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Sublethal Effects of Waterborne Herbicides in Tropical Freshwater Fish

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Abstract The study evaluated the sublethal effects of the herbicides glyphosate (Roundup) and diuron (Hexaron) and the mixture of them, used extremely in agriculture, through biomarkers in fish. The glutathione S-transferase activity increased (74%) and catalase activity decreased (37%) at the higher exposure concentration of Hexaron in comparison to the control group, suggesting an activation of this metabolism route. Membrane damage was observed at the higher exposure of Roundup and in the mixture group compared to the control group, which can be related to the nuclear alterations observed in these exposed groups. The cholinesterase activity was also inhibited (37%) in mixture group compared to the control group and no gill morphology damage was found. The results suggested a potential synergic effect in some analysed parameters.

Keywords *Astyanax* sp. · Bioassay · Biomarkers · Freshwater fish · Herbicides

Herbicides used in agriculture can be transported to surface or ground waters, possibly causing adverse ecotoxicological effects on aquatic life. The herbicides used in this study were glyphosate under the tradename Roundup and diuron under the tradename Hexaron WG widely used in sugarcane cultures and hence found in the environment.

Roundup is a glyphosate-based herbicide (480 g L^{-1}, corresponding to 356 g L^{-1} of the acid equivalent), including water and a surfactant polyoxyethyleneamine as inert ingredients in its formulation. Due to its high water solubility and extensive usage (especially in shallow water systems), the exposure of nontarget aquatic organisms to this herbicide is a major concern. However, commercial glyphosate formulations are more acutely toxic than the active ingredient glyphosate (Peixoto 2005). The aquatic biodegradation period of glyphosate under aerobic and anaerobic conditions is less than 14 days and between 14 and 21 days, respectively (WHO 1994).

Diuron (468 g kg^{-1}) and hexazinone (132 g kg^{-1}), are the active ingredients of Hexaron WG, and a further 400 g kg^{-1} of inert ingredients are present in the formulation. Diuron [3-(3,4-Dichlorophenyl)-1,1-dimethylurea] belongs to the chemical group of phenylureas, and its physical and chemical properties include solubility up to 42 mg L^{-1} in water at 25°C, is neither ionized nor volatile, and its half-life time in soil is 328–212 days (Souza et al. 2000). Hexazinone [3-cyclohexyl-6-(dimethylamino)-1-methyl-1,3,5-triazine-2,4(1H,3H)-dione] is highly water-soluble (33 g L^{-1}) and mobile in soil, which confers a great potential for leaching, and it is considered to be among the most likely pesticides to contaminate ground water (Wang et al. 2005). Zhonglin et al. (1998) observed that hexazinone hydrolyzed very slowly, with half-lives of more than 1 year in buffered solutions at pH levels from 5 to 9.

In spite of the wide application of glyphosate and diuron in Brazil, only a limited amount of information is available about its toxicity to native freshwater fish. *Astyanax* sp.

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(Order Characiformes, Family Characidae) is a Brazilian native fish, rarely exceeding 10 cm in total length and showing a relatively varied coloration, which are mainly insectivorous, represent a species well suited for environmental monitoring due to their sensitivity to water-quality variations.

This work aimed to evaluate the sublethal effects in *Astyanax* sp. after acute exposure to glyphosate, diuron, and a mixture of them through biochemical, genetical, and histopathological biomarkers.

Materials and Methods

Astyanax sp. (10 ± 5 g body weight) were collected from a pisciculture farm, transported to the laboratory, and acclimatized for 15 days in 15-L aquaria under controlled temperature (22 ± 2°C), constant aeration, and 12-h-light/12-h-dark photoperiod. The animals were fed daily with commercial food (Alcon Guppy Co.), except during the experiments. All the procedures in this study were carried out in accordance with the Guidelines for the Care and Use of Laboratory Animals (Canadian Council on Animal Care) and the animal protocol was approved by the ethical committee for animal experimentation of the Paraná Federal University.

The bioassays were performed to evaluate the sublethal effects in *Astyanax* sp. of the commercial formulation of glyphosate (Roundup) and diuron (Hezaron WG), Milenia Agrociências S/A. The fish were distributed randomly into six groups of 20 individuals each (15-L aquaria each–reconstituted with water): control-water (CO), Roundup-0.003 mL L^{-1} (RA), Roundup-0.006 mL L^{-1} (RB), Hexaron-15 mg L^{-1} (H15), Hexaron-30 mg L^{-1} (H30), and a group exposed to high concentrations of a mixture of the herbicides (MT). Previous experiments were conducted to determine the sublethal concentrations.

After 96 h of exposure, the fish were killed through medullar section, and the blood was collected for the micronucleus test. The livers were grouped in numbers of two, forming 12 pools, and stored in a freezer at −70°C for biochemical analysis of glutathione S-transferase (GST), catalase (CAT), and lipoperoxidation (LPO). A fragment of muscle was stored at −20°C for measurement of acetylcholinesterase (AChE) activity. Samples of muscle and liver were homogenized in 0.1 M phosphate buffer at pH values of 7.5 and 6.5, respectively, and centrifuged at 10,000×*g* for 20 min at 4°C. The muscle supernatants were used for AChE analysis, and the liver supernatants were processed for CAT, GST, and LPO analysis. For light microscopy, gills samples were fixed in Alfac solution (70% ethanol, 4% formaldehyde, 5% glacial acetic acid) for 16 h.

The AChE activity was measured spectrophotometrically according to the method of Ellman et al. (1961), modified for use in the microplate by Sturm et al. (1999), at 415 nm. The CAT activity was measured at 240 nm according to the method described by Aebi (1984). The GST activity was measured at 340 nm according to the method described by Keen et al. (1976). The analysis of LPO was carried out using the ferrous oxidation–xylenol assay at 570 nm (Jiang et al. 1992). The protein concentration was determined by using Bradford' method (1976), with bovine serum albumin as the standard.

The preserved gills were dehydrated in a graded series of ethanol baths and embedded in Paraplast Plus® (Sigma). Sections of 5 μm were obtained, stained with Hematoxylin/Eosin and observed under observed under a Zeiss Axiophot photomicroscope. The criteria to establish the histopathological findings were according to Bernet et al. (1999).

The method proposed by Heddle (1973) was used for the piscine micronucleus test. Blood samples, obtained from the caudal vein of the specimens, were distended on clean slides. After fixation in ethanol for 30 min, the slides were left to air-dry and stained with 10% Giemsa solution for 10 min. For each fish, 2,000 erythrocytes were examined under 1,000× magnification and scored for the presence of both typical micronuclei and nuclear alterations manifested as changes in the normal elliptic shape of the nuclei.

The data analysis was preceded by the Kolmogorov–Smirnov normality test. The data on biochemical markers were analyzed using the one-way analysis of variance (ANOVA), followed by the Bonferroni posthoc tests. For the genetic alterations, the Kruskal–Wallis test was used. All tests were regarded as statistically significant when $p < 0.05$.

Results and Discussion

The specific activity of GST in the groups exposed to the herbicide Roundup (RA and RB) and herbicide mixture (MT) showed no difference compared to the control group (CO) (Fig. 1a). The H30 group showed an increase in the GST activity compared to the CO, MT, and H15 groups (Fig. 1a). GSTs are a group of enzymes that catalyze the conjugation of reduced glutathione (GSH) with a variety of electrophilic metabolites and are involved in the detoxification of both reactive intermediates and oxygen radicals. The GST activity increased only in the H15 group suggesting an increase in the metabolism due to the herbicide exposure, such as carcinogenic compounds and metabolites of pesticides that are eliminated through glutathionization. Isik and Celik (2008) observed an increase in GST activity in rainbow trouts (*Oncorhynchcus mykiss*) exposed to pesticides such as methyl parathion and diazinon. Yi et al.

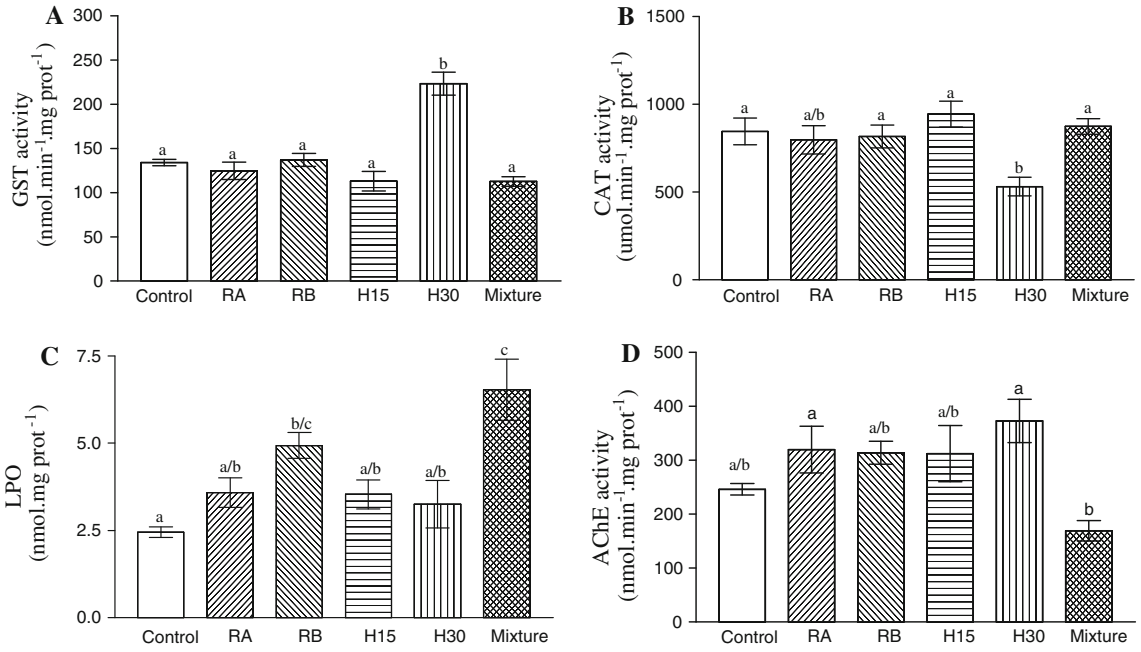

Fig. 1 Biochemical biomarkers in *Astyanax* sp. exposed to Roundup and Hexaron and to the both (mixture). **a** Glutathione S-transferase specific activity. **b** Catalase specific activity. **c** Lipoperoxidation. **d** Acetylcholinesterase specific activity. The *bars* are the mean ± mean standard error. *RA*: Roundup 0.003 mL L^{-1}, *RB*: Roundup 0.006 mL L^{-1}, *Mixture*: Roundup 0,006 mg L^{-1} + Hexaron 30 mg L^{-1}, *H15*: Hexaron 15 mg L^{-1}, *H30*: Hexaron 30 mg L^{-1}. *Mixture*: Roundup 0.006 mg L^{-1} + Hexaron 30 mg L^{-1}. *Different letters* mean statistical differences; $p < 0.05$ (ANOVA, Bonferroni)

(2007) found the same result in crucian carp (*Carassius auratus*) exposed to alochlor at different concentrations over 60 days. However, the fish exposed to the herbicide glyphosate showed no variation in the liver GST activity. Langiano and Martinez (2008) also did not find alterations of this enzyme in *Prochilodus lineatus* after exposure to glyphosate in commercial formulations, which might indicate that the metabolism of the compounds present in Roundup occurs by other biotransformation pathways.

Compared with the CO group, the specific activity of CAT was not different in the groups exposed to the herbicide Roundup (RA and RB) and the MT (Fig. 1b), only the H30 group activity was decreased when compared to CO, MT, and H15 (Fig. 1b). Many environmental pollutants can induce both the production of reactive oxygen species (ROS) and enhancement in the activities of antioxidant enzymes such as CAT, which have been proposed as indicators of pollutant-mediated oxidative stress. In the group exposed to the herbicide diuron at higher concentrations, the CAT activity was reduced as described for exposure to diuron (Teisseire and Vernet 2000) and other pesticides (Ballesteros et al. 2009). It can be the result of the sequestration inhibition essential metals, such as calcium, iron, copper, and zinc, because CAT is a heme protein and hence needs, for example, iron for its synthesis (Filipak Neto et al. 2008). The group that showed significantly higher GST activity (Hexaron 30 mg L^{-1}) was the same with reduced CAT activity, also described by Teisseire and Vernet (2000) in their experiment with *Lemma minor* exposed to 0.25 mg L^{-1} of diuron. Studies regarding the use of these biomarkers in organisms exposed to the herbicide hexazinone, also present in the formulation Hexaron, were not found in literature.

The hidroperoxide concentration was higher in the RB and MT groups when compared with the control group, and MT also had higher values than the H30 group (Fig. 1c). In the experiment with the herbicide Hexaron, only the animals from the MT group showed higher hidroperoxide concentrations than the other groups (Fig. 1c). Several studies have shown an increase in the levels of lipid peroxidation in some fish tissues exposed to organic pollutants; for example, *Jenynsia multidentata* exposed to endosulfan (Ballesteros et al. 2009), channel catfish (*Ictalurus punctatus*) and brown bullhead (*Ameiurus nebulosus*) exposed to *t*-butyl hidroperoxide (Ploch et al. 1999). In the present study, the fish exposed to the mixture of herbicides showed higher concentration of hidroperoxides than the animals exposed to single concentrations of herbicides, indicating a synergetic effect that increases the damage to biological membranes.

The AChE activity was not statistically different in the groups exposed to the herbicide Roundup, but the animals exposed to MT showed an enzymatic inhibition compared to the groups RA and RB (Fig. 1d). Individuals exposed to

the H30 showed an increase of the AChE activity when compared with CO and MT groups. Neurological and behavioral activities of animals are extremely sensitive to environmental contamination. The measurement of AChE activity is frequently used as a biomarker for exposure to certain groups of contaminants, such as organophosphorates and carbamates; however, other compounds also can influence the activity of this enzyme. In the present work, the AChE activity increased at the higher concentrations of diuron, indicating a stimulation that could interfere with the energetic demand and cause other physiological disturbances, in addition to affecting the locomotion and balance. Therefore, a greater inactivation of acetylcholine might be taking place at the synapses. Saglio and Trijasse (1998) observed alterations in the behavior of swimming in fishes (*Sarotherodon mossambicus*) exposed to 0.55 g L^{-1} diuron for 60 days. In the present study, similar signals were observed, and their intensity was higher in the H15 group. The action mechanism of the increase in the activity of AChE is not explained by the present study, so it must be investigated in future. The muscle AChE activity was also not inhibited in *Astyanax* sp., in contrast to that observed when the fish were exposed to both herbicides simultaneously, the latter showing a synergetic effect. This plays a role for sugarcane culture, for example, since the herbicides are used in association in field situations.

The group exposed to the mixture (MT) showed a large number of morphological nuclear alterations and micronuclei in the erythrocytes compared to the CO group. The fish exposed to the highest concentration of Roundup showed difference compared to the control group. No difference was found in the groups exposed to Hexaron (Fig. 2).

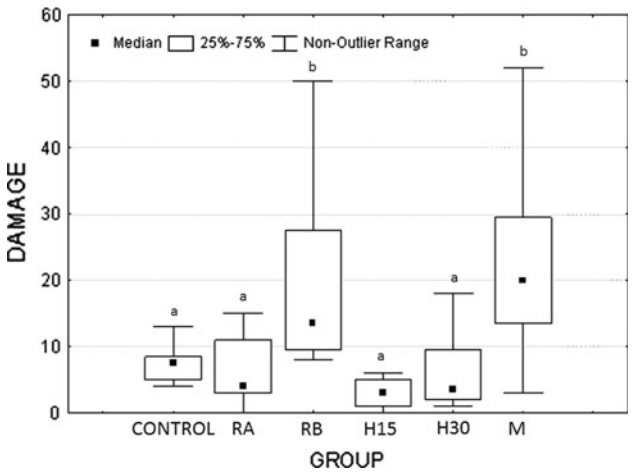

Fig. 2 Nuclear alterations and micronucleus (Median and quartis) in erythrocytes of *Astyanax* sp. exposed to Roundup and Hexaron and to the both (mixture). *RA*: Roundup 0.003 mL L^{-1}, *RB*: Roundup 0.006 mL L^{-1}, *H15*: Hexaron 15 mg L^{-1}, *H30*: Hexaron 30 mg L^{-1}, *Mixture (M)*: Roundup 0.006 mg L^{-1} + Hexaron 30 mg L^{-1}. *Different letters* indicate statistical differences; $p < 0.05$ (Kruskal–Wallis)

ROS, such as hydrogen peroxide (H_2O_2), hydroxyl radicals ($OH^{\cdot}$) and superoxide anions (O_2^{-}) have been shown to damage chromosomal DNA and other cellular components, resulting in DNA degradation, protein denaturation, and lipid peroxidation. However, the mechanisms of these cellular effects are rather complex and are not yet fully understood. DNA damage induced by oxygen radicals occurs by oxidative modification of the bases in nucleic acids and by scission of the DNA strands. However, the implications of lipid peroxidation for ROS-induced DNA damage remain to be elucidated. Therefore, the micronucleus can develop after an acute exposure to a genotoxic substance. Grisolia (2002) also found an increase in the micronucleus frequency in erythrocytes of *Tilapia rendalli* exposed to different concentrations of glyphosate in the commercial formulation Roundup. In this study, only the mixture group showed high frequency of nuclear alteration; this occurrence can be related to the high hidroperoxide concentrations. Thus, the loss of membrane integrity favors the attack on DNA.

The absence of damage on the gills of individuals exposed to herbicides in the current work showed that apparently the waterborne exposed animals were not affected. In general, the morphological alterations found in gills of fish after acute exposure to pollutants are aneurisms and lamellar fusion. These findings potentially disrupted the gas exchange and osmoregulation functions of the gills as already demonstrated under experimental conditions to in fish species exposed to other pollutants (Akaishi et al. 2004). The acuteness of exposure and the low concentrations of the herbicides as evaluated in the current study could explain the absence of lesions, implying that the results could be different under chronic exposure. Nevertheless, it is important to investigate the bioavailability of the compounds tested and chronic exposures should be further investigated to understand the preferential route of uptake.

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