/L of the herbicide glyphosate, corresponding to the maximum concentration allowed in potable water (Class 1 waters), as established by CONAMA Resolution No. 375 of 2005.

2 METHODOLOGY

This study was approved by the Animal Research Ethics Committee (CEUA) – UNESPAR, under registration number 007/2023. The fish species *Danio rerio* were exposed to 65 µg/L of glyphosate in the original Glyphosate Roundup® VR DI formulation, composed of di-ammonium salt of N-(phosphonomethyl) glycine (glyphosate) at 445 g/L (44.5% w/v) and acid equivalent of N-(phosphonomethyl) glycine (glyphosate) at 370 g/L (37.0% w/v; São Paulo, Brazil) for 96 hours.

The study involved two distinct exposure experiments, both following the same initial parameters. The first experiment focused on behavioral analysis related to predation, while the second assessed predator avoidance and detection evasion behaviors, using a light/dark box model.

For each experiment, 15 adult *D. rerio* fish were used, with an average weight of 0.180 ± 0.08 g and an average length of 3.107 ± 0.26 cm. The specimens were obtained from a commercial aquarium shop in the city of União da Vitória, Paraná, Brazil. The batch was transported to the Multidisciplinary Research Laboratory of the Biological Sciences Program at the State University of Paraná (UNESPAR), União da Vitória Campus, for the bioassays. The fish were acclimated for a period of 48 hours upon arrival at the laboratory. The organisms were randomly distributed into two aquaria (n = 7) containing 20 liters of mineral water, previously adjusted for pH and maintained under constant aeration. After acclimation, one of the aquaria was contaminated with 65 μg/L of glyphosate (Roundup®) at its commercial concentration, constituting the experimental group (EG). The concentration used corresponded to the maximum value allowed for Class 1 freshwater, as established by CONAMA Resolution No. 357 of 2005 (Brazil, 2005). The exposure period to the herbicide lasted 48 hours, characterizing an acute exposure.

The fish were fasted for 24 hours prior to the predation assays. After 48 hours of exposure, a test was conducted to analyze reaction distance. The experiment consisted of two treatments: a) Individual placement of *D. rerio* in a non-contaminated test aquarium with three *Chironomidae* larvae; b) Individual placement of *D. rerio* in a contaminated test aquarium with three *Chironomidae* larvae (Figure 3:1).

2.1 BEHAVIOR ANALYZES

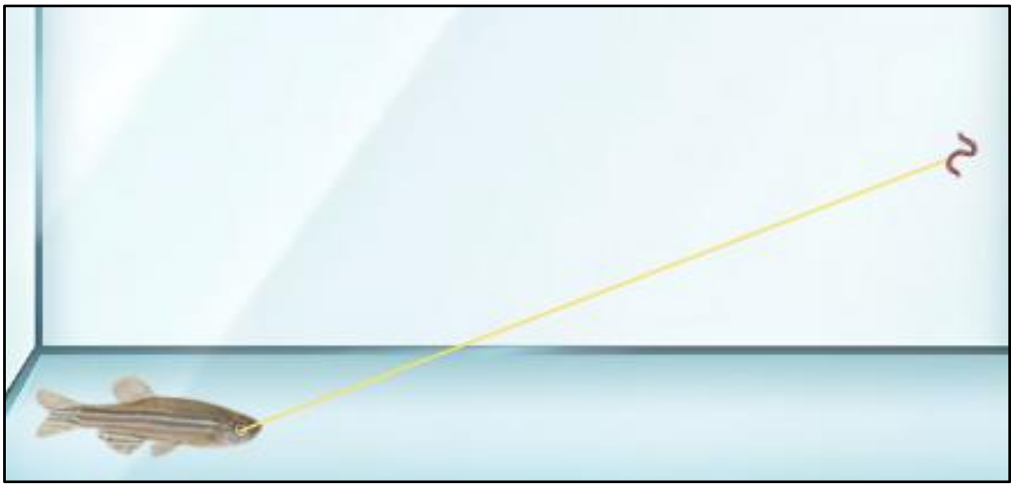
For the observation of predation and reaction distance, a test aquarium measuring 30 × 15 × 16 cm (length, height, and width) was used, containing non-contaminated mineral water previously adjusted for pH.

A recording setup was assembled with a white background, including a camera (Canon PowerShot SX530 HS) positioned parallel to the aquarium to record the fish's behavior and hunting activity. A millimeter paper was fixed to the side of the aquarium to allow subsequent image calibration.

One fish at a time was introduced into the test aquarium and allowed to acclimate for three minutes. Subsequently, three *Chironomidae* larvae were introduced on the right side of the aquarium, and the fish's behavior was recorded for five minutes after removing the net, in order to observe predation and reaction distance. After this period, the fish and any remaining *Chironomidae* larvae were removed from the aquarium. The reaction distance was determined as the maximum distance between the fish's eye and the prey at the moment immediately preceding the first attack (Grzesiuk *et al*, 2023), using the software **ImageJ 1.37 for Windows.**

Figure 1

Manual measurement of the distance from the fishs eye to the larva using the ImageJ software



Source: the authors.

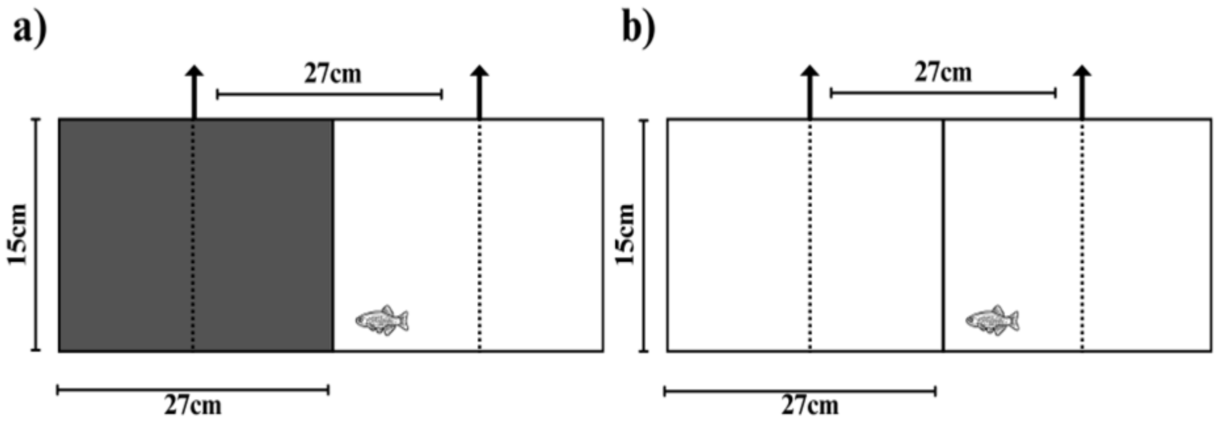
2.2 DETECTION AVOIDANCE BEHAVIOR: LIGHT/DARK BOX MODEL

For the detection avoidance behavior test, a 20 L tank was used, equally divided into white and black compartments (light/dark configuration). The fish were introduced into an intersection area closed by sliding doors. After five minutes of habituation, the doors were removed, allowing free exploration of the tank (Fig. 1). The behavior of each individual was recorded for ten minutes.

A completely white tank was used as a negative control (light–light configuration). The average time spent in each compartment was considered a measure of light preference (Sandoval-Herrera et al., 2019).

Figure 2

Configuration of the light/dark box test used to evaluate detection avoidance behavior in D. rerio



Source: the authors.

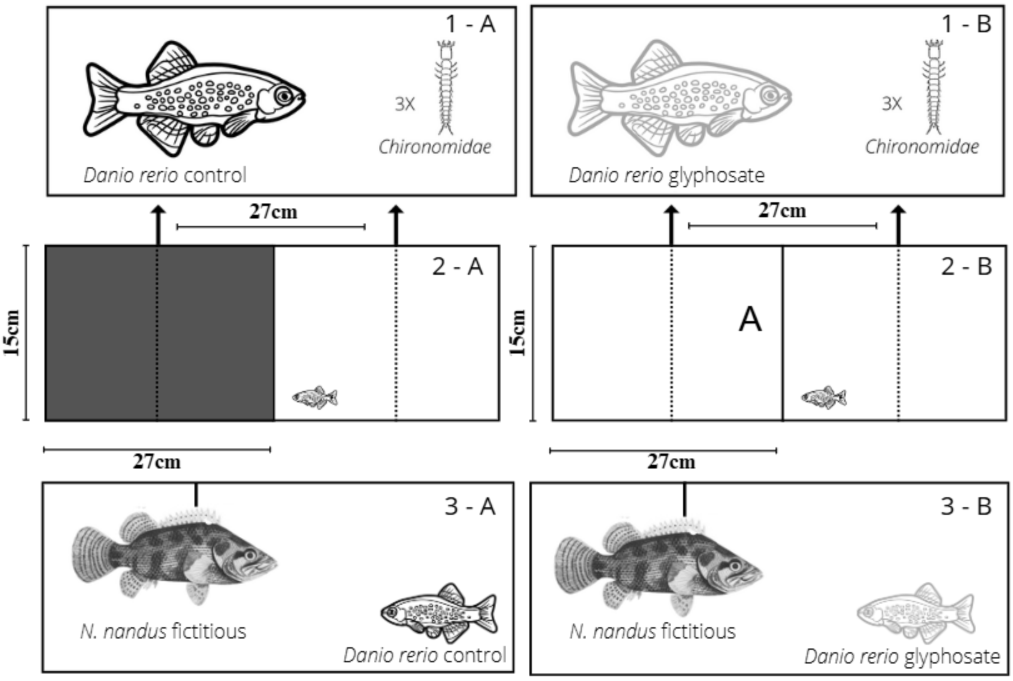
2.3 ESCAPE RESPONSE ANALYSIS TO A SIMULATED PREDATOR

For the predator escape response test, a 3D model representing the Indian Leaf Fish (*Nandus nandus*), a sympatric predator of *D. rerio*, was used (Bass and Gerlai, 2008). After the detection avoidance test, the fish were transferred to another experimental aquarium containing non-contaminated water.

In each trial, one fish was placed on the left side of the aquarium, and a simulated predator attack was generated by a vibration produced through the movement of the artificial predator (Fig. 3). The reaction time to the threat was recorded and analyzed using **Movavi Video Converter** software, with the footage played in slow motion (10% of the original speed).

Figure 3

Visual diagram of the treatments and arrangement of organisms in the aquarium for the reaction distance test. 2–Configuration of the light–dark box test used to evaluate detection avoidance behavior in D. rerio.*3–Visual diagram of the treatments and arrangement of organisms in the aquarium for the analysis of antipredator behavior*



Source: the authors.

2.4 STATISTICAL ANALYSES

First, Shapiro-Wilk test and Levene's test were carried out to obtain normality and variance homogeneity. Statistical analyses were performed separately, comparing first the biochemical biomarkers and then the morphological abnormalities of erythrocytes. Significant difference level relative to control with the test group (exposed to glyphosate) was analyzed with T-test (normal data distribution) and Wilcoxon test (Wilcoxon 1945) (non-normal data distribution), assuming the significance level of $p< 0.05$. All analyses were performed in the R environment using the Vegan package (version 4.4-1, R Core Team 2022).

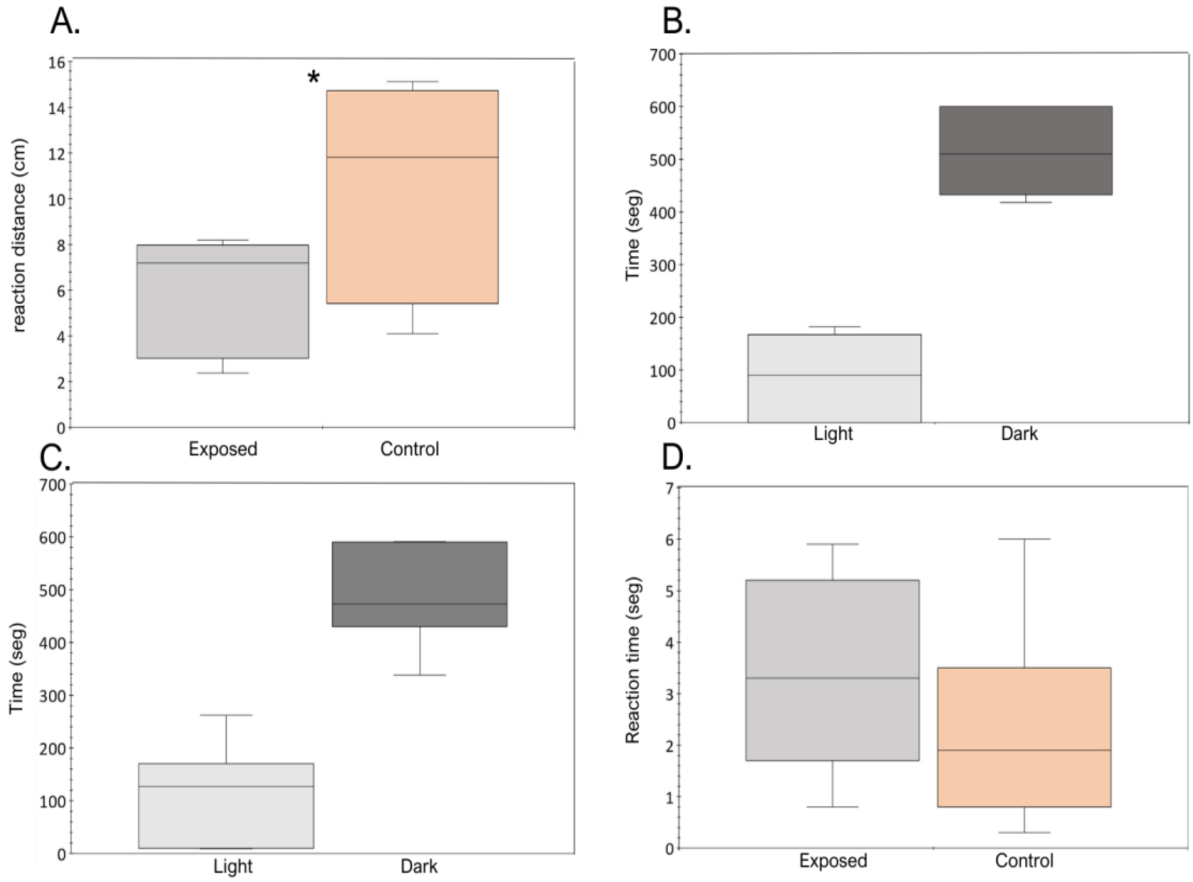
3 RESULTS

3.1 BEHAVIOR ANALYSIS

Fish exposed to glyphosate exhibited a significantly shorter reaction distance compared to the control group ($F = 5.207$; $df = 9.757$; $p = 0.05$) (Fig. 4A). Regarding the detection avoidance test, both groups showed a significant preference for the dark compartment ($F = 58.11$ for the control group and $F = 120.8$ for the glyphosate group; $df = 12$; $p < 0.05$) (Fig. 4B–C). Exposure to glyphosate did not significantly alter the escape response time to a simulated attack (F = 0.8207; df = 11.99; p > 0.05), although a trend toward a slower biological response was observed in the exposed group compared to the control group (Fig. 4D).

Figure 4

Behavioral biomarker responses of D. rerio *exposed to an environmental concentration of glyphosate.(A) Reaction distance of exposed and control individuals in relation to* Chironomidae *larvae. (B) Time spent by control group individuals in the dark and light compartments during the detection avoidance behavior test. (C) Time spent by exposed individuals in the dark and light compartments during the detection avoidance behavior test. (D) Reaction time of exposed and control group individuals in response to a simulated attack by a fictitious predator. Error bars indicate the standard deviation of the mean*



Source: the authors.

4 DISCUSSION

The results of this study demonstrated several behavioral alterations in *D. rerio* when exposed to glyphosate. This response pattern is not exclusive to exposure to this substance, as behavioral changes have been widely observed in several fish species exposed to different classes of potentially cytotoxic compounds.

The reduction in motor activity in *D. rerio* found in our study corroborates the findings of Pandey *et al*. (2024), who observed similar effects in *Clarias batrachus* exposed to zinc sulfate, and of Ridha and Mohsin (2024) with *Cyprinus carpio* treated with diazinon. Despite the differences between compounds and species, these similarities suggest the existence of toxicity mechanisms that affect common neuromotor pathways in fish. The alterations in exploratory behavior in *D. rerio* observed in our study were also described by Toussaint *et al*. (2025) however, they observed an anxiolytic effect in individuals exposed to the biopesticide *Bacillus thuringiensis israelensis* (Bti-B2). Similarly, Melefa and Nwani (2021) reported comparable results when exposing African catfish (*Clarias gariepinus*) to the drug clotrimazole, reinforcing the idea that the behavioral sensitivity of fish to chemical stressors constitutes a common ecotoxicological response. Although our study did not evaluate reproductive behavioral effects, such alterations have been reported by Scott and Sloman (2004) as changes in spawning site selection and other reproductive behaviors that may compromise the reproductive process. Wierzbicki *et al*. (2025) also describe, in their review, a wide range of behavioral alterations in various fish species, corroborating the findings reported here.

Magalhães and Ferrão Filho (2008) reported that behavioral alterations in fish exposed to chemical contaminants occur prior to detectable physiological responses, and Pandey et al. (2024) reinforced that such alterations are sensitive and early indicators of toxic exposure. These changes may reveal sublethal effects of pollutants, contributing to environmental quality assessment and ecological risk evaluation. Scott and Sloman (2004) further observed that, although acute lethality tests are useful for establishing guidelines to prevent physiological death in aquatic animals, they do not account for *ecological death*, which may occur at much lower exposure concentrations, impairing the organisms ecological functionality. These behavioral dysfunctions have the potential to cause population imbalances, negatively affecting growth, survival, and reproduction of predatory species (Weis *et al*, 2001). Toussaint *et al*. (2025) emphasize the importance of considering dosage in ecotoxicological assessments, since behavioral responses may precede more severe effects at the population and ecosystem levels.

The results obtained reinforce the hypothesis that bioassays based solely on lethality tests may be insufficient to assess the real impact of aquatic pollutants. Considering the widespread use of glyphosate and other potentially cytotoxic agents in monocultures and their presence in aquatic ecosystems, the present study highlights the need to implement behavioral monitoring in ecotoxicological assays as a complementary and essential tool for more accurate diagnoses of sublethal effects.

5 CONCLUSION

The results of this study indicated that exposure of *D. rerio* to glyphosate at a concentration of 65 µg/L was capable of inducing measurable behavioral alterations. It is noteworthy that this concentration corresponds to the maximum value permitted by CONAMA Resolution No. 357 of 2005, considered environmentally acceptable for Class 1 waters.

Behavioral alterations proved to be sensitive and effective tools for the early detection of environmental stress, serving as important ecotoxicological indicators. These parameters contribute to understanding the sublethal effects of chemical agents and xenobiotics on fish.

Based on these findings, there is a reinforced need to review the currently accepted environmental tolerance levels established by Brazilian biomonitoring and regulatory agencies, in order to mitigate the impacts caused by the excessive use of such chemical agents.

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