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Biochemical and genotoxic effects of a commercial formulation of the herbicide tebuthiuron in *Oreochromis niloticus* of different sizes

M.F. Franco-Bernardes; L.R. Maschio; M.T.V. de Azeredo-Oliveira & E.A. de Almeida

Universidade Estadual Paulista Júlio de Mesquita Filho. Rua Cristóvão Colombo, 2265. 15054-000.
São José do Rio Preto, SP. Brazil.

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Abstract

Pesticides are serious contaminants because they are designed to eliminate pests, but they also affect non-target species. The present study aimed to evaluate the biochemical and genetic effects of the herbicide tebuthiuron in *Oreochromis niloticus* of different sizes. Thus, we analyzed biomarkers in small and large *O. niloticus* specimens exposed to 62.5, 125 and 250 mg L^{-1} of tebuthiuron for 72 hours. Fish exposed to 250 mg L^{-1} had high mortality rates; therefore, the data could not be used. The results showed an increase in EROD activity in fish exposed to 125 mg L^{-1}, but no GST alteration. Antioxidant enzymes GPx and CAT were altered only in the liver of treated fish compared to the control group: CAT decreased in large fish, and GPx increased in small fish. The MDA analysis did not evidence lipid peroxidation. High DNA damage in exposed small fish (not in large fish) was observed using comet assay, but a micronucleus test did not show mutagenicity. Moreover, a comparison between control groups with specimens of different sizes revealed that small fish are more susceptible than large fish to the tebuthiuron effects, since increased comet scores was observed only for smaller fish.

Keywords: aquatic toxicology; biomarkers; ecotoxicology; fish; pesticide.

INTRODUCTION

According to estimates from the Brazilian agency known as UNICA (Sugarcane Industry Union) (Sugarcane Industry Union, 2013), the Center-South region of Brazil will produce 589 million tons of sugarcane during the 2013/2014 harvest. This impressive productivity is supported by the extensive use of several agrochemicals for pest control that, as a consequence, can result in significant negative impacts on the environment. In the case of herbicides alone, more than 30 compounds present in different formulations are used in sugarcane cultivation in Brazil (Martini & Durigan, 2004), such as 2,4-D, acetochlor, ametryne, clomazone, atrazine, diuron, hexazinone, glyphosate and tebuthiuron, though the toxic effects of some of these compounds on non-target organisms are not known (Oruç & Üner, 2000; Cattaneo *et al*., 2008; Xiao *et al*., 2006; Paiva and Takahashi, 1996; Sikkema & Shropshire, 2007; Nwani *et al*., 2011; Akcha *et al*., 2012; Mei *et al*., 2012; Lancetõt *et al*., 2013; Bicalho & Langenbach, 2013). Tebuthiuron (1-(5-tert-Butyl-1,3,4-thiadiazol-2-yl)-1,3-dimethylurea) is a pre- and post-emergent substituted urea herbicide used for the control of broadleaf and woody weeds, grasses and brush through the inhibition of photosystem II. In Brazil, it is widely used on sugarcane crops (Tofoli *et al*., 2009), and it is present in different formulations. In some cases, it is present in combination with other herbicides. Tebuthiuron has a long half-life of about one year in soils (Helling, 2005), especially in high-carbon or low-rainfall environments (Chang & Strizke, 1977), but may have a half-life as low as 20 days under specific conditions (Cerdeira *et al*., 2007). Due to its high water solubility, relatively strong persistence, and low absorption in soil particles, it is expected that tebuthiuron can reach aquatic environments, thus causing negative effects on non-target organisms.

According to data from EPA (2004) and the U.S. National Library of Medicine (2013), tebuthiuron is slightly to practically non-toxic to fish and other aquatic species, with reported 96-hour LC50 values of 87 to 144 mg L^{-1} in rainbow

*Corresponding author: Eduardo Alves de Almeida; e-mail: ealmeida@ibilce.unesp.br

trout, and 87 to 112 mg L^{-1} in bluegill sunfish; however, the sublethal effects of tebuthiuron on fish metabolism are not yet completely known. Due to its intense use in agriculture, especially in sugarcane cultivation in Brazil, any additional information on the sublethal effects of tebuthiuron to fish are important for a better understanding of the risks that this herbicide poses to fish communities that inhabit aquatic environments near agricultural zones. In this context, an assessment of biochemical biomarkers can be useful, since it generally represents the first line of metabolic responses of cells to the contaminants.

The activities of biotransformation enzymes such as cytochrome P450 isoforms or glutathione S-transferases are commonly evaluated as biomarkers in fish, since they can be significantly induced by several contaminants and therefore indicate a response from the organism to the pollutant (Van Der Oost *et al*., 2003). Oxidative stress markers, such as the activities of the antioxidant enzymes superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT), or the levels of oxidative damage to macromolecules, are also useful biomarkers. Several environmental contaminants can lead cells to increase their rates of oxygen reactive species (ROS) generation due to increases in biotransformation reactions, cyclic redox reactions and/or increases in oxygen uptake by mitochondria for energy production. If not counteracted by antioxidant defense systems, ROS can oxidize macromolecules, thus leading to oxidative damages to proteins, lipids and nucleic acids and generating oxidative stress (Oruç & Üner 2000; Van Der Oost *et al*., 2003). Furthermore, the genotoxic effects of environmental pollutants can be monitored using a broad range of assays, such as the micronucleus test and the comet assay (Çavas & Könen, 2007).

Although numerous studies exist in the literature that report oxidative stress and genotoxic effects caused by different photosystem inhibitor herbicides (i.e. diuron, atrazine and paraquat) in fish, there is no such data available regarding the effects of tebuthiuron. Due to both the intense use of tebuthiuron formulations in sugarcane cultivation in Brazil and the lack of studies regarding the sublethal effects of this herbicide in fish, this work considered whether tebuthiuron affects some classic biochemical biomarkers in Nile Tilapia (*Oreochromis niloticus*): the phase I enzyme ethoxyresorufin-O-deetilase (EROD, indicative of CYP1A), the phase II enzyme glutathione S-transferase (GST), the antioxidant enzymes SOD, CAT and GPx, the levels of oxidative damage to lipids (lipid peroxidation, as malondialdehyde levels, MDA), and levels of genotoxic markers (micronucleous tests, comet assay and nuclear abnormality levels).

Because biomarker responses can vary significantly according to the size of the fish (Peixoto & Santos, 2009), a factor that can indicate differences in the organism's susceptibility to the pollutant, we were also interested in evaluating the discrepancies in biomarker responses among fish of the same species (*O. niloticus*) but with significant differences in body length and weight. The sizes of aquatic animals have been shown to play an important role in tissue pollutant loads. Recently Kanak *et al*. (2014) showed that younger tilapias were affected from metal exposure much more than large ones (two fold in length and five fold in weight), as their antioxidant parameters significantly decreased even in controls. Therefore, although EPA reported low toxicity of tebuthiuron to fish, we hypothesize that this herbicide can poses important metabolic alterations in tilapias, which can be helpful to understand its effects in fish, and that smaller fish are more susceptible to the tebuthiuron effects than larger ones.

MATERIAL AND METHODS

Chemicals

All reagents were purchased from Sigma Chemical. In our study, the herbicide was tested as the complex commercial mixture because this is the form in which it is routinely applied in agriculture and introduced into the environment. The commercial preparations of 1-(5-tert-butyl-1,3,4-thiadiazol-2-yl)-1,3-dimethylurea (tebuthiuron) (known as Combine* 500SC) was used. It is composed of the herbicide (50% w / v, or 500 g / L) and inert ingredients (63.3% w / v, or 633g / L).

Animals and experimental design

Adult nile tilapia (*O. niloticus*) of both sexes were obtained from the Aquaculture Center of Universidade Estadual Paulista, Jaboticabal Campus, and transferred to the Laboratory of Animal Ecology, at Universidade Estadual Paulista, Campus of São José do Rio Preto. The specimens were kept in tanks with clean tap water for at least one week before the experiment. A total of 48 animals were used; 24 were small fish (mean length 11.68 ± 1.23 cm, mean weight 47.19 ± 15.5 g) and 24 were large fish (mean length 20.74 ± 1.79 cm, mean weight 284.16 ± 77.95 g).

Fish were placed individually into 48 aquariums of 17 L in eight groups of six fish each (N=6, real replicas). Male and female was not separated. For each fish size, the animals were divided into four groups of six animals: the control group and three groups with different concentrations of the herbicide. The nominal concentrations of tebuthiuron that were used were 62.5, 125 and 250 mg L^{-1} (corresponding to 0.125, 0.25 and 0.500 mL L^{-1} of the commercial Combine *500SC). Due to the lack of studies regarding subcellular effects of tebuthiuron on fish, and due to the reported low toxicity of this herbicide in fish according to the EPA pesticide database, we chose to test concentrations near the LC50 values reported for rainbow trout (143 mg L^{-1}) and bluegill sunfish (106 mg L^{-1}). Because we used a formulation of tebuthiuron instead of the pure standard, we also considered the LC50 values reported by EPA for formulated tebuthiuron products (20 to 80%) for fathead minnow (>180 mg L^{-1}). We also considered the fact that the fish in our study were exposed to the formulated tebuthiuron for only 72 h. According to the EPA pesticide

database, chronic exposure (45 days) of rainbow trout to 52 mg L^{-1} of tebuthiuron caused no mortality, but only effects on growth. This further justifies the elevated concentrations used in our study.

The aquariums were kept under constant aeration and temperature. No food was supplied to the fish during the experiment, and the animals were exposed to the herbicide for 72h. After this period, they were anesthetized with benzocaine for removal of the liver, gills and blood. The liver and gills were frozen at -80° C for subsequent biochemical analysis. The blood was used for micronucleus tests and comet assays on the same day.

Preparation of samples for Biochemical analysis

For the analysis of enzymes EROD, GST, SOD, GPx and CAT, tissues (liver and gills) were homogenized (1:4, w:v) in Tris-HCl 20 mM, pH 7.5 containing 0.5M of sucrose, 1mM of etylenediamine tetraacetic acid (EDTA), 1mM of dithiothreitol (DTT), and 0.15M of KCl containing 1 mM of the protease inhibitor phenylmethylsulfonyl fluoride (PMSF). The homogenate was centrifuged at 10,000g for 30 min at 4ºC, and the resulting supernatants were centrifuged at 50,000g for 60 min at 4ºC. The supernatant obtained after this second centrifugation was used for the analyses of GST, SOD, GPx and CAT, and the pellet of the liver samples was re-suspended in 100 ml of 0.1-M Tris-HCl, pH 7.5, containing 1mM of EDTA, 1mM of DTT, and 0.1 M of KCl and used to analyze EROD activity. The prepared samples were aliquoted for later analysis. For the lipid peroxidation analysis, tissues (liver and gills) were homogenized (1:3 weight: volume) in 0.1M of Tris HCl, pH8.0. Prepared samples were analyzed on the same day.

BIOCHEMICAL ANALYSIS

EROD, GST, SOD, GPx and CAT

The EROD assay measures the 0-dealkylation, mediated by CYP1A, of non-fluorescent substrate 7-ethoxy-resorufin in resorufin, a fluorescent product detected by fluorimeter (λexcit = 537 nm λemiss = 583 nm) (Sarasquete and Segner, 2000). The assay mixture contained 80 mM of potassium phosphate buffer (pH 7.4), 335 lM 7-ethoxyresorufin, 20 mM of NADPH, and microsomal liver extract (prepared sample). The reaction was observed for 3 min at 30 ºC. EROD activity (pmol min^{-1} mg^{-1} of protein) was calculated based on a previously prepared resorufin standard curve.

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

The method for analyzing SOD uses a system of superoxide (xanthine / xanthine oxidase) generation coupled with cytochrome c reduction by the superoxide anion radical, which causes an increase in absorbance at 550 nm at 25 ° C. The addition of the sample containing SOD promotes an inhibition of cytochrome c reduction, because the enzyme competes with cytochrome c by superoxide (Mccord & Fridovich, 1969).

GPx activity was evaluated according to Sies *et al*. (1979). In the analysis, the consumption of NADPH was monitored at 340 nm. NADPH is used by glutathione reductase (GR) for the reduction of glutathione, which had been previously oxidized by GPx in the conversion of t-butyl hydroperoxide in its corresponding alcohol.

CAT activity was measured following the method reported by Beutler (1975), in which the rate of decomposition of hydrogen peroxide by the enzyme is quantified through the decrease in absorbance at 240 nm at 30 °C.

Protein levels were measured following the method of Bradford (1976). In a microtube, the Bradford reagent and the sample were combined. After 40 min in the dark, the samples were measured in a spectrophotometer at 595 nm. The protein concentration was calculated using the calibration curve prepared from bovine serum albumin (BSA) and the Bradford reagent.

Lipid peroxidation

Lipid peroxidation levels were determined by measuring the product formed from the combination of malondialdehyde, and thiobarbituric acid (TBA) through High Performance Liquid Chromatography (HPLC) and UV/Vis detection. After homogenizing the sample, the TBA was dissolved in 0.2 M of HCl and the solution was added to the sample. The reaction mixture was then heated at 90 ^{o}C for 60 min. The colored derivative was extracted with butanol and quantified using HPLC at 532 nm, in terms of a malondialdehyde (MDA) standard calibration curve that had been previously prepared using the same procedure used for the samples. The HPLC system consisted of an ESA584 pump and an ESA526 UV/Vis detector. The column used was an ACE 5 C18 (250_x 4.6 mm, 5 μm). Chromatogram monitoring and peak identification and quantification were performed using the EZChrom Elite software (Agilent Technologies). The mobile phase was 0.05 M of KH_2PO_4, pH 7.0, with 40% methanol, and was pumped at an isocratic flow of 1 mL min^{-1}.

GENOTOXIC EFFECTS

Micronucleus test

In the micronucleus test (Al Sabti, 1986; Al Sabti and Metcalfe, 1995), a cardiac puncture was performed to remove 3 cc of blood from each animal. The smear technique was used to prepare three slides for each fish. The material was fixed in absolute methanol and stained through a Feulgen reaction. A total of 1000 erythrocytes were analyzed in each slide, totaling

3000 erythrocytes per fish. This analysis was performed with an optical microscope under 100 x magnification, and the frequency of micronuclei in treated animals was compared to the negative control. The frequencies of nuclear abnormalities (notched, lobed, broken eggs and bebbled, as described by Carrasco 1990), was also determined. No positive control was used. Since all cells in the field of vision are counted, the total number of cells analyzed per animal can vary. Thus, the total number of cells counted was transformed into percentage values. Therefore, the frequency is measured as a percentage.

Comet assay

The comet assay was performed followed the method described by Singh *et al*. (1988). Blood samples were each diluted in 1000 µl of saline solution. Slides were made with 10 uL of this cell suspension and 120 µl of low melting point agarose (0.5%) at 37 °C. The slides remained in a lysis solution (1 mL of Triton X-100, 10 mL of DMSO and 89 mL of lysing solution stock, pH 10.0 - stock solution: 2.5 M of NaCl, 100 mM of EDTA100, 10 mM to 1 L of Tris) in the refrigerator for one hour. After their time in the lysis solution, the slides were placed inside a horizontal electrophoresis system for 20 minutes at 25 V, 300 mA. The slides were neutralized with 0.4M of Tris (pH 7.5), for 15 minutes and fixed in ethanol for 10 minutes. Cells with no DNA damage migrate homogeneously, while cells with damaged DNA form fragments of different sizes, and the smaller ones migrate faster during electrophoresis, thus forming the tail of a comet.

From each fish, two slides were prepared. On each slide, 50 nucleoides were analyzed. The slides were stained with ethidium bromide (0.002 mg mL^{-1}). The analysis was performed with a fluorescence microscope, filter B - 34 (excitation: λ = 420-490 nm, barrier λ = 520 nm) under 40 x magnification. The comets were classified according to the comet length using the visual parameters proposed by Kobayashi *et al*. (1995): class 0 (no damage), class 1 (slight damage); class 2 (moderate damage), class 3 (major damage). The injury score was calculated by multiplying the total number of cells in each class by class value (0 -3). No positive control was used.

Statistical analysis

To verify the effect of the herbicide on the animals, the treatments were compared to the control. The small fish group was analyzed separately from the large fish group. Tests for normality (Shapiro–Wilk) and homogeneity of variances (Levene) were applied. For the parametric data, the one-way ANOVA was used, followed by the Tukey test. For non-parametric data, the Kruskal–Wallis test was used, followed by multiple comparisons of mean ranks.

To see whether the size of the fish influences the effect of the biomarkers studied, the differences between small and large fish and their respective control groups were calculated. For the parametric data, the Student T-test was used, and for non-parametric data, the Mann-Whitney test was used. Significant differences were accepted only when $p < 0.05$. Analyses were performed with the Statistica 7.0 software.

RESULTS

Mortality

During the exposure period, a high mortality rate of the animals was observed in aquariums containing 250 mg L^{-1} of tebuthiuron. In aquariums containing small fish, four of them died, leaving only two animals to be analyzed. In aquariums containing large fish, two of them died, leaving four for analysis. Due to this mortality rate, statistical analyses were infeasible with the group of fish exposed to the high concentration of the herbicide, and for this reason, data for the fish exposed to 250 mg L^{-1} of the herbicide were not considered any further. None of the fish died in the other treatments.

Biotransformation enzymes

The results regarding EROD and GST activities are shown in Figure 1. EROD activity was measured only in the liver, due to a lack of gill samples available for measurement. The activity of the enzyme increased in small and large fish exposed to 125 mg L^{-1} when compared to their respective controls. The analysis did not show significant difference between animals of different sizes. With respect to GST activity, no differences were observed between the treated groups and the respective control groups in neither the liver nor the gills from fish of either size. When the GST activity of fish of different sizes was compared, it was noted that GST activity in the liver was higher in large fish than in small fish, but no such difference was observed in the gills.

Oxidative stress parameters

Oxidative stress parameters (SOD, CAT, and GPx activities, and MDA levels) measured in fish from the exposure experiment are shown in Figure 2. The hepatic SOD activity showed no significant differences in small and large fish treated with tebuthiuron in comparison to their respective control groups. The analysis also showed no significant differences between *O. niloticus* of different sizes. The SOD activity in the gills did not differ significantly in exposed groups when compared to their controls; however, the enzyme activity was higher in large fish than in small fish.

GPx activity was significantly higher in the liver of small fish exposed to 125 mg L^{-1} than in the control group, though no significant difference was found in large fish treated with the herbicide when compared to their control group. No difference was observed in GPx activity among animals with different sizes. In the gills, the GPx activity did not differ between treated and control fish in either large or small fish groups, nor when large and small fish were compared.

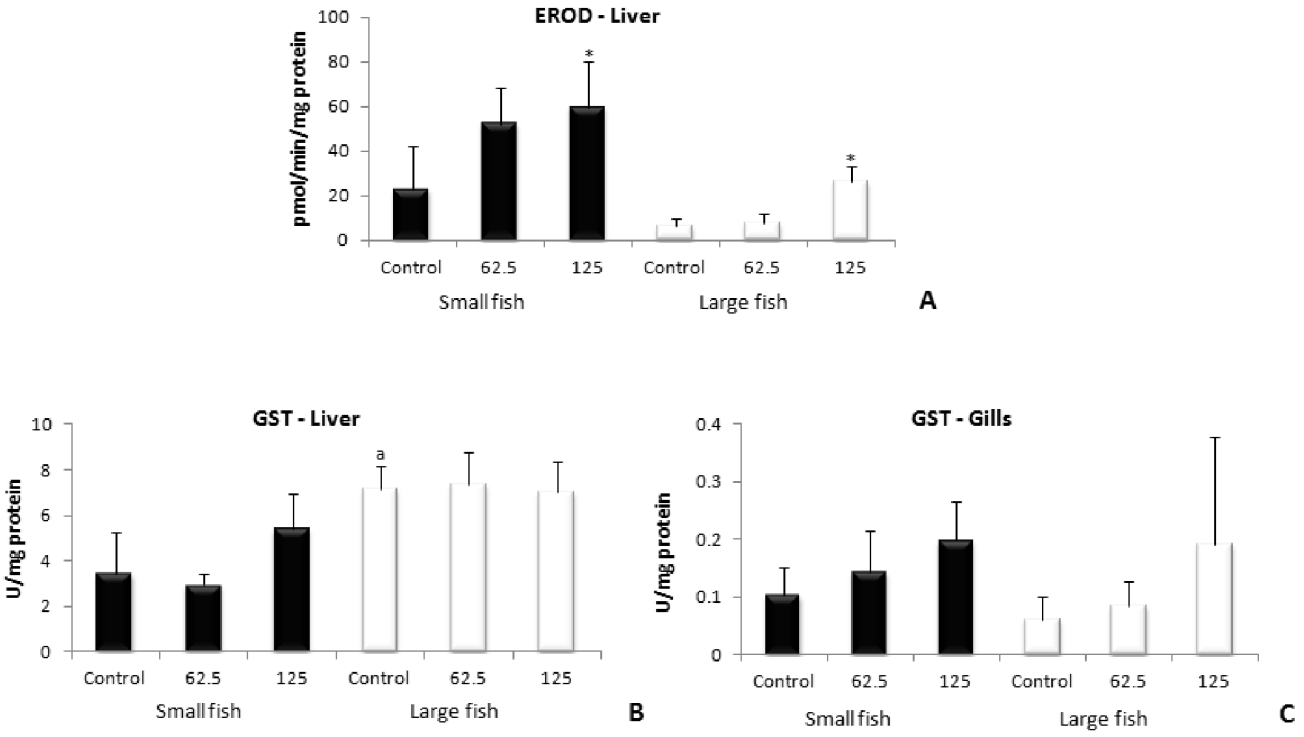

Figure 1 - Activity of biotransformation enzymes EROD in liver (A) and GST in liver (B) and gills (C) of small and large *O. niloticus* exposed to tebuthiuron for 72 h at concentrations of 62.5 and 125 mg L^{-1}. *: Significant difference compared to the respective control group; **a**: Significant difference compared to the smaller control group.

In the liver, the CAT activity did not differ significantly in small *O. niloticus* exposed to herbicide when compared to the control. However, large fish treated with 62.5 mg L^{-1} had lower CAT activity than the control group. There was no significant difference between the control groups of large or small animals. The CAT activity of the gills did not differ significantly in exposed groups compared with their controls, nor was there any significant difference between fish of different sizes.

No significant difference was observed in MDA levels in livers of fish exposed to the herbicide compared to the control groups. However, large *O. niloticus* showed a higher level of lipid peroxidation than small specimens. The MDA levels in the gills remained unchanged when treated and control animals were compared. There was also no significant difference between the lipid peroxidation levels in animals of different sizes.

Genotoxic markers

The micronucleous frequency in erythrocytes from *O. niloticus* specimens of both sizes exposed to tebuthiuron did not differ from their respective controls (Fig. 3). On the other hand, large fish had lower micronucleous frequency than small fish. Some nuclear abnormalities were also observed, being the notched the most predominant, but no differences were observed between treated and control groups. The nuclear abnormalities did not differ between small and large fish, either. The comet assay showed that small fish exposed to 125 mg L^{-1} of the herbicide presented a higher level of damaged erythrocytes than the control group. Furthermore, the analysis revealed that large fish had a higher comet score than small fish.

DISCUSSION

Although studies regarding the biological effects of pesticides have increased substantially over the last few years, there are no studies on the effects of tebuthiuron on biochemical biomarker in fish. For this reason, the present work brings important information about the susceptibility and responses of fish to a commercial formulation of this herbicide, which has been extensively used in sugarcane agriculture in Brazil. EROD activity was higher in the group of large fish exposed to 125 mg L^{-1} of the tebuthiuron formulation when compared to the controls. Numerous field studies have demonstrated a significant increase in hepatic CYP1A activity in many species of fish from polluted environments, though no studies exist regarding the effects of tebuthiuron on this enzyme. Nevertheless, Schoket & Vincze (1990) observed a significant increase in EROD activity in rats intragastrically treated with diuron (0.73, 1.62, 2.43 and 3.65 mmol/kg) and chlorotoluron (2.35, 5.22, 7.83 and 11.74 mmol/Kg), both phenylurea herbicides, for 3 days. As demonstrated in this study, the herbicide tebuthiuron was also able to induce EROD activity in tilapia, an increase that was not followed by the phase II enzyme GST. This result suggests that GST is not involved in the metabolism of tebuthiuron. Morton & Hoffman (1976) reported that the main tebuthiuron metabolite excreted by

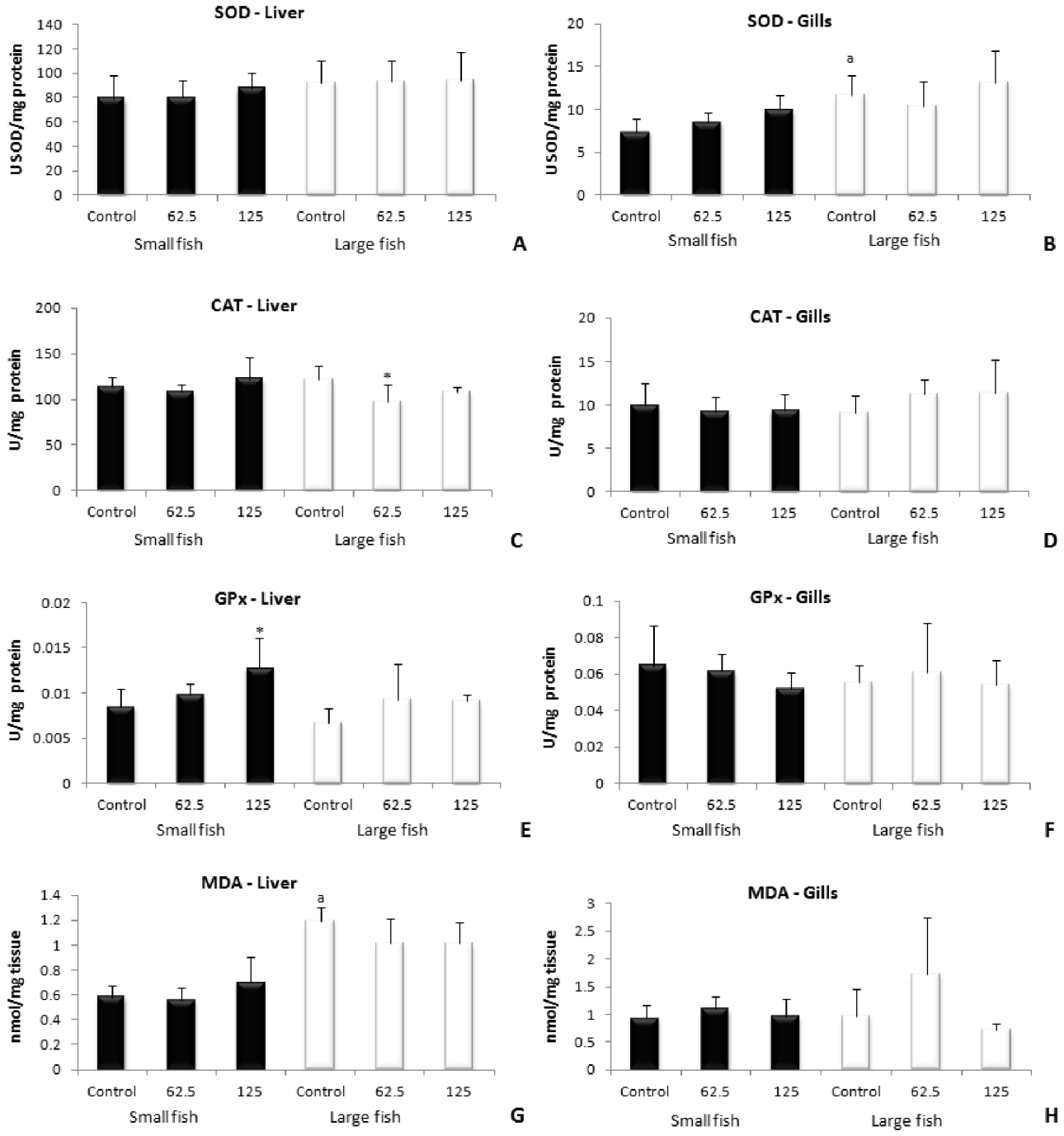

Figure 2 - Oxidative stress parameters: SOD in liver (A) and gills (B), CAT in liver (C) and gills (D), GPx in liver (E) and gills (F) and MDA in liver (A) and gills (B) of small and large *O. niloticus* exposed to tebuthiuronfor 72 h at concentrations of 62.5 and 125 mg L^{-1}. *: Significant difference compared to the respective control group; **a**: Significant difference compared to the smaller control group.

fish is formed by N-demethylation of the substituted urea side chain; but in our work, no conjugated compounds were found, which is consistent with EROD induction and the lack of GST effects on tilapias. Interestingly, neither EROD nor GST activities varied according to the size of the fish, and both large and small fish presented similar responses to the pesticide exposure in terms of these enzymes.

SOD activity did not vary in treated fish of either size compared to their controls, in the liver nor in the gills. This result indicates that elevated levels of anion radical are not being formed because of tebuthiuron exposure, which is consistent with the lack of increase in MDA levels. Nevertheless, the GPx activity was higher in the livers of small *O. niloticus* specimens exposed to 125 mg L^{-1} of tebuthiuron, which may indicate the production of reactive intermediates as a result of tebuthiuron exposure. Even though a small but significant decrease in CAT activity in the liver of large fish exposed to the lower concentration of the herbicide formulation could increase the susceptibility of fish to oxidative stress, the increase in GPx activity can also be considered to be a protective

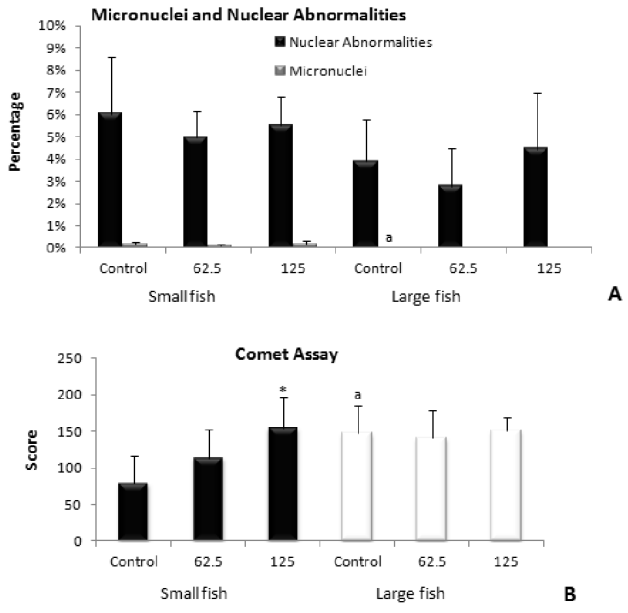

Figure 3 - Genotoxic markers: Micronuclei, Nuclear Abnormalities (A) and Comet Assay (B) in erythrocytes of and large *O. niloticus* exposed to tebuthiuron for 72 h at concentrations of 62.5 and 125 mg L^{-1}. *: Significant difference compared to the respective control group; **a**: Significant difference compared to the smaller control group.

response of hepatocytes against ROS. This response would explain the lack of increases in lipid peroxidation levels. No studies were found regarding MDA levels in fish exposed to photosynthesis inhibitor herbicides, but an investigation with male *Wistar* rats exposed to atrazine (300 mg/kg) through gavage for 7, 14 and 21 days showed a significant increase in hepatic lipid peroxidation (Singh *et al*., 2011). It is likely that the concentrations of herbicide used in the present study were not enough to generate oxidative stress. Nevertheless, it should be mentioned that the concentrations used herein were very high, and were close to both the concentrations that are recommended for agricultural use and to the LC50 values reported for other fish species. In any case, these concentrations are not expected to be found in natural aquatic environments. According to data from the EPA , tebuthiuron was found in several surface water samples from the U.S. in the '80s and '90s at levels of 1 to 200 ng L^{-1} (Stavola, 2013) – much lower than the concentrations used in the present study. Therefore, considering the fact that there was no evidence of oxidative stress in fish in the present study, we can suppose that tebuthiuron is not able to generate oxidative stress in fish at environmentally relevant concentrations, after the duration of exposure used in the present work.

With respect to DNA damage, our results also showed no difference in the frequency of micronuclei and nuclear abnormalities in treated animals compared to the control, which indicates that tebuthiuron poses no potential genotoxic effects on fish, as previously reported by the EPA. However, small fish exposed to 125 mg L^{-1} had a higher comet score than the control. This data shows that, in the concentrations tested, tebuthiuron may cause DNA damage, though likely not at realistic environmental concentrations. Again, more studies are necessary to verify the genotoxic effects after longer exposure periods. Indeed, the presence of increased comet scores without increases in micronucleous levels suggests that the herbicide caused genotoxicity, but not mutagenicity. In a study with atrazine in erythrocytes of Channa punctatus, Nwani *et al*. (2011) observed an increase in micronuclei and an increase in DNA damage in treated animals through comet assay, results which are consistent with our findings.

Comparisons were also made between biomarker responses in fish of different sizes. In general, larger tilapias presented higher GST activity in the liver and higher SOD activity in the gills, results which are consistent with higher levels of MDA. Similarly, DNA damage levels measured by the comet assay were higher in larger fish. Smaller fish, on the other hand, presented higher micronuclei frequencies than larger fish. This data suggests that larger fish may be more susceptible to lipid peroxidation and DNA damage, and they therefore possess higher activities of protective enzymes, such as SOD and GST. Higher GST activity and EROD induction may also indicate that larger fish present a better biotransformation response to tebuthiuron compared to smaller fish. In addition, the higher micronucleous frequency in small fish compared to large fish can indicates that larger fish also present better DNA repair mechanisms than smaller fish. All of this data indicates that small fish could be more susceptible to tebuthiuron exposure than large fish, as hypothesized. Small fish were also more responsive than large fish in terms of the number of biomarkers that responded to the herbicide exposure. EROD increased in both small and large fish exposed to the 125 mg L^{-1} concentration. In fact, in the case of large fish, this was the only alteration observed among the biomarkers tested. In small fish, in addition to EROD induction, a decrease in CAT activity, an increase in GPx activity, and an increase in comet score were also observed. Larger fish, with larger body mass and larger organs, such as the liver, have more bioaccumulation and biotransformation capacity compared to smaller fish, which can explains these differences. Nevertheless, it is also known that the metabolic activity of a young individual is normally higher than the older individual (Kanak *et al*., 2014), so the most frequent variations observed in biomarkers from smaller fish compared to large fish could be also due to a better response capacity in smaller fish, an hypothesis that remains to be clarified. Indeed, it should be considered that both experiments with small and large fish were done in a aquariums with the same water volume, so both small and large fish received the same amount of the herbicides that, proportionally to the fish size, implies that there was more thebutiuron available in water to small fish than to larger ones, which can also account for the increased biomarker responses in smaller fish.

CONCLUSIONS

In conclusion, this study shows that, at the concentrations and exposure duration tested, tebuthiuron can increase the fase I biotransformation enzymes in fish of both sizes, and

can increase the production of reactive intermediates and generate genotoxicity in small fish. Larger fish seem to be less susceptible to the effects of tebuthiuron compared to smaller fish, which were more responsive, a result that agrees with a study recently published in this same journal by Kanak *et al*. (2014), in which small tilapias were more susceptible to metal effects compared to larger ones.

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