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# Acute toxicity of mixture of sugarcane herbicides to tilapia fingerlings

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## Abstract

Sugarcane cultivation is the most important agricultural activity in the State of São Paulo, which is responsible for over half of Brazilian production of the crop. Eutrophication and contamination of hydric resources caused by agricultural chemicals are major problems related to this crop. In the case of herbicides used to control weeds, aquatic organisms can be exposed to more than one toxic agent, and combinations of such pollutants can have different effects on biological systems. The aim of this work was to evaluate the toxic effects of mixtures of herbicides widely used in sugarcane cultivation, namely tebuthiuron (TBUT), ametryn (AMT), and Velpar K (a commercial mixture of diuron (DIU) and hexazinone (HZN)), to tilapia (*Oreochromis niloticus*) fingerling. The herbicides showed low to moderate toxicity and AMT was notably more toxic then TBUT and DIU+HZN. The mixtures were found to be moderately toxic to the tilapia fingerling (LC$_{50}$;96h were: TBUT+AMT: 10.76 mg L$^{-1}$; (DIU+HZN)+TBUT: 43.09 mg L$^{-1}$; and (DIU+HZN)+TBUT+AMT: 11.90 mg L$^{-1}$), and a slight antagonism was observed between the components tested. These findings could contribute to the establishment of maximum permissible levels for the herbicides in Brazilian continental water bodies.

*Keywords:* Ametryn, diuron, hexazinone, *Oreochromis niloticus*, tebuthiuron.

### INTRODUCTION

Brazil is the major global sugarcane producer and the State of São Paulo has been the most important representative of this sector (Lourenzani & Caldas, 2014). Among the environmental impacts generated from the intensive sugarcane production, Corbi *et al*. (2006) cited the application of herbicides, pesticides and fertilizers during the different stages of cultivation, combined with the devastation of riparian forests, which led to the contamination of soil, surface water and groundwater in areas adjacent to the plantations.

The impacts on water quality due to the global use of herbicides have been widely discussed. Armas *et al*. (2007) detected the presence of triazine herbicides (ametryn, atrazine, and simazine), hexazinone, glyphosate, and clomazone in surface waters and in the sediments of the subbasin of the Corumbataí River (São Paulo State, Brazil), in a region with extensive sugarcane cultivation.

In the environment, organisms are often exposed simultaneously to a range of toxic agents, and different pollutants can act synergistically in biological systems (Tallarida, 2001). The toxicity of mixtures is not always the sum of the toxic activities of the individual compounds, due to the effects of synergism and antagonism among the components of a complex mixture (Nelson & Kursar, 1999).

Franco-Bernardes *et al.* (2014) evaluated the biochemical and genetic effects of the herbicide tebuthiuron in tilapia (*Oreochromis niloticus*) and concluded that this herbicide can increase the fase I biotransformation enzymes, the production of reactive intermediates and generate genotoxicity in fish. The herbicide diuron revealed a genotoxic potential to *Danio rerio*, another tropical species, at realistic environmental concentrations (4.3 nM or 1.00233 μg L$^{-1}$) (Bony *et al*., 2010). In relation to ametryn, Tesolin *et al*. (2014) evaluated the toxicity of the commercial formulation Gesapax 500® (500g L$^{-1}$ of active

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ingredient) to *D. rerio* and concluded that the herbicide was moderately toxic.

Sugarcane producers use the herbicide tebuthiuron (TBUT) in mixtures with ametryn (AMT) as well as with the commercial product Velpar K WG®, which is a mixture of diuron (DIU) and hexazinone (HZN). No $LC_{50}$;96h values have been established for the mixture of these herbicides and the Brazilian legislation (Resolution CONAMA nº 357/2005) did not establish their maximum permissible levels, according to the use of the water. Data are therefore needed in order to be able to estimate the environmental risks caused by the use of the compounds individually or in the form of mixtures.

The aim of this work was to evaluate the toxic effects of the herbicides TBUT, AMT, and Velpar K (DIU+HZN), used individually and in mixtures, in tilapia (*O. niloticus*), an economically important species due to its widespread use in commercial fisheries (Santos *et al.*, 2007). This fish species is extensively used in ecotoxicological assays due to its robustness, sexual precocity, and easy adaptation to laboratory conditions (Jordaan *et al.*, 2013).

## MATERIALS AND METHODS

The herbicide formulations tested individually and in combination were TBUT (Combine 500 SC; concentrated suspension; 500 g $L^{-1}$), AMT (Gesapax 500, concentrated suspension; 500 g $L^{-1}$), and Velpar K, which consists of dispersible granules containing DIU (468 g $kg^{-1}$) and HZN (132 g $kg^{-1}$). All these formulations were obtained from commercial outlets.

### Test organism and exposure to the herbicides

The fish used were tilapia fingerling (*O. niloticus*) with average total weight of $3.24 \pm 1.58$ g and average standard length of $4.50 \pm 0.82$ cm, obtained from the Brumado fishery in Mogi Mirim (São Paulo State). The fish were acclimatized for seven days in a 1000 L tank containing dechlorinated water, at pH 7.5 and temperature of 26 ºC, with constant aeration (6 mg $L^{-1}$ dissolved oxygen). The animals were fed twice daily with commercial fish food.

On the day prior to the assays, the fry were transferred to 10 L aquaria, using a stocking rate of 2.0 g $L^{-1}$. Static conditions were employed, and the fish were submitted to fasting for the period from 24 hours prior to the exposure and during the assays. The experimental systems were kept in a climate-controlled room, using a photoperiod of 16h: 8h (light: dark) and temperature of $26 \pm 2$ ºC, with constant aeration.

The water used was obtained from ground water and had the following characteristics: pH = 7.7; dissolved oxygen = 6.2 mg $L^{-1}$, electrical conductivity = 3.8 mS $cm^{-1}$; total hardness = 53.6 mg $L^{-1}$ $CaCO_3$. The water quality parameters were measured at the end of the assays using WTW Models 330i and 3210 CellOx 325 probes.

The tests were based on the OECD Guideline 203 acute toxicity protocol (OECD, 1992). The proportions of each active ingredient in the mixtures tested were in accordance with those used in sugarcane plantations: TBUT+AMT: 1.2+2.0 kg a.i. $ha^{-1}$; (DIU+HZN)+TBUT: 2.0+1.2 kg a.i. $ha^{-1}$; (DIU+HZN)+TBUT+AMT: 2.0+1.2+1.5 kg a.i. $ha^{-1}$.

Preliminary tests were used to define the nominal concentrations employed in the subsequent assays. The concentrations of the test solutions used in the assays were: AMT: 0, 1.0, 1.8, 3.2, 5.6, and 10.0 mg $L^{-1}$; TBUT: 0, 100.0, 126.0, 158.0, 199.0, and 250.0 mg $L^{-1}$; (DIU+HZN): 0, 3.0, 5.0, 9.0, 17.0, and 30.0 mg $L^{-1}$. In the case of the mixtures, the concentrations used were: TBUT+AMT: 0, 3.0, 5.0, 9.0, 16.0, and 30.0 mg $L^{-1}$; (DIU+HZN)+TBUT: 0, 22.0, 40.0, 74.0, 132.0, and 240.0 mg $L^{-1}$; (DIU+HZN)+TBUT+AMT: 0, 3.0, 5.0, 9.0, 17.0, and 30.0 mg $L^{-1}$. The nominal concentrations of the active ingredients in the formulations followed a geometrical series with concentrations increasing by factors <1.8. The tests were performed in triplicate for each concentration tested, using five fish from each aquarium (N=30 for each replicate).

Although OECD protocols recommend that tests with substances that have $LC_{50}$;96h $\geq$ 100 mg $L^{-1}$ should not be performed believing it is a waste of material (sacrificing animals and unnecessary waste generation). However, we decided to test those herbicides given its wide use in sugarcane crops, as well as because there is no limit stablished for such herbicides in the Brazilian Legislation (CONAMA 357/05), and we believe it is important to establish its toxicity in single and mixture exposures.

At each 24 hours of exposure, the numbers of dead organisms were recorded, enabling to determine the concentrations that affected 50% of the population exposed during this period ($LC_{50}$), in terms of the concentrations of the active agents; as well as to compare how the toxicity of each herbicide formulation changes throughout the 96h of exposure. The experimental procedure was authorized by the Ethics Commission for Animal Experimentation of Embrapa Environment (Record no 002/2012).

## DATA ANALYSIS

### Statistical analysis

The mortality results were analyzed using the Probit Regression analysis (Statpoint Technologies, 2014) which enabled the determination of the $LC_{50}$ values and the corresponding 95% confidence intervals. The $LC_{50}$;96h values were considered significantly different when there was no overlap of the 95% confidence intervals (Czuczwar *et al.*, 2001).

### Analysis of combined effects

The combined effects of the herbicide mixtures were evaluated based on the $LC_{50}$;96h values for the individual and mixed formulations. This was achieved using the methodology of Marking (1985) to calculate the sum of the contributions,

followed by application of the additive index (AI), with positive values (+) indicating synergism and negative values (-) reflecting antagonism. Values equivalent to 0.0 (without any positive or negative sign) represented additivity. The degree of magnification of the effect was determined by means of the procedure described earlier (Marking, 1985).

### RESULTS AND DISCUSSION

In single tests, the average values of the water quality parameters were: pH 7.721 ± 0.065; dissolved oxygen 6.214 ± 0.168 mg $L^{-1}$; electrical conductivity 3.800 ± 0.124 μS $cm^{-1}$. In mixture tests, the average values of the water quality parameters were: pH 7.728 ± 0.058; dissolved oxygen 4.470 ± 0.087 mg $L^{-1}$; electrical conductivity 3.799 ± 0.076 μS $cm^{-1}$. No differences between the treatments were observed.

In Table 1 we can see the decrease in $LC_{50}$ values during the experiment, which clearly shows the importance of the time factor for the manifestation of toxic effects. This phenomenon is observed more markedly for mixing (DIU+HZN)+TBUT where there was a 5.7 fold increase in toxicity in the period of 24 h to 96 h of exposure.

The acute toxicity $LC_{50}$;96h values (with 95% confidence intervals) determined individually for each herbicide are presented in Table 2. According to the classification of Zucker (1985), AMT showed moderately toxicity to tilapia, while DIU+HZN was slightly toxic and TBUT was practically non-toxic.

Tesolin *et al*. (2014) working with zebrafish (*Danio rerio*) embryos exposed to the herbicides AMT and (DIU+HZN) determined the $LC_{50}$;96h values as, 53.23 ± 3.25 mg $L^{-1}$ and 37.45 ± 1.741 mg $L^{-1}$, respectively. In the present work we

observed that tilapia was more sensitive than *D. rerio* in which both herbicides were slightly toxic. For some toxicants, the chorion of the egg may act as a barrier of embryo protection (Oliveira *et al.*, 2009), which may explain this lower sensitivity of zebrafish embryos in relation to tilapia fingerlings. Botelho *et al.* (2009) evaluated the toxicity of atrazine, another member of the triazine herbicide family and one of the most world widely used herbicides, for tilapia and determined a $LC_{50}$;96h of 5.02 mg $L^{-1}$, similar to the value found in this study for AMT.

Franco-Bernardes *et al*. (2014) couldn't establish the $LC_{50}$;96h of TBUT in their work, as no mortality was observed in concentrations of 62.5 and 125 mg $L^{-1}$ of Combine 500SC; and tilapia exposed to 250 mg $L^{-1}$ had high mortality rates in 72 hours of exposition. The results obtained in the present work were therefore in agreement with the literature data, indicating low acute toxicity of this compound to fish.

Herbicides, as a variety of environmental contaminants, can induce apoptosis, necrosis, or autophagy in vertebrates; and this is dependent of both, cell type and exposure dose. It is apparent that multiple cell death programs can be activated during toxicity. It seems more likely that several death-executing routines may be activated concomitantly within injured cells and that one or the other becomes predominant, depending on the energy requirement, signaling molecules or the intensity of the injury. This latter is associated with the capacity of herbicides, by itself or their metabolites or the secondary products of oxidative stress, interacting with biomolecules such proteins and DNA (Severi-Aguiar *et al.*, 2014). In this context, antioxidant enzymes such as catalase, superoxide dismutase, glutathione peroxidase and glutathione-S-transferase have been studied as biomarkers of oxidative

**Table 1.** $LC_{50}$;96h (mg $L^{-1}$) (95% C.I.*) of sugarcane herbicides to tilapia fingerling (*Oreochromis niloticus*) at different exposure times.

| | Herbicides | Exposure time (hours) | | | |
| --- | --- | --- | --- | --- | --- |
| | | 24 | 48 | 72 | 96 |
| Single | AMT | 7.63 (6.27 – 9.57) | 4.92 (4.07 – 6.15) | 4.41 (3.63 – 5.26) | 4.41 (3.63–5.26) |
| | TBUT | 260.51 (-) | 255.44 (-) | 251.65 (-) | 245.51 (205.59 –256.10) |
| | (DIU+HZN) | - | 46.37 (-) | 21.38 (17.53 – 26.53) | 18.77 (15.27 – 23.97) |
| Mixture | TBUT+AMT | 18.19 (-) | 12.52 (10.27 – 15.82) | 11.46 (9.43 – 14.17) | 10.76 (8.54–14.28) |
| | (DIU+HZN)+TBUT | 245.38 (-) | 95.39 (76.78 – 123.98) | 47.03 (-) | 43.09 (33.85–56.61) |
| | (DIU+HZN)+TBUT+AMT | 28.24 (22.61 – 37.29) | 15.01 (13.40 – 16.62) | 13.28 (11.50 – 14.69) | 11.90 (9.25–15.56) |

* 95% of Confidence Interval; (-) means it was not possible to calculate.

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stress caused by herbicides in fish (Stara *et al.*, 2012; Franco-Bernardes *et al.*, 2015) and other organisms present in the aquatic environment (Mofeed & Mosleh, 2013). Commonly, it is observed an increase of the enzymes activities in order to neutralize the reactive oxygen species effects (Moraes *et al.*, 2007). However, due to the oxidative damage, the reduction in the enzyme activity is also possible (Crestani *et al.*, 2007), leading to the suppression of antioxidant defenses and loss of compensatory mechanisms.

The fish exposed to AMT concentrations $\geq 5.6$ mg L$^{-1}$ showed increased opercular beating and expansion in the abdominal region, which caused a loss of equilibrium so that the animals remained floating at the surface of the water. The animals exposed to the highest concentration of TBUT (250 mg L$^{-1}$) presented the same behavioral pattern, and in the case of the DIU+HZN mixture, the fish exposed to concentrations $\geq 9$ mg L$^{-1}$ showed lethargy, loss of equilibrium, and in some cases paralysis (when only opercular beating occurred).

Behavioral changes in fish exposed to herbicides have not generally been reported in the literature, although some studies have described the effects of triazine compounds. We observed effects similar to those found in the work of Velisek *et al.* (2008), with loss of movement coordination in rainbow trout exposed to metribuzin (a triazinone herbicide). The same behavior was described for the species *Chrysichthyes auratus* and *Carassius auratus* exposed to atrazine (Saglio & Trijasse, 1998).

The acute toxicity values obtained for the herbicide mixtures are also shown in Table 2, together with the effects of the combined compounds. It can be seen that there were small effects that were lower than additive (x<0, indicating antagonism) for the three mixtures studied. The AI and magnification values indicated that the mixtures did not result in major reductions in toxicity, relative to the individual compounds.

The combined effects of herbicides and other agrochemicals have been discussed previously in terms of the toxicity to aquatic organisms. According to Xing *et al.* (2012), atrazine had almost no effect on the toxicity of the insecticide chlorpyrifos to common carp (LC$_{50}$;96h = 0.58 mg L$^{-1}$) when a 1:1 mixture was used (LC$_{50}$;96h = 0.56 mg L$^{-1}$). In contrast, Wacksman *et al.* (2006) reported that atrazine could increase the toxicity of chlorpyrifos by between two-fold and seven-fold. Nonetheless, in these studies, no such effects were reported for bluegill fish, while synergism between the two compounds was observed for toxicity to the amphibian *Xenopus laevis*. It was hypothesized that interspecies variations in sensitivity could be explained by the differences in the physiological systems of each species, which affected the biotransformation and metabolism of the xenobiotics.

Overall, the LC$_{50}$;96h values obtained in the present work indicated that the toxicity of the compounds followed the order: AMT > TBUT+AMT = (DIU+HZN)+TBUT+AMT > DIU+HZN > (DIU+HZN)+TBUT > TBUT. All treatments with AMT were more toxic to tilapia fingerling than the others two herbicides, as well as its mixtures tested in this study.

This difference can be explained by the n-octanol/water partition coefficient (Kow), a physic-chemical parameter which determines the distribution of pesticides among

**Table 2.** Acute toxicities of sugarcane herbicides to tilapia fingerling (*Oreochromis niloticus*).

| | Herbicides | LC$_{50}$-96h (mg L$^{-1}$) (95% C.I.*) | Class.[1] | AI[2] | MF[3] (times) |
|---|---|---|---|---|---|
| Single | AMT | 4.41 (3.63–5.26) | moderate | - | - |
| | TBUT | 245.51 (205.59–256.10) | practically non-toxic | - | - |
| | (DIU+HZN) | 18.77 (15.27–23.97) | slightly toxic | - | - |
| Mixture | TBUT+AMT | 10.76 (8.54–14.28) | moderate | - 0.541 | 0.65 |
| | (DIU+HZN)+TBUT | 43.09 (33.85–56.61) | | - 0.245 | 0.80 |
| | (DIU+HZN)+TBUT+AMT | 11.90 (9.25–15.56) | | - 0.129 | 0.88 |

* 95% of Confidence Interval; [1]Classification of acute aquatic toxicity levels: moderately toxicity: >1 <10 mg L$^{-1}$; slightly toxic: >10 <100 mg L$^{-1}$; practically non-toxic: >100 mg L$^{-1}$. (ZUCKER, 1985); [2]Aditive index; [3]Magnification factor.

environmental compartments and the bioaccumulation in both animals and plants (Finizio *et al.*, 1997). Herbicides with high Kow are more susceptible to be absorbed by cells, but this process also depends on the acid dissociation constant (pKa), the pH at which 50% of the molecules are ionized (Roman *et al.*, 2007). AMT has a Kow = 427 and a pKa = 4.1. With the exception of HZN (Kow = 15.0), the others herbicides evaluated have Kow values with similar order of magnitude (DIU – Kow = 589; TBUT – Kow = 671) of AMT-Kow. However, HZN, DIU and TBUT are in non-ionized forms (pKa = 0). These physico-chemical properties differences might help to explain the greater toxicity of AMT.

The $LC_{50}$;96h values of the mixtures can be checked against the maximum concentration estimated in water (MCE-H20) according to the proposed by SETAC (1994) and Zagatto & Bertoletti (2006) for risk assessment of pollutants in the aquatic environment. This estimate considers the resulting concentration of a direct application, in its application rate (kg a.i. ha$^{-1}$), in a water depth of 0.3 meters deep (depth adopted in European Union). This height of water column adopted for calculate the MCE-H20 is considered a fairly protective condition to Brazilian reservoirs, since the average depth of these environments is approximately 8.0 meters (Zagatto & Bertoletti 2006). Thus the MCE-H20 values calculated were equivalent to 1.06; 1.06 and 1.55 mg L$^{-1}$, respectively for the mixtures TBUT+AMT; (DIU+HZN)+TBUT and (DIU+HZN)+TBUT+AMT. Although Brazilian legislation (Resolution CONAMA nº 357/2005) did not establish a maximum permissible levels for these herbicides in aquatic environments, we believe that these concentrations do not represent a risk in terms of acute toxic effects since they are well below the values of the ratio $LC_{50}$;96h / 3. According to Gherardi-Goldstein *et al.* (1990), the application of a safety factor of 3 is a practical measure that can be adopted to prevent these adverse effects.

The relative toxicities of the formulations tested are shown in Table 3. The greatest difference between the calculated toxicity parameter values was obtained for AMT and TBUT evaluated individually, with AMT being approximately 56 times more toxic than TBUT. The mixtures TBUT+AMT and TBUT+AMT+(DIU+HZN) presented virtually identical toxicities, while the TBUT+AMT mixture was four times more toxic than TBUT+(DIU+HZN), with the $LC_{50}$;96h values being significantly different (at the 95% confidence level).

## CONCLUSIONS

The results of this work indicated that the herbicides evaluated presented moderate toxicity (ametryn) to slightly toxicity (diuron+hexazinone) to tilapia, as well the mixture of those pesticides presented moderate toxicity to this species. Whether in the isolated exposure to each component, or in mixture, ametryn was always more toxic to tilapia than the other herbicides evaluated. A chronic exposure to those compounds would be necessary in order to determine a long term effects on the aquatic community. No potentiation of effects was observed for interaction of the active agents, which suggests that use of the mixtures would not result in increased environmental risks due to synergism, in terms of the acute exposure of fish. Nevertheless, it is essential to study the effects of complex mixtures in risk assessment studies, as it is virtually impossible to find just a single contaminant in the environment and a single exposure approach may underestimated the risk that these compounds represent. The present findings provide information to agriculturalists concerning the use of herbicides, and should assist regulatory authorities in establishing maximum permissible levels for these compounds in continental water bodies.

## ACKNOWLEDGEMENTS

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**Table 3.** Relative toxicities (dimensionless) of the individual and combined herbicides to tilapia fingerling (*Oreochromis niloticus*).

| | AMT | TBUT | DIU+HZN | TBUT +AMT | TBUT+(DIU+HZN) | TBUT+AMT+ (DIU+HZN) |
|---|---|---|---|---|---|---|
| **AMT** | - | (1) 55.8* | (1) 4.2* | (1) 2.4* | (1) 9.8* | (1) 2.69* |
| **TBUT** | (2) 55.8* | - | (2) 13.2* | (2) 22.9* | (2) 5.7* | (2) 20.7* |
| **(DIU+HZN)** | (2) 4.2* | (1)13.2* | - | (2) 1.7* | (1) 2.3* | (2) 1.6* |
| **TBUT+AMT** | (2) 2.4* | (1) 22.9* | (1) 1.6* | - | (1) 4.0* | (1) 1.1 |
| **TBUT+(DIU+HZN)** | (2) 9.8* | (1) 5.7* | (2) 2.3* | (2) 4.0* | - | (2) 3.6* |
| **TBUT+AMT+(DIU+HZN)** | (2) 2.7* | (1) 20.7* | (1) 1.6* | (2) 1.1 | (1) 3.6* | _ |

(1) The toxicity of the compound in the vertical column is "x" times greater than that of the compound in the horizontal line; (2) The toxicity of the compound in the horizontal line is "x" times greater than that of the compound in the vertical column; *95% confidence level (p<0.05).

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