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Acute and chronic toxicity of glyphosate to native fish from San Luis province, Argentina

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ABSTRACT

Public concern on the risks of glyphosate exposure has risen in Argentina, where glyphosate is used massively. Fish are widely used as models to assess the effects of contaminants. In this study we estimated the toxic effects of glyphosate (Roundup) on *Cheirodon interruptus*, *Australoheros facetus* and *Jenynsia multidentata*, three native fish species from San Luis, Argentina. Adult mortality was registered after acute exposure to Roundup (0 to 100 µL/L). The minimum concentration causing 100% mortality (MC100%M) and the maximum concentration with no mortality detected (MC0%M) were recorded. Biomarkers (hepatic enzymes and acetylcholinesterase) were assessed in fish that where chronically exposed to a sublethal concentration of Roundup. Inter-specific differences in sensitivity were not detected. The MC100%M was 50 µL/L and the MC0%M was 25 µL/L for the three species. Exposed fish had significantly higher hepatic enzymes and significantly reduced acetylcholinesterase activity levels compared to controls. This study provides valuable evidence on the impacts of glyphosate on native fish species.

KEYWORDS: Roundup, native fish, toxic effects, mortality, exposure, biomarkers

ABBREVIATIONS

POEA	:	polyoxyethyleneamine
MC100%M	:	minimum concentration causing 100% mortality
MC0%M	:	maximum concentration with no mortality detected
AST	:	glutamic oxaloacetic transaminase
ALT	:	glutamic pyruvic transaminase
AChE	:	acetylcholinesterase

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INTRODUCTION

The province of San Luis, located in central-west Argentina, has two clear areas of sierras belonging to the Pampean Sierras system. The central area, with a maximum height over 2000 m, is the most important highland of the province and serves as a spread center of water courses. Its gentle and extended oriental slope favors the development of an important hydrographic net, where two rivers, the Quinto River and the Conlara River, with permanent regimen stand out [1]. The hydrographic basin of Quinto River, which also extends over the south of Córdoba province, originates in La Florida Reservoir and receives tributaries from other subbasins, shaping the most important fluvial course of the province that covers a surface of around 1800 km^2.

The province of San Luis accounts for about 83% of the total 15 fish species cited for the Quinto River basin (80% native and 20% introduced). In accordance with the studies of zoogeography of fish, the province of San Luis is included in the Brasilic subregion of the Paranense Dominium [2, 3, 4]. Recent studies on the collection and identification of fish species in the Quinto River basin found 15 species (12 native and 3 introduced), grouped in 15 genus, 11 families and 6 orders [5]. Among the most common and more widely distributed species are *Cheirodon interruptus*, *Australoheros facetus* and *Jenynsia multidentata*.

Argentina is a country that produces and exports agricultural commodities (raw materials). Currently one of the most important farming products in the region is soybean, an oilseed crop with high economic value, that has largely incremented its production. In order to increase crop yields, soybean transgenic varieties resistant to the herbicide glyphosate are used. Glyphosate (N-phosphonomethylglycine) is highly soluble in water (10,500 mg/L), with a half-life time that can reach up to 90 days [6]. Its mode of action as herbicide is achieved through the inhibition of 5-enol-pyruvylshikimate-3-phosphate synthase, the enzyme that catalyzes amino acid biosynthesis in plants [7].

Glyphosate has been extensively used in Argentina during the past years. During 2012, 300-million liters of glyphosate were applied over the total area under soybean cultivation, a considerable amount if it is compared with the 13.9-million liters applied in 1996. The massive and uncontrolled use of glyphosate has given rise to public concern about the toxicological risks, especially in rural and periurban areas nearby soybean-cultivated fields.

This herbicide is sold in multiple formulations; however the most popular and widely used is commercialized under the trade name Roundup, which comprises glyphosate isopropylamine salt, and polyoxyethylenamine (POEA) as a surfactant agent. POEA is a compound belonging to the family of polyethoxyalkylamines which is synthetized from animal fatty acids and added to herbicides to improve their efficacy [8, 9]. A large amount of evidence identifies POEA as the principal compound responsible for the toxic effects of glyphosate formulations [10].

Biomarkers are biological responses related to the impact of contaminants at organization levels below individuals (enzymes, cells, tissues, organs and systems) [11]. Whereas, bioindicators are responses at levels of organisms, populations, communities and ecosystems [12]. The response of organisms to contaminants is analyzed at several scales (biomarkers and bioindicators), and this alternative is an improvement over the classical physical-chemical methods designed for environmental quality analysis. Some vertebrates such as fish and amphibians have been promptly used as experimental models of biomarkers or bioindicators for a considerable period of time, as a way to measure the environmental impact of different pollutants [13, 14]. Fish have been used as models in acute toxicity studies where mortality is the end point evaluated [10], and also in chronic toxicity evaluations that include measuring the levels of hepatic transaminases (glutamic oxaloacetic and glutamic pyruvic transaminase) and acetylcholinesterase, enzymes whose level variations indicate exposure to xenobiotics [15]. Previous studies in our laboratory have used fish with the aim of evaluating toxicity of novel drug compounds from natural and synthetic sources [16, 17, 18].

The objective of this study was to evaluate the acute and chronic toxic effects of a commercial formulation of the herbicide glyphosate (Roundup) on three native fish species from the River Quinto Basin, San Luis, Argentina: *Cheirodon interruptus*, *Australoheros facetus* and *Jenynsia multidentata*. Mortality was registered in order to evaluate acute toxicity. As biomarkers of chronic toxicity, the levels of hepatic enzymes glutamic oxaloacetic transaminase (AST), glutamic pyruvic transaminase (ALT), and acetylcholinesterase (AChE) were assessed.

MATERIALS AND METHODS

Herbicide

For acute and chronic exposure experiments, the commercial formulation of the herbicide Roundup® (active ingredient: 48% glyphosate, Monsanto) purchased from retail suppliers was used.

Study organisms

About 80 individuals of each of the three native fish species assayed, *Cheirodon interruptus*, *Australoheros facetus* and *Jenynsia multidentata*, were captured from La Florida reservoir (S 33° 06′ 43.3′′ W66° 02′ 26.0′′) using a net and immediately transported to the lab, 30 Km away from the field site. Individuals from each species were kept separately in 50 L tanks, with tap water, for 21 days, at 23 °C, in order to accustom them to laboratory conditions. During this acclimation period, fish were fed once a day with fish food flakes (Tetramin®), continuous aeration was provided and water was replenished every 3 days. Adult fish that demonstrated signs of favorable acclimation, normal movement, fin position and general external morphology, were selected for the exposure experiments.

Acute toxicity assays

Acute exposures were conducted, following the US Fish and Wildlife Service protocols for 96-h static, non-renewal toxicity testing, without feeding and aeration [19]. Mascotti *et al.* (2008) have modified this protocol in our laboratory in order to run assays with small amounts of chemicals.

Ten adult individuals from the three fish species kept in tanks of 20 L were exposed to five different concentrations of the herbicide Roundup (6.25; 12.5; 25; 50 and 100 µL/L). Besides, a control group not exposed to herbicides was used. Mortality was registered and dead individuals were removed from the tanks every 24 hours. Percent mortality was evaluated after 96 hours of exposure.

The minimum concentration that caused 100% mortality (MC100%M) and the maximum concentration at which no mortality was detected (MC0%M) were recorded.

Chronic toxicity exposure

Fish were exposed for 30 days to a sublethal concentration of 25 µL/L of Roundup (12 mg/L of glyphosate salt), which was the maximum concentration that did not produce mortality in acute toxicity exposures. Besides, a control group not exposed to herbicides was used. Ten individuals from each species were exposed to treatments in 20 L tanks, in triplicate, at a temperature of 23 °C, with controlled aeration and daily feeding of fish flake-granules (Tetramin®). Water was renewed every 3 days.

Enzymatic assays

After the period of chronic exposure, 3 individuals from each species were euthanized by decapitation using a scalpel. To perform enzymatic assays, viscera of fish, both treated and control were separated and macerated. From these macerated viscera, the enzymatic activities of AST and ALT were determined using a Metrolab 1000 Analizer UV/VIS Spectrophotometer with a 320-980 nanometer detection limit. The protocols followed were those specified by the manufacturer (Wiener Lab) based on the use of a specific enzymatic colorimetric method (transaminases 200, Wiener) and already implemented in other toxicity studies conducted by our research group [20, 21]. For the AChE, muscle tissue flow area was used, and the method used is indicated in the protocol specified by the manufacturer (Wiener Lab), which is based on the use of a kinetic method at 405 nm (Cholinesterase, Wiener). The enzymatic assays were performed using the internal quality controls provided by Wiener Lab.

Statistical analysis

Mortality frequencies obtained from acute toxicity tests were compared among concentrations and among species using Chi Square test.

The results of enzymes activities were analyzed using one-way analysis of variance, after square root transformation of data, in order to homogenize variances. Post hoc comparisons were performed using Tukey's t-test. In all cases, the significance level was 95%. All data were analyzed using the statistical software Graph Pad Instat.

RESULTS AND DISCUSSION

Acute toxicity

The three species assayed showed the same sensibility to the action of the herbicide Roundup (Table 1). The MC100%M was 50 µL/L and the MC0%M was 25 µL/L, equivalent to 24 mg/L and 12 mg/L of glyphosate salt.

These results agree with previous studies [22, 23]. The observed effect had an "all or nothing" action; all fishes died when exposed to 50 µL/L Roundup (24 mg/L of glyphosate salt) ($p \leq 0.000001$), with no differences in mortality at concentrations over this value and below 12 mg/L ($p \geq 0.95$) (Table 1).

Chronic toxicity

a. Hepatic enzymes

AST and ALT activity levels were significantly higher in fish exposed to Roundup, as compared to control fish (Table 2). These values are indicators of increased hepatocyte activities or destruction, related to the toxic effects caused by the herbicide. Differences in the control values of AST and ALT activities among species were not significant.

Fish liver is involved in the metabolism of xenobiotics [24], and as the primary place of biotransformation of strange compounds, it is particularly vulnerable to them. Liver detoxifies and eliminates many of these compounds principally as conjugates, and some of them accumulate to toxic

Table 1. Mortality (%) in native fish exposed to Roundup.

Species	Roundup exposure concentration (µL/L)					
	Control	**6.25**	**12.5**	**25**	**50**	**100**
Cheirodon interruptus	0	0	0	0	100	100
Australoheros facetus	0	0	0	0	100	100
Jenynsia multidentata	0	0	0	0	100	100

Table 2. Activity (average ± SD) of biomarkers, hepatic enzymes (AST and ALT) and acetylcholinesterase in native fish exposed to Roundup.

Species		AST (U/L)	ALT (U/L)	AChE (U/L)
Cheirodon interruptus	Control	50.33 ± 2.52	51 ± 1	13.23 ± 1.84
	Roundup	69.67 ± 6.1	77.67 ± 2.52	4.67 ± 1.03
Australoheros facetus	Control	53.33 ± 2.08	52 ± 3.61	21.50 ± 1.25
	Roundup	77.33 ± 4.04	80.67 ± 8.74	10.93 ± 1.27
Jenynsia multidentata	Control	42.33 ± 1.53	42 ± 2.65	22.50 ± 0.82
	Roundup	94.67 ± 5.68	102 ± 8.39	14.07 ± 1.05

levels or are bio-activated to reactive intermediates that may damage the liver or produce other disorders. Hepatic transaminases ALT and AST catalyze the conjugation of reduced glutathione (GSH) with a variety of electrophilic metabolites involved in detoxification. They are widely distributed in several organs, but their activity in serum is very low or negligible. Increased serum levels of these enzymes are indicative of damage to several organs or tissues, namely liver, muscle, heart and kidneys.

The activity of liver transaminases changes in fish exposed to diverse contaminants such as herbicides, which may drive an increase [25] or decrease [23] in their liver activity.

b. Acetylcholinesterase (AChE)

AChE belongs to a family of serine esterases that are able to hydrolyze choline esters as Acetylcholine. Cholinesterases are found in several organisms such as microorganisms, plants, vertebrates and invertebrates. Among the organisms mentioned, cholinesterases appear before synaptogenesis, during the early stages of embryonic development of invertebrates, and prevail in the muscles and nervous system. The inhibition of AChE in brain and other tissues such as muscle is considered as the specific biomarker more sensitive to the effects of carbamate and organophosphate insecticides on marine and fresh water fish [26, 27, 28]. When the enzymes are inhibited, the hydrolysis of acetylcholine to acetic acid and choline does not progress, thereby generating a permanent nervous impulse transmission that leads to an overstimulation of nervous cells that may cause tetanus, respiratory failure and death [29]. Glyphosate concentrations used in agricultural practices may cause changes in the metabolic and enzymatic parameters of fish, leading to the inhibition of AChE, increase in alkaline phosphatase, lipid peroxidation and protein catabolism, as demonstrated in studies on silver catfish (*Rhamdia quelen*) by other authors [30].

AChE activity levels of fish exposed to the commercial herbicide were significantly reduced when compared with control fish (Table 2). The reduced activity led to the overstimulation of nervous cells and caused diverse functional alterations such as increased muscular contractions and impediment or altered swimming performances, all alterations consistent with toxicity effects. *C. interruptus* showed lower control levels than the other two species (F GL $p \leq 0.001$), which had similar values.

CONCLUSION

This study reveals the effects of acute and chronic toxicity of the commercial herbicide Roundup, formulated with glyphosate, on native fish from the Río Quinto basin, San Luis, Argentina.

Acute toxicity was similar for the three species assayed, that showed the same MC100%M and MC0%M values (24 mg/L and 12 mg/L, respectively). Chronically exposed fish showed an increase in the activity of AST and ALT, enzymes that indicate increased hepatocytes activity, and a decrease in the activity of AChE, that lead to the overstimulation of nervous cells and may have deleterious consequences in muscle functioning.

This study contributes in understanding the impacts of herbicides formulated with glyphosate on native aquatic species. While it is difficult to generalize our findings with other fish species, they add valuable information to the previous studies that reported similar effects. Considering the wide use of glyphosate formulations and the increase in soybean cultivated surfaces in South American countries, it is fundamental to broaden our knowledge on the effects of these compounds on native species.

CONFLICT OF INTEREST STATEMENT

The authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest, or non-financial interest in the subject matter or materials discussed in this manuscript.

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