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The use of biomarkers to study the effects of the mixture of diuron and hexazinone on small and large *O. niloticus*

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Abstract

Pesticides have made possible a safer and plentiful supply of food; however, the ultimate sink for many of these contaminants is the aquatic environment. We analyzed the commercial mixture Velpar K® WG, which is composed of the pesticides diuron (46.8% m/m) and hexazinone (13.2% m/m), as well as inert ingredients (40.0% m/m). The present study aimed to evaluate the effects of the herbicide mixture on *Oreochromis niloticus* of different sizes. To this end, we analyzed biomarkers in small and large *O. niloticus* exposed to a mixture of herbicides at 125, 250 and 500 ug L^{-1} for 72h. EROD increased activity in small fish exposed to the herbicide mixture at 250 and 500 ug L^{-1}. The GST activity and levels of the antioxidant enzymes GPx and CAT remained the same in the treated fish, compared with the control. The level of the antioxidant enzyme SOD measured in the fish gills was changed in animals exposed to the herbicide mixture at 250 ug L^{-1}. MDA analysis did not show lipid peroxidation. The comet assay evidenced widespread DNA damage, but the micronucleus test did not show mutagenicity. Hepatosomatic (HSI) analysis did not indicate any alterations in liver morphology. The biomarkers response in the fish depended on the size of the individuals.

Keywords: biomarkers; diuron; environmental toxicology; hexazinone.

INTRODUCTION

Herbicides play a crucial role in weed control, which is very important for improving agricultural production and yield. However, the excessive use of these substances may impact non-target organisms, especially aquatic fauna and flora and their environment (Nwani *et al*., 2011).

In Brazil, herbicides are the most used pesticide in agriculture, constituting 59% of all the classes of pesticides used in the agricultural sector, particularly in the soybean, corn, and sugarcane crops (Bortoletto *et al*., 2009; Alves *et al*., 2012). In 2011, herbicide delivery reached 403,600 tons, representing an increase of 44% as compared with the 279,200 tons recorded in 2006 (Freitas Jr, 2012).

To control weeds in sugarcane cultures, a sustainable source of ethanol fuel, a pre- and post-emergent herbicide consisting of a mixture of diuron and hexazinone is widely used (Bicalho *et al*., 2010). The commercial mixture Velpar K® WG, composed of diuron (46.8% m/m, or 468 g Kg^{-1}), hexazinone (13.2% m/m, or 132g Kg^{-1}), and inert ingredients (40.0% m/m, or 400g Kg^{-1}), is routinely applied in agriculture and introduced into the environment.

Diuron belongs to the group of substituted ureas, and hexazinone belongs to the group of triazinones. Both compounds act by inhibiting the photosystem II of photosynthesis (Mei *et al*., 2012). Studies have shown that herbicides displaying this mode of action in plants (e.g., atrazine, diuron, and simazine) are highly mobile and can end up in stream networks (Lewis *et al*., 2009). Moreover, phenyl-urea derivatives are among the most widely used herbicides in agriculture (Barbash *et al*., 2001). However, removing these herbicides from the environment takes weeks, or even months (Mackay *et al*., 1997; Benitez *et al*., 2006).

Diuron (3-(3,4-dichlorophenyl)-1,1-dimethylurea) is a non-ionic compound with moderate water solubility – about 36-42 mg L^{-1} at 20-25 ºC (Cabrera *et al*., 2010). This

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herbicide persists in the environment, and its breakdown in ground and surface waters is extremely slow (Madhum *et al*., 1987; Abass *et al*., 2007). Hexazinone (3-cyclohexyl-6-(dimethylamino)-1-methyl-1,3,5-triazine-2,4 (1H, 3H)-dione) tends not to undergo hydrolysis or photolysis, so it can persist in aquatic systems (Mei *et al*., 2012). This herbicide is highly water soluble (33 g L^{-1} at 25 ◦C) and significantly mobile in soil, so it can potentially leach into ground water and run off into surface water (Mei *et al*., 2012; Wang *et al*., 2005).

Regarding the concentrations found in the environment, in canals of south Florida, United States, 76 ug L^{-1} of diuron and 4.5 ug L^{-1} of hexazinone were found in surface waters (Miles & Pfeuffer, 1996). Besides, Lewis *et al.* (2009) analyzed water samples collected from river and creek sites in three geographical regions of the Great Barrier Reef catchment area (Australia). Sampling sites were selected based on land use in the upstream catchment area, including sugar cane cultivation (areas with >10% sugar cane). In these sites, it was found diuron at concentrations up to 20 ug L^{-1} and hexazinone at concentrations up to 5 ug L^{-1}.

The effects of the herbicides diuron and hexazinone in target organisms are already known, but given that they reach the aquatic environment after their application in agriculture, it is important to study the effect that these herbicides can cause in non-target animals, such as fish. Also, bearing in mind that agriculture uses commercial mixtures, not the pure pesticide, tests with the most commonly applied commercial mixtures are crucial to understanding the real impacts in the environment.

Fish serve as a good bioindicator for assessing environmental pollution: they are directly exposed to chemicals and mutagens resulting from agricultural production via surface runoff, or indirectly exposed to contaminants through the food chain of the ecosystem (Nwani, 2010). Nile Tilapia (*Oreochromis niloticus*) is a teleost fish that is distributed worldwide. This fish can function as a model for conducting toxicological studies and assessing environmental pollution (Min & Kang 2008).

To determine whether these herbicides affect non-target organisms, analysis of changes in enzyme activities (biotransformation and antioxidant enzymes) and lipid peroxidation offers a rapid means of assessing the toxic impact of pesticides on these organisms (Altinok *et al*., 2012). A wide range of biomarker assays, such as the micronucleus test and the comet assay, can also be used to monitor the effects of environmental pollutants (Çavas & Könen, 2007). As a complement to the micronucleus test, many authors examine whether morphological changes – known as erythrocytic nuclear abnormalities (ENAs) - occur in the erythrocyte nucleus, as these changes are indicators of genotoxicity (Ayllon & Garcia-Vazquez, 2001; Cavalcante *et al*., 2008). Morphological parameters, such as hepatosomatic indices (HSI), also help to evaluate the effects of xenobiotics.

Given that biomarker responses can vary significantly according to the size of the animal (Peixoto & Santos, 2009), a factor that can indicate differences in the organism's susceptibility to the pollutant, the present study uses Nile Tilapias with differences in body length and weight in order to analyze the biomarker responses according to the animal's size.

This study aimed to investigate the biochemical and genetic effects of increasing concentrations of diuron and hexazinone, in the form of the commercial mixture Velpar K® WG, on Nile tilapia of different sizes. To this end, we exposed the animals to the commercial herbicide mixture at concentrations of 125, 250, and 500 ug L^{-1} for 72 h. We then analyzed the activities of EROD (in the liver) and GST, SOD, CAT, and GPx, as well as the levels of lipid peroxidation (in the liver and gills) in *O. niloticus*. We also evaluated genome damage on the erythrocytes of the animals, through the micronucleus test and the comet assay. We hypothesize that this herbicide can induce metabolic and genetic alterations in Nile tilapias, and that smaller fish are more susceptible to the effects of diuron and hexazinone than larger fish.

MATERIAL AND METHODS

Chemicals

The reagents were purchased from Sigma Chemical. In our study, the herbicide was tested in the form of the complex commercial mixture, as this form is routinely applied in agriculture and introduced into the environment. The commercial mixture of diuron and hexazinone (Velpar K® WG), composed of 46.8% m/m (or 468g Kg^{-1}) of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (diuron), 13.2% m/m (or 132g Kg^{-1}) of 3-cyclohexyl-6-dimethylamino-1-methyl-1,3,5-triazine-2,4 (1H, 3H)-dione (hexazinone), and 40.0% m/m (or 400g Kg^{-1}) of other ingredients, was selected for the study. We analyzed the concentrations of 125, 250 and 500 ug L^{-1} of the commercial mixture, which is equivalent to 58.5, 117, and 234 ug L^{-1} of diuron and 16.5, 33, and 66 ug L^{-1} of hexazinone, respectively.

Animals and experimental design

Adult Nile tilapia (*O. niloticus*), of both sexes, were obtained from the aquaculture center of Universidade Estadual Paulista (State University of São Paulo - UNESP), of Jaboticabal Campus, São Paulo, Brazil, and transferred to the animal ecology laboratory of the same University, São José do Rio Preto Campus, São Paulo, Brazil. The fish where kept in tanks for at least one week before the experiment, to acclimatize. There were a total of 48 fish: 24 small (mean length 10.73 ± 1.72 cm, mean weight 43.00 ± 13.42g) and 24 large (mean length 18.76 ± 2.15 cm, mean weight 182.51 ± 58.39 g), as shown in Figure 1.

For each size of fish tested in this study (small and large), a control group and three experimental groups were assayed, with a total of eight groups. A different concentration of the herbicide mixture was used in each experimental group, namely 125, 250, and 500 ug L^{-1}, corresponding to diuron

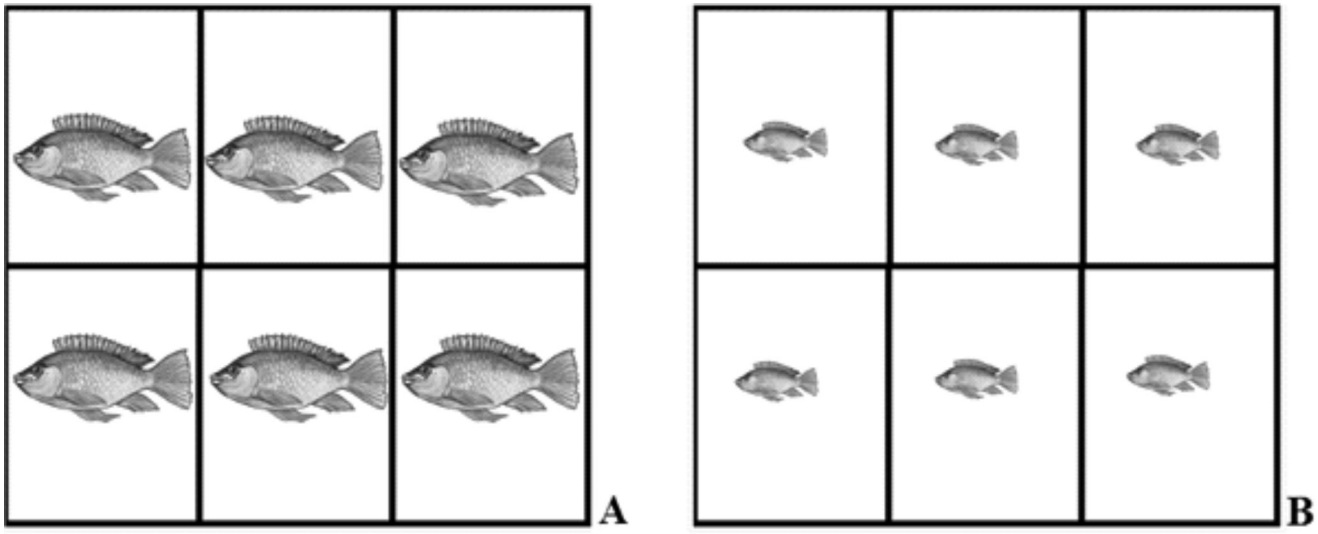

Fig 1. Figure of the experiment showing the compartments. A: Large fish (mean length 18.76 ± 2.15 cm, mean weight 182.51 ± 58.39 g). B: Small fish (mean length 10.73 ± 1.72 cm, mean weight 43.00 ± 13.42g)

concentrations of 58.5, 117, and 234 ug L^{-1} and hexazinone concentrations of 16.5, 33 and 66 ug L^{-1} respectively. The concentration of 500 ug L^{-1} corresponded to a 1000x dilution of the solution recommended for use in agriculture, therefore the amounts used in the present study are comparable to those found in the environment, as shown by Lewis *et al*. (2009) and Miles & Pfeuffer (1996).

For the experiment, eight 100 L-capacity aquaria were used. Each aquarium had six 17 L compartments. Each compartment contained only one fish, with a total of six fish per group. Having isolated each fish in its compartment, we were able to guarantee the occurrence of replicates with n = 6.

The aquaria were kept under constant aeration and temperature. No food was supplied to the fish during the experiment. The animals were exposed to the herbicide mixtures for 72 h; after this period, they were anesthetized with benzocaine; the liver, gills and samples of blood were removed. The liver and gills were frozen at -80°C for subsequent biochemical analysis. With the blood, histological slides were made for micronucleus test and comet assay in the same day.

Preparation of samples for biochemical analysis

To analyze the enzymes EROD, GST, SOD, GPx, and CAT, the tissues (liver and gills) were homogenized (1:4 weight:volume) in Tris-HCl 20 mM, pH 7.5, containing sucrose 0.5 M, etylenediamine tetraacetic acid (EDTA) 1 mM, dithiothreitol (DTT) 1 mM, and KCl 0.15 M as well as protease inhibitor phenylmethylsulfonyl fluoride (PMSF) 1 mM. The homogenate was centrifuged at 10,000 g for 30 min, at 4 ºC, and the resulting supernatants were centrifuged at 50,000 g for 60 min, at 4 ºC. The supernatant obtained after the second centrifugation was used to analyze GST, SOD, GPx, and CAT. The pellet of the liver samples was re-suspended in 100 mL of Tris-HCl 0.1 M, pH 7.5, containing EDTA 1 mM, DTT 1 mM and KCl 0. 1 M and used to analyze EROD activity. The prepared samples could then be aliquoted for later analysis. For lipid peroxidation analysis on basis of malondialdehyde (MDA) quantification, the tissues (liver and gills) were homogenized (1:3 weight: volume) in Tris HCl 0.1 M, pH 8.0. The prepared samples had to be analyzed on the same day.

Biochemical analysis

EROD, GST, SOD, GPx, and CAT

The EROD assay measures the O-dealkylation, mediated by CYP1A, of the non-fluorescent substrate 7-ethoxy-resorufin in resorufin, a fluorescent product detected by fluorometry (λ_{excit} = 537 nm, λ_{emiss} = 583 nm) (Sarasquete and Segner 2000). The assay mixture contained potassium phosphate buffer 80 mM (pH 7.4), 7-ethoxyresorufin 335 l M, NADPH 20 mM and microsomal liver extract (prepared sample). The reaction was observed 30 ºC, for 3 min. The EROD activity (pmol min^{-1} mg^{-1} of protein) was calculated on the basis of a previously prepared resorufin standard curve.

GST activity was assessed according Keen *et al*. (1976). The assay mixture contained potassium phosphate buffer 0.2 M, pH 6.5, the substrate 1-chloro-2,4-dinitrobenzene (CDNB), GSH, and the sample containing GST. The activity was determined by measuring the absorbance at 340 nm, on a spectrophotometer.

The method used to analyze SOD relied on a system that generated superoxide (xanthine/xanthine oxidase) coupled to cytochrome c reduction by the superoxide anion radical; this reaction intensified the absorbance at 550 nm, at 25 ° C. Addition of the sample containing SOD promotes an inhibition of cytochrome c reduction, because the enzyme competes with cytochrome c for superoxide (Mccord & Fridovich, 1969).

GPx activity was measured according to Sies *et al*. (1979), by following the decrease in the absorbance at 340 nm on a spectrophotometer, due to the consumption of NADPH. Glutathione reductase (GR) used NADPH to reduce

glutathione, previously oxidized by GPx during conversion of t-butyl peroxide-OOH to its corresponding alcohol.

CAT activity was measured by the method of Beutler (1975), through quantification of the rate of hydrogen peroxide decomposition, as evidenced by the decrease in absorbance at 240 nm at 30°C.

Protein levels were determined by the method of Bradford (Bradford, 1976) in a microtube containing the sample and the Bradford reagent. After 40 min in the dark, the samples absorbance was read at 595 nm. The protein concentration was calculated on the basis of the calibration curve prepared from bovine serum albumin (BSA) and the Bradford reagent.

Lipid peroxidation

Lipid peroxidation levels were measured on the basis of the product originating from the combination of malondialdehyde, a product of lipid peroxidation, and thiobarbituric acid (TBA), as analyzed by High Performance Liquid Chromatography (HPLC) and UV-Vis detection. TBA was dissolved in HCL 0.2 M, and the resulting solution was added to the homogenized sample. The reaction mixture was heated at 90 ºC, for 60 min. The colored derivative was extracted with butanol and quantified by HPLC at 532 nm, on the basis of a malondialdehyde (MDA) standard calibration curve that had been previously constructed by using the same procedure employed for the samples. The HPLC system consisted of an ESA584 pump and an ESA526 UV-Vis detector. The column was an ACE 5 C18 (250 _ 4.6 mm, 5 lm). The EZ Chrom Elite software (Agilent Technologies) was used to monitor the chromatograms and to identify quantify the peaks. The mobile phase consisted of KH_2PO_4, 0.05 M, pH 7.0, with 40% methanol, pumped at an isocratic flow of 1 mL min^{-1}.

Genotoxic and mutagenic analysis

Micronucleus test

For the micronucleus test (Al-Sabti, 1986; Al-Sabti & Metcalfe, 1995), a cardiac puncture was performed to remove 3 cc of blood from each animal. Three slides were prepared for each fish by means of the smear technique. The material was fixed in absolute methanol and stained by the Feulgen reaction. A total of 1000 erythrocytes were analyzed in each slide, which amounted to 3000 erythrocytes per fish. This analysis was conducted with the aid of an optical microscope, under 100 x magnification. The frequency of micronuclei and nuclear abnormalities was calculated. Because all the cells in the field of view were counted, the total number of cells analyzed per animal may have varied. Hence, the total number of counted cells was transformed into a percentage of the numbers of micronuclei and abnormalities, and the frequency was measured as a percentage.

Comet assay

The comet assay was performed according to the method described by Singh *et al.* (1988). Blood samples were diluted in 1000 µL of saline solution. Slides were prepared with 10 uL of this cell suspension and 120 µL of low melting point agarose (0.5%) at 37°C. The slides were kept in a lysis solution (1 mL of Triton X-100, 10 mL of DMSO and 89 mL of lysing stock solution, pH 10.0 - stock solution: NaCl 2.5 M, EDTA100 mM, and Tris 10 mM diluted to 1 L) in the refrigerator for 1h. After lysis, the slides were placed on a horizontal electrophoresis box for 20 min, at 25 V, and 300 mA. The slides were neutralized with Tris 0.4 M (pH 7. 5) for 15 min, and fixed in ethanol for 10 min. The cells with no DNA damage migrated homogeneously. The cells with damaged DNA formed fragments of different sizes – the smaller ones migrated faster during electrophoresis, giving rise to a tail that resembled the tail of a comet.

Two slides were prepared from each fish. Fifty nucleoids were analyzed from each slide, according to the procedure described by Kobayashi *et al.* (1995). The slides were stained with ethidium bromide (0.002 mg mL^{-1}). The analysis was conducted under a fluorescence microscope, filter B - 34 (excitation: λ = 420-490 nm, barrier λ = 520 nm), under 40 x magnification. The nucleoides were classified visually, according to the migration of the fragments: class 0 (no damage), class 1 (little damage), class 2 (medium damage), and class 3 (major damage). The injury score was calculated by multiplying the total number of cells in each class by the class value (0 -3).

Morphological analysis

Hepatosomatic index (HSI)

Fish were killed and their liver weight (LW) and body weight (BW) were recorded. The HSI for each fish was calculated by the formula: HSI = [liver weight (g) / body weight (g)] x 100.

Statistical analysis

To verify the effect of the herbicides on the animals, the treated fish were compared with control fish. The groups of small fish and large fish were analyzed separately. Tests for normality (Shapiro–Wilk) and homogeneity of variances (Levene) were applied. For the parametric data, one-way ANOVA was used, followed by the Tukey test. The Kruskal-Wallis test for nonparametric data was used, followed by Multiple comparisons of mean ranks. Significant differences were accepted only when $p < 0.05$. The analyses were performed using the software Statistica 7.0.

RESULTS

Biochemical analysis

Table 1 lists the biochemical parameters (EROD, GST, SOD, GPx, and CAT activities, and MDA levels). The activity of EROD, measured in the liver, differed in exposed fish as compared with the control fish. Small *O. niloticus* treated

Table 1. Activities of the enzymes EROD (pmol/min/mp of protein), GST, SOD, GPx, and CAT (U/mg of protein), and MDA levels (nmol g^{-1} of tissue) in control fish and fish exposed to a mixture of the herbicides diuron and hexazinone at 125, 250, and 500 ug L^{-1} for 72 h. * indicates significant difference ($p < 0.05$) between the treatment and control groups.

Velpar (ug L^{-1})	Fish size	Tissue	EROD	GST	SOD	GPx	CAT	MDA
0 (control)	Small	Liver	17.49 + - 7.78	0.76 +- 0.29	16.93 +- 4.34	0.03 +- 0.00	52.14 +- 17.95	0.75 +- 0.18
		Gill	-	0.16 +- 0.03	15.00 +- 3.30	0.03 +- 0.00	11.94 +- 1.26	0.96 +- 0.25
	Large	Liver	68.44 + - 64.71	0.77+- 0.11	13.78 +- 5.35	0.04 +- 0.00	46.13 +- 12.33	0.54 +- 0.16
		Gill	-	0.20 +- 0.04	10.93 +- 1.03	0.03 +- 0.00	13.06 +- 1.49	1.57 +- 1.10
125	Small	Liver	44.35 + - 33.07	0.61 +- 0.21	15.06 +- 4.13	0.02 +- 0.01	34.23 +- 8.95	0.66 +- 0.14
		Gill	-	0.20 +- 0.05	13.12 +- 3.50	0.04 +- 0.01	13.13 +- 4.31	0.94 +- 0.16
	Large	Liver	66.92 + - 87.51	0.67 +- 0.26	15.07 +- 7.50	0.03 +- 0.00	43.75 +- 19.75	0.47 +- 0.08
		Gill	-	0.15 +- 0.04	10.13 +- 1.31	0.03 +- 0.00	11.33 +- 1.44	1.21 +- 0.29
250	Small	Liver	68.31 + - 23.47 *	0.69 +- 0.11	16.41+- 4.54	0.03 +- 0.00	42.15 +- 9.05	0.64 +- 0.17
		Gill	-	0.21 +- 0.02	9.20 +- 0.66 *	0.03 +- 0.00	13.50 +- 2.22	0.71+- 0.15
	Large	Liver	20.63 +- 14.51	0.661+- 0.20	13.79 +- 4.28	0.03 +- 0.01	36.35 +- 9.55	0.65 +- 0.20
		Gill	-	0.14 +- 0.03	11.54 +- 2.42	0.03 +- 0.00	12.44 +- 1.452	1.70 +- 0.94
500	Small	Liver	61.91 + - 27.34 *	0.68 +- 0.26	20.21 +- 5.02	0.03 +- 0.01	59.64 +- 19.81	0.52 +- 0.16
		Gill	-	0.20 +- 0.05	10.46 +- 2.01	0.03 +- 0.00	13.92 +- 2.25	1.13 +- 0.42
	Large	Liver	27.75 +- 10.87	0.79 +- 0.22	13.65 +- 3.70	0.03 +- 0.00	44.30 +- 20.29	0.50 +- 0.11
		Gill	-	0.15 +- 0.02	10.74 +- 1.37	0.02 +- 0.00	13.61+- 2.27	1.07 +- 0.45

with commercial mixture at 250 and 500 ug L^{-1} showed higher EROD activity than the control group.

The activity of GST in the liver and gills of treated groups did not differ from the control group, in both small and large fish.

The activity of SOD in the livers of small fish treated with the commercial herbicides did not differ significantly from the activity of SOD observed in the control fish, as well as the activity of SOD in the livers of large fish.

In the gills, the activity of SOD in small fish exposed to the commercial herbicide mixture at 250 ug L^{-1} was lower than the activity of SOD in the control fish.

Regarding hepatic GPx activity, the treated animals (large and small) did not differ significantly from the control. GPx in the gills of the treated fish, both large and small, did not differ from the control.

CAT analysis did not reveal any significant differences in the liver and gills of small and large exposed fish as compared with the control.

Quantification of MDA, the product of lipid peroxidation, did not evidence any differences in the treated animals compared with the control, either in the liver or in the gills.

Genotoxic and mutagenic analysis

The micronucleous test performed on the blood of *O. niloticus* revealed just one cell with a micronucleous. Therefore, statistical analysis could not be performed (data not shown). However, nuclear abnormalities existed in the analyzed cells of all the studied groups, with no significant differences between exposed *O. niloticus* and control fish. The comet assay, performed on the blood of *O. niloticus*, did not show any differences between small fish and the control. However, large fish exposed to 125 and 500 ug L^{-1} of the mixture of diuron and hexazinone had more damage than the control. The results for nuclear abnormalities and the comet assay are shown in figure 2.

Morphological analysis

The HSI, both in liver and gills, did not differ significantly in small and large exposed fish compared to the control (Fig. 3).

DISCUSSION

The mixture of herbicides diuron and hexazinone constitutes a selective herbicide for some plant species, because these compounds act by inhibiting photosystem II of photosynthesis. Unfortunately, several authors have reported that selective pesticides can damage non-target organisms (Freemark & Boutin, 1995; Bony *et al*., 2008; Stephen *et al*., 2011). Although studies regarding the biological effects of pesticides have increased in recent years, there are no studies on the effects of the herbicide mixture diuron and hexazinone in biomarkers of *O. niloticus*.

The concentrations of Velpar K® WG used in present work were 125, 250 and 500 ug L^{-1}, which corresponds to diuron concentrations of 58.5, 117, and 234 ug L^{-1} and hexazinone concentrations of 16.5, 33 and 66 ug L^{-1} respectively. Lewis *et al*. (2009), showed, in the Great Barrier Reef, Australia, that diuron reached 20 ug L^{-1} and hexazinone reached 5 ug L^{-1} in sites were land use in the upstream catchment area is sugar cane cultivation (areas with >10% sugar cane), and Miles & Pfeuffer (1996), analyzing the surface waters of the canals of south Florida, United States, found the presence of diuron at a concentration of 76 ug L^{-1} and hexazinone at a concentration of 4.5 ug L^{-1}. These data show that the amounts of the herbicides used in the present study are close to those found in the environment.

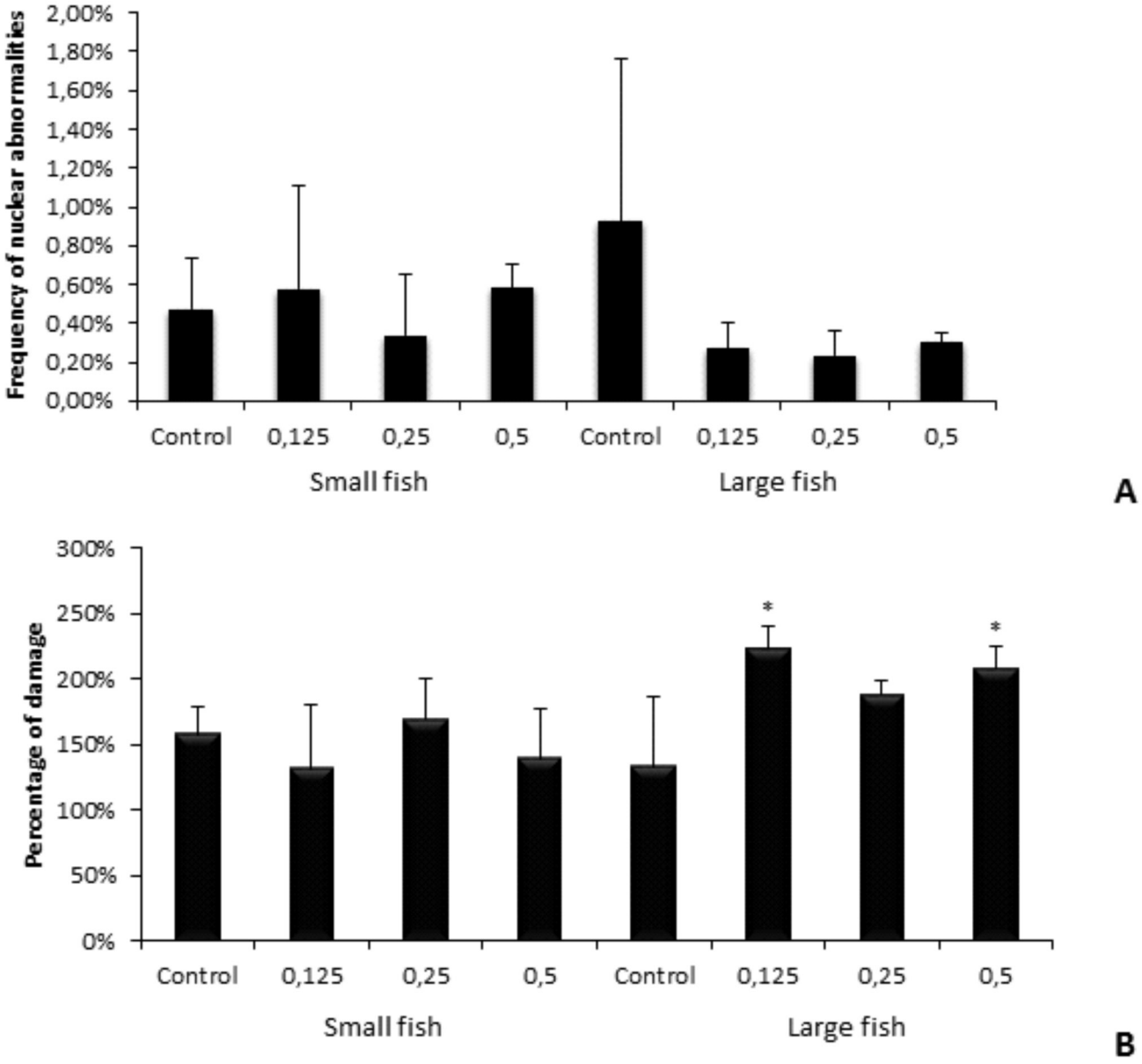

Fig 2. Genotoxic and mutagenic markers: Nuclear Abnormalities (A) and Comet Assay (B) on the blood of small and large *O. niloticus* exposed to tebuthiuron at concentrations of 125, 250, and 500 ug L^{-1} of a mixture of the herbicides diuron and hexazinone. * indicates significant difference ($p < 0.05$) between the treatment and control groups.

In this work, *O. niloticus* exposed to the mixture of the herbicides diuron and hexazinone, Velpar K® WG, at 250 and 500 ug L^{-1} showed higher EROD activity than the control. Numerous studies have already demonstrated significantly increased hepatic CYP1A protein levels and activity in many fish species from polluted environments (Van Der Oost *et al*., 2003), and in fish exposed to herbicides, like alachor (Mikula *et al*., 2009). Furthermore, Schoket and Vincze (1990) analyzed Wistar rats treated intragastrically with diuron (0.73, 1.62, 2.43, and 3.65 mmol kg^{-1}) and chlorotoluron (2.35, 5.22, 7.83, and 11.74 mmol Kg^{-1}), and observed greater P450 monooxygenase activity, including EROD, for both herbicides. Rudzok *et al*. (2009) showed that diuron potently induced CYP1A1 mRNA transcription in HepG2 and elicited pronounced CYP1A1 enzyme activity when analyzed based on EROD activity. Therefore, our data are consistent with the results of these studies in that they reveal increased basal phase I biotransformation.

However, in the present work, there was no difference in the activity of GST in exposed fish as compared with the control. According to the Van Der Oost *et al*. (2003) and Andersson *et al*. (1985), compared with phase I systems, the induction responses of phase II enzymes are generally less pronounced, which may explain the induction of EROD, and the lack of response of GST in the present study.

Regarding antioxidant enzymes, higher SOD indicates increased O_2.- production. In contrast, O_2.- radicals, either in the form of radicals or after transformation to H_2O_2, can oxidize

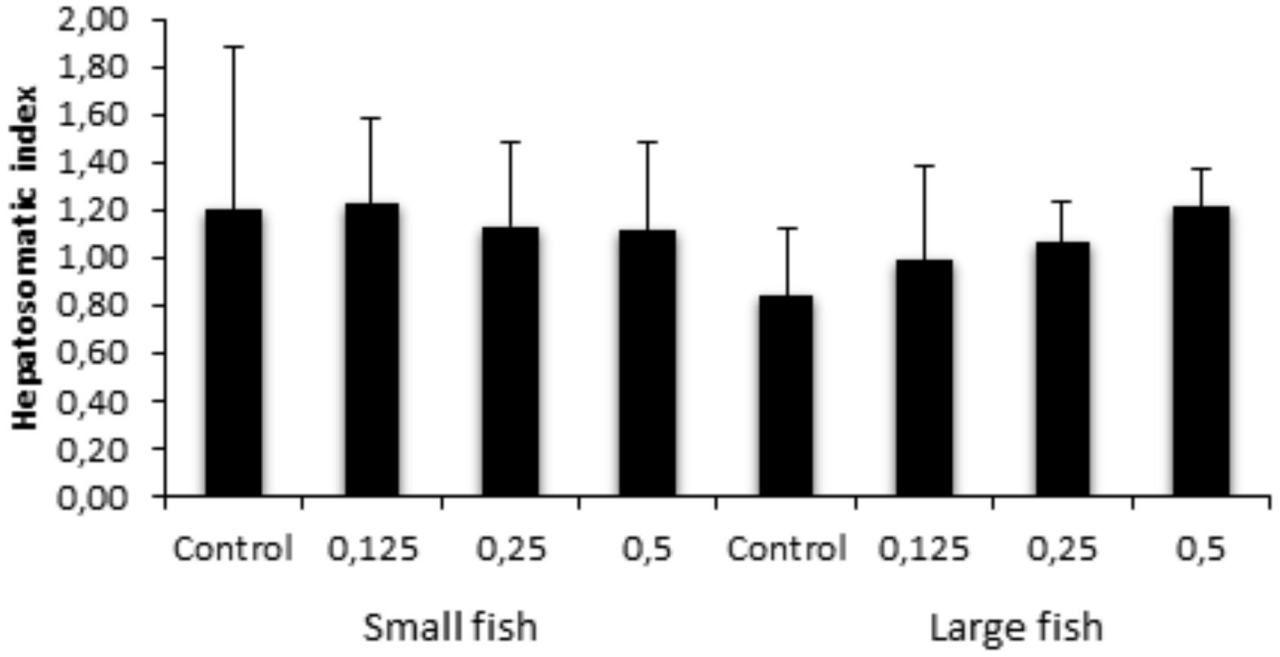

Fig 3. Hepatosomatic index in small and large O. niloticus exposed to a mixture of the herbicides diuron and hexazinone at 125, 250, and 500 ug L^{-1}. * indicates significant difference ($p < 0.05$)

cysteine in the enzyme and reduce the level of SOD activity (Min & Kang, 2008; Bartkowiak *et al*., 1981; Dimitrova *et al*., 1994). In this study, the activity of SOD in the gills of *O. niloticus* exposed to a mixture of diuron and hexazinone at 250 ug L^{-1} was lower than the activity of SOD in the control fish, which suggested oxidation in the SOD cysteine. In a study on *O. niloticus* exposed to sublethals concentrations (0.3 and 0.6 mg L^{-1} at 7, 14, and 21 days) of oxyfluorfen, Peixoto *et al*. (2006) observed significantly suppressed SOD activity in treated fish, as verified in the present work.

The activities of GPx and CAT in animals exposed to diuron and hexazinone did not differ from the control. Hence, there was no defense against oxidative stress at the tested concentrations. Similarly, Oruç & Üner (2000) did not detect any difference in the activity of CAT in the liver of *Oreochromis niloticus* exposed to 27 ppm of 2,4-D, indicating the absence of protection against oxidative stress.

The absence of protection against oxidative stress suggests no alteration in ROS production, which is consistent with the lack of lipidic peroxidation, as indicated by the MDA levels. Oruç & Üner (2000) studied fish exposed to 27 ppm of 2,4-D for 24, 48, 72, and 96 h. These authors showed that exposure to the pesticide did not affect the MDA level in the liver of the fish. In the present study, it is probable that no oxidative stress occurred.

With respect to DNA damage, our results did not show the formation of micronuclei in the control or treated fish. Nuclear abnormalities did not differ significantly in exposed *O. niloticus* when compared with the control. However, the comet assay demonstrated significantly more damage in large animals exposed to a mixture of diuron and hexazinone at 125 and 500 ug L^{-1} of diuron + hexazinone than in the control. These data showed that the herbicide is genotoxic but not mutagenic. The absence of a significant increase in damage shown by the comet assay in fish exposed to 250 ug L^{-1} may be due to the low number of replicates. However, an experiment with more individuals would be not viable.

The micronucleus test and the comet assay conducted on various fish species exposed to a number of genotoxicants indicated that the micronucleus test was much less sensitive than the comet assay (Petras *et al*., 1995; Lee & Steinert, 2003), which explains the damage indicated by the comet assay and the lack of damage indicated by the micronucleous test in the present work. Moretti *et al*. (2002) studied Terbutryn (up to 100 or 150 μg mL^{-1}) in vitro using freshly isolated human peripheral blood leukocytes; they did not observe any significant increase in the number of micronuclei, but the comet assay evidenced DNA damage. The comet assay also identified a significant genotoxic effect of diuron on oyster spermatozoa exposed to the herbicide at concentrations higher than 0.05 μg L^{-1} (Akcha *et al*., 2012).

Analysis of HIS did not identify any differences between treated fish and the control, attesting that the herbicide mixture did not alter the liver morphology. Similarly, Mikula *et al*. (2009) studied *Cyprinus carpio* exposed to alachlor (42% w/v) at 2400 μg L^{-1} and found no differences in HIS among the groups.

Analyzing the response of biochemical biomarkers in Nile tilapia, we can see that one of the biotransformation enzymes analyzed, EROD, indicated toxicity, as hypothesized. However, unlike what was expected, the study did not indicate the formation of reactive species, either by analysis of antioxidant enzymes or the formation of lipid peroxidation.

This result may be because the Nile tilapia is a relatively resistant fish. However, Nile tilapia are widespread, including in Brazil, making it a suitable species for use as a biomarker. Moreover, if an animal considered resistant can do show alterations under exposure to a contaminant, we can be sure about this effect.

With respect to damage to the genetic material, as hypothesized, this study showed that the herbicide mixture induces damage at concentrations of 125 and 500 ug/L according to the comet assay. This test did not show a dose-dependent induction, since fish exposed to 250 ug/L did not differ significantly from the control. This is probably due to the low number of replicas. It is possible that with a larger number of replicas, effects might be observed at a concentration of 250 ug/L, but an experiment with a larger number of individuals would not be viable.

Regarding the morphological parameters, the results did not show any changes in the liver morphology, as was expected. This may be have been due to the abovementioned resistance of Nile tilapia.

The present study also compared the number of biomarkers that responded to exposure of small and large fish to the herbicide mixture. In this context, the biomarkers EROD (liver) and SOD (gills) were changed in small fish as compared with the control. Therefore, the herbicide mixture affected the biochemical biomarkers in small fish more than in large ones, indicating that small *O. niloticus* were more responsive to the mixture of diuron and hexazinone than large fish regarding, in terms of biochemical biomarkers, which represent the first line of metabolic response of cells to the contaminants. Biochemical biomarkers varied more widely in small fish probably because of the higher metabolic activity in these individuals as compared with large fish (Kanak *et al*., 2014).

Regarding the genetic biomarkers, the present work showed that the herbicide mixture induced more DNA damage in large fish than in small ones, indicating that large fish are more responsive to genetic biomarkers.

Previous studies by our group (Franco-Bernardes *et al*., 2014) showed similar data for Nile Tilapia exposed to the herbicide tebuthiuron, and so did a study by Kanak *et al*. (2014), in which the authors exposed this species to metal using antioxidant parameters. Both studies revealed greater responsiveness in small fish, corroborating the results of our work.

CONCLUSIONS

In conclusion, this study shows that at the concentrations and exposure times tested, despite the absence of induction of reactive species and changes in morphology of the liver, the induction of EROD and genotoxic damage in Nile tilapia, a relatively resistant fish, indicates that the mixture of the herbicides diuron and tebuthiuron, Velpar K® WG is toxic to aquatic organisms. Moreover, small fish are more susceptible to biochemical biomarkers, which represent a first line of metabolic responses of cells to the contaminants. This shows that small fish are more susceptible to the herbicide mixture. However, large fish are more susceptible to genotoxic damage, and represents a better model for use in genetic damage studies.

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CONFLICT OF INTERESTS

There are no conflicts of interest.

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