Artigos Científicos Residual de Agrotóxicos

Sumário

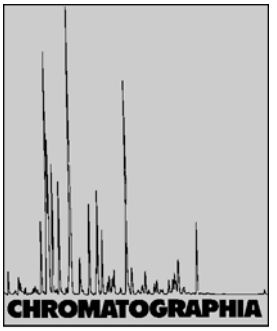
Persistência dos herbicidas imazethapyr e clomazone em lâmina de água do arroz irrigado

Acetylcholinesterase enzyme activity in carp brain and muscle after acute exposure to diafuran

Toxicological and metabolic parameters of the teleost fish (leporinus obtusidens) in response to commercial herbicides containing clomazone and propanil

Acetylcholinesterase activity in the brain and muscle of cyprinus carpio and aristichthys nobilis exposed to azimsulfuron and metsulfuron-methyl

Monitoring of the Herbicide Clomazone in Environmental Water Samples by Solid-Phase Extraction and High-Performance Liquid Chromatography with Ultraviolet Detection



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Key Words

Column liquid chromatography
Clomazone
Pesticides
Water analysis

Summary

Evaluation of a simple and rapid method for determination of clomazone in environmental water samples, by high-performance liquid chromatography with ultraviolet detection, is described. After solid-phase extraction with C_{18} extraction cartridges clomazone was separated on a C_{18} column with 65:35 (*v/v*) methanol-water, pH 4.0, as mobile phase at a flow-rate of 1.0 mL min^{-1}. After optimization of the extraction and separation conditions, the method was used for determination of clomazone residues in rivers and agricultural waters of the central region of the Rio Grande do Sul province. The results revealed that clomazone persists in agricultural water at least for 130 days, and was present in 90% of the river water samples analyzed.

Introduction

Pesticide contamination of environmental waters as a result of agricultural use has been well documented. Several hundred pesticides of different chemical structure are used world-wide in agriculture. Although these pesticides are considered to be essential for agricultural development, some can cause serious environmental contamination, primarily of water [1–5].

EEC Directive 80/778 concerning the quality of water designated for human consumption establishes the maximum permissible concentration of individual pesticides as 0.1 μg L^{-1} and the total amount of pesticides at 0.5 μg L^{-1} [1]. The detection of many organic compounds in surface water is typically required at levels of 1–3 μg L^{-1} [2, 3]. This strict standard for water purity requires the availability of suitable analytical methods with high sensitivity, selectivity, accuracy, and precision.

Herbicides are potential contaminants of environmental waters because they are applied directly to the soil and can be leached by the surface water and transported into ground water [4]. According to the literature a pesticide can contaminate ground water if its solubility in water is higher than 30 mg L^{-1}, its K_{oc} (organic carbon partition coefficient) is less than 300 mL g^{-1}, its K_d (distribution adsorption constant) is less than 5 mL g^{-1} and its soil half-life is longer than 3 weeks [4, 5]. Other relevant factors are described in a review by Barceló [6].

The herbicide clomazone [2-[(2-chlorophenyl)methyl]-4,4-dimethyl-3-isoxazolidinone] (Figure 1) is particularly widely used against species of annual broadleaf weeds and grass. Clomazone is currently used for weed control in the cultivation of soybeans, cotton, rice, sugar cane, corn, tobacco, and a variety of other vegetable crops [7]. It is highly soluble in water (1100 mg L^{-1}), and has a K_{oc} of 150 mL g^{-1} and a K_d value from 0.47 to 5.30 mL g^{-1}. The field dissipation half-life of clomazone, determined from field studies with several types of soil ranged from 4 to 12 weeks [8]. The properties of clomazone thus indicate its potential for groundwater contamination.

High-performance liquid chromatography (HPLC) and gas chromatography (GC) are suitable techniques for monitoring herbicides in water [4, 5, 9–13], and a review covering this use of these methods has recently been published [9]. HPLC is preferred to GC for the most polar pesticides, which are of low volatility and are thermally unstable, because GC methods

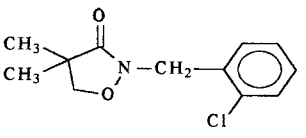

Figure 1. The chemical structure of clomazone.

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Table I. Within-batch recovery and repeatability (RSD_r) for the herbicide clomazone in river water spiked at five different levels.[a]

Clomazone concentration (μg L^{-1})		Recovery (%)	RSD_r (%)
Spike level	Found (mean ± *SD*)		
0.1	0.087 ± 0.0005	87.3	0.66
	0.087 ± 0.001		1.15
	0.088 ± 0.0005		0.65
0.5	0.492 ± 0.002	104.0	0.51
	0.528 ± 0.010		1.82
	0.539 ± 0.022		4.07
1.0	0.960 ± 0.010	95.5	1.11
	0.953 ± 0.008		0.93
	0.951 ± 0.012		1.33
3.0	2.684 ± 0.009	87.9	0.33
	2.589 ± 0.006		0.22
	2.645 ± 0.018		0.68
5.0	4.771 ± 0.052	92.1	1.09
	4.528 ± 0.128		2.84
	4.512 ± 0.077		1.71

[a] The number of replicates at each level, *n*, was 9 (three extractions with three injections each). All were performed under the same conditions on the same day.

Table II. Between-batch recovery and reproducibility (RSD_R) for the herbicide clomazone in river water spiked at five different levels.[a]

Clomazone concentration (μg L^{-1})		Recovery (%)	RSD_r (%)
Spike level	Found (mean ± *SD*)		
0.1	0.080 ± 0.0005	86.3	0.72
	0.099 ± 0.001		1.01
	0.080 ± 0.0005		0.72
0.5	0.487 ± 0.012	96.4	2.64
	0.480 ± 0.005		1.03
	0.478 ± 0.003		0.67
1.0	0.891 ± 0.048	94.4	5.38
	0.982 ± 0.016		1.62
	0.958 ± 0.010		1.10
3.0	2.631 ± 0.044	87.9	1.67
	2.560 ± 0.015		0.58
	2.724 ± 0.005		0.19
5.0	4.589 ± 0.018	91.3	0.40
	4.511 ± 0.047		1.05
	4.594 ± 0.027		0.60

[a] The number of replicates at each level, *n*, was 9 (three extractions with three injections each). Each extraction and series of injections was performed on three consecutive days.

can be used only after a derivatization step [14]. Although many HPLC methods have been developed for the determination of clomazone in soil [7, 8, 15–17] and in soybeans [18, 19], only one reference [20], from our team, was found on the determination of clomazone in water by HPLC.

Because of rigorous water-purity regulations, methods for extraction and preconcentration of the pesticides present in water have become necessary. For analysis of pesticides at trace levels in water samples a preconcentration step is usually performed before analysis. For this purpose, solid-phase extraction (SPE) is replacing traditional methods [21] such as liquid-liquid extraction (LLE), and has been widely used for extraction of water samples before analysis. SPE reduces sample handling, labor, and solvent consumption [9, 13, 22]. The most popular SPE adsorbent for pesticides in water is octadecyl- (C_{18}) bonded silica because of its extremely retentive nature for non-polar compounds.

In this work, a simple, relatively fast, and efficient HPLC-UV method has been developed for the determination of clomazone in environmental water, because a very important aspect of the development of new analytical methods for pesticides is their application to real samples [12]. To achieve efficient preconcentration with good reproducibility and accuracy solid-phase extraction on C_{18} was used. Finally, the proposed procedure was validated [23, 24] by evaluation of calibration and linearity, limits of detection and quantification, precision (repeatability and reproducibility), and accuracy (recovery).

Experimental

Chemical and Reagents

Clomazone standard (99,6%) was obtained from FMC (Uberaba, MG, Brazil). Methanol of chromatographic grade was from Mallinckrodt (Phillipsburg, NJ, USA). Phosphoric acid of analytical grade was from Merck (Darmstadt, Germany). Water was purified with a Milli-Q water-purification system (Millipore, Bedford, MA, USA). The extraction tubes were BondElut C_{18} (3 mL, 200 mg) from Varian (Harbour City, CA, USA).

Instrumentation

HPLC was performed with a Shimadzu (Kyoto, Japan) LC-10AD pump, a Rheodyne (Cotati, CA, USA) 7125 six-port valve with 20-μL loop, and a Shimadzu SPD-10AV UV-visible absorbance detector connected to a Shimadzu Model C-R6A integrator for data acquisition. Mobile phase pH was measured with a Cole Palmer series 500 pH meter. Compounds were separated on a 250 mm × 4.6 mm i. d., 5 μm particle, Bondesil C_{18} analytical column from Varian.

Reproducibility studies were performed with a Varian model 9002 HPLC system equipped with a spectrophotometric detector (Varian 9050).

Procedure

The analytical column was operated at room temperature. The mobile phase was methanol-water, 65:35 (*v*/*v*) adjusted to pH 4.0 with phosphoric acid. It was prepared volumetrically from separately measured volumes of methanol and water and was degassed for 15 min in an ultrasonic bath before use. The flow-rate was set at 1.0 mL min^{-1} and quantification was performed by UV detection at 220 nm. The HPLC system was conditioned by passage of mobile phase for 1 h at a flow-rate of 1.0 mL min^{-1}.

A stock standard solution (2000 mg L^{-1}) of clomazone was prepared by dissolution in methanol and was stored at −18 °C. Calibration standards were prepared by appropriate dilution with the mobile phase and stored under refrigeration (4 °C).

The extraction procedure was that previously developed in our laboratory [20].

The precision (repeatability and reproducibility) and recovery of the procedure were investigated by use of river water samples containing no clomazone. The results are given in Tables I and II. River water samples (200 mL) were fortified by addition of appropriate volumes of clomazone stock solution ($2.0\,mg\,L^{-1}$) to furnish five levels of fortification – 0.1, 0.5, 1.0, 3.0 and $5.0\,\mu g\,L^{-1}$. After fortification the samples were mixed well and immediately filtered through a 0.45 µm PTFE filter. Before sample application, the SPE column was conditioned by consecutive passage of 3 mL methanol, 3 mL Milli-Q water, and 3 mL Milli-Q water at pH 3. After adjustment of the pH to 3 by addition of phosphoric acid, to increase clomazone retention, the samples were mixed well and passed through the SPE column under vacuum at $3\,mL\,min^{-1}$. Immediately after passage of the sample the column was washed with 3 mL Milli-Q water, the eluate was discarded, and the adsorbent bed was dried under vacuum for 2 min. Drying of the adsorbent after elution of water-soluble impurities contained in the samples was necessary if high recovery and reproducibility were to be achieved – if the adsorbent is moist extraction can occur inhomogeneously and more impurities can be eluted, disturbing the analysis. After drying the analyte was eluted with 1 mL ($2 \times 500\,\mu L$) methanol. The solvent was evaporated to dryness under a gentle stream of nitrogen and the residue redissolved in 0.5 mL mobile phase and injected into the chromatograph.

Environmental water samples were extracted by use of the SPE procedure described above and analyzed by HPLC. Samples were collected in 2-L glass bottles approximately 1 m from the shore and 10 cm below the water surface. Immediately after collection the samples were acidified to pH 3.0 with phosphoric acid and filtered through a 0.45 µm PTFE filter to remove particulate mater. Samples were stored at 4 °C before extraction, which was normally performed within 48 h.

Area Description

The samples of river water were collected in the central region of the Rio Grande do Sul province, where the herbicide clomazone is used extensively in rice cultivation. Normally approximately ten days after planting, the water containing the herbicide is released to enable development of the plant. A few days later the area is re-irrigated and remains in this state until harvest. The sampling points are shown in Figure 2. Water samples were collected during the rice-production season from the rivers Ibicuí, Ibicuí-Mirim, Vacacaí-Mirim, Jacuí, and Vacacaí once a month from December 1999 to March 2000, when clomazone application was heaviest. Sampling was repeated during the same period of 2000/2001. The criterion for selection of the sampling sites was the presence of major agricultural activity. Water samples were also collected in the river Tigre located in the region of Nonoai, RS, where the herbicide clomazone is not used. The samples were analyzed by the method described above; the results are shown in Table III.

To evaluate the aquatic dissipation of clomazone under field conditions samples of agricultural waters were collected from experimental rice fields situated at the Federal University of Santa Maria. Two independent rice fields, described as repetition I and II, were used for this study. In these fields the recommended dose ($0.50\,kg\,ha^{-1}$) of the herbicide clomazone (formulated material) was used. The samples were collected 1 h (T_0) and 7, 14, 21, 28, and 130 days after application of the herbicide in the month of December. This study was repeated in the two subsequent years.

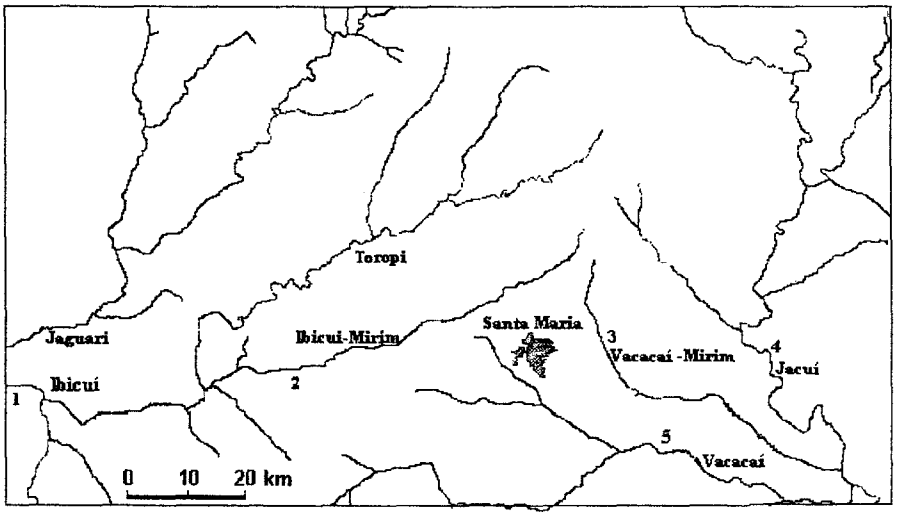

Figure 2. The area of the Rio Grande do Sul province studied, showing the location of sampling sites. All the rivers sampled receive irrigation from the rice fields.

Table III. Results from analysis of river-water samples.

River	Dec 1999 to Mar 2000		Dec 2000 to Mar 2001	
	Concentration ($\mu g\,L^{-1}$)	*RSD* (%)	Concentration ($\mu g\,L^{-1}$)	*RSD* (%)
Ibicuí	1.72	0.6	0.69	1.3
Ibicuí-Mirim	1.02	0.7	0.60	0.9
Vacacaí-Mirim	0.60	0.9	1.03	2.1
Jacuí	0.31	1.2	n. d.	–
Vacacaí	1.21	0.4	1.15	0.3
Tigre	n. d.	–	n. d.	–

Results and Discussion

General Considerations

Reversed-phase HPLC with UV detection is highly suitable for clomazone determination because no derivatization step is needed. Chromatographic separation on C_{18} columns provides good results and detection at 220 nm furnishes chromatograms suitable for the quantification of clomazone in real samples.

Mobile phases containing methanol and water in different proportions were evaluated to determine optimum peak shape, retention time, and chromatographic separation from interfering analytes. A 65:35 (*v*/*v*) methanol-water mixture at a flow-rate of $1.0\,mL\,min^{-1}$ proved to be the most suitable mobile phase.

Under the conditions chosen the retention time of clomazone was 12.0 ± 0.1 min and the peak was completely separated from those of interfering analytes present in the water samples.

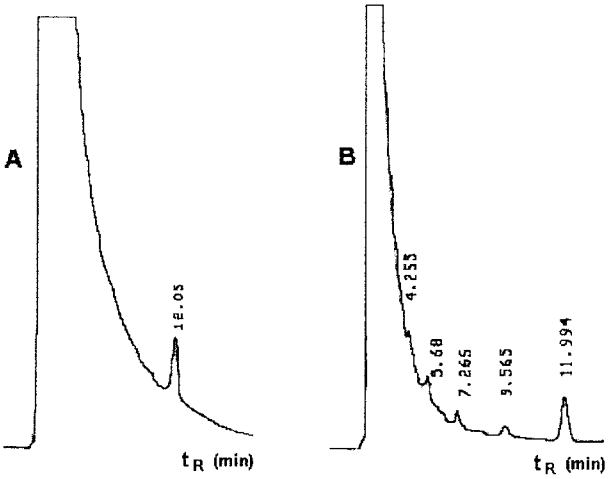

Figure 3. HPLC-UV chromatograms obtained after extraction of 200-mL samples of river water (**A**) and experimental rice field water (**B**). The retention time of clomazone is 12.05 min in **A** and 11.96 min in **B**.

Table IV. Results from analysis of experimental rice-field-water samples.

Days after application	December 1999[a]		December 2000[a]	
	Concentration ($\mu g\,L^{-1}$)[b]	*RSD*	Concentration ($\mu g\,L^{-1}$)[b]	*RSD*
T_0[c]	505	4.5	377	1.4
7	262	4.0	238	5.2
14	34.5	4.6	7.3	6.6
21	3.0	5.2	3.1	4.2
28	2.2	6.3	1.6	5.7
130	0.9	10.4	0.2	8.3

[a] The month the experiment started. [b] Mean from two replicate analyses – two extractions with two injections each. [c] One hour after application.

The regression equation for clomazone calibration under these chromatographic conditions was:

$$y = -4913.3 + 153818.2x \; (r = 0.9998)$$

where y = peak area, x = clomazone concentration ($mg\,L^{-1}$), and r is the correlation coefficient.

For clomazone the response was linearly dependent on concentration for concentrations up to $10\,mg\,L^{-1}$. By comparison of the response with the baseline noise the limit of detection was found to be $0.012\,mg\,L^{-1}$ and the limit of quantification was $0.036\,mg\,L^{-1}$. As shown by Figure 3, the effective limits of detection and quantification in the surface water samples, after 400-fold preconcentration by SPE were 0.03 and $0.1\,\mu g\,L^{-1}$, respectively.

Instrument precision was measured by comparing the standard deviation of the response from injection in triplicate of eleven different calibration standard solutions (0.025 to $10.0\,mg\,L^{-1}$). The *RSD* ranged from 0.6 to 4.5% and the mean instrument precision was 2.1%.

No breakthrough of the analyte occurred after preconcentration of 200 mL of river water fortified with clomazone at $5.0\,\mu g\,L^{-1}$. When the recovery obtained from drinking water and real environmental water samples, both fortified at $5.0\,\mu g\,L^{-1}$, was compared no interference from the water matrix was observed.

The repeatability of the method was determined by adding clomazone to blank surface water at five different concentrations. The within-batch recovery and repeatability (RSD_r) for surface water spiked with clomazone at 0.1, 0.5, 1.0, 3.0, and $5.0\,\mu g\,L^{-1}$ are summarized in Table I. The precision (repeatability) ranged from 0.22 to 4.07%, with a mean value of 1.27%. The results are fairly good for the concentration levels investigated.

The reproducibility of the method was determined by analysis of spiked surface water samples under a variety of test conditions (different analysts, different instruments, and different days). The between-batch recovery and reproducibility (RSD_R) for several levels are given in Table II. The precision (reproducibility) ranged from 0.19 to 5.38%, with a mean value of 1.29%. The reproducibility (RSD_R) values are very good because all measurements should be within ±15% at all concentrations [23, 25].

The average recovery obtained for clomazone for all the concentrations and conditions investigated (Tables I and II) was found to be 92.3 %, which is highly satisfactory.

Analysis of Real Samples

The main problem in the analysis of water samples is the presence of organic substances, principally humic and fulvic substances (humic and fulvic acids). Some difficulties were encountered in the analysis of samples of environmental waters collected from rivers and rice fields, because of the large amounts of compounds, probably organic, which remained in the SPE cartridges and resulted in light orange-yellow extracts.

The chromatograms obtained from the water samples (Figure 3) contained a large peak at the beginning of each chromatogram and then other peaks of low intensity. These peaks did not interfere with qualitative and quantitative analysis of the herbicide.

River Waters

The results obtained from analysis of river water samples collected during the period of rice-production when the irrigation water was released to the environment are listed in Table III. The results reflect agricultural practice – clomazone residues were detected in 90% of samples taken from rivers of the rice-producing regions.

Experimental Rice-Field Waters

The results presented in Table IV show that the clomazone concentration is much lower in the 14-day sample than in that collected on the 7th day, because of the release of the irrigation water on the 10th day to enable development of the rice plants. Irrigation is restored on the 13th day and is maintained until harvest (approximately 130 days) when the water is released to the environment. At this point the mean clomazone concentrations for both periods studied were 0.9 and $0.2\,\mu g\,L^{-1}$.

The *RSD* values obtained for these analyses show that the method used is precise – all the values are well below 15%.

Conclusion

The results obtained for calibration, linearity, precision, and accuracy (recovery) show that this is a rapid and efficient method for quantification of clomazone in environmental water samples. The *LOD* and *LOQ* were, respectively, 0.012 and 0.036 mg L^{-1} and the effective *LOD* and *LOQ* for the samples after the SPE preconcentration step were 0.03 and 0.1 μg L^{-1}, respectively.

The results obtained from analysis of water from the experimental rice field show that the herbicide clomazone is persistent – 28 days after application the concentration was still above the limit normally stipulated for environmental waters (1 to 3 μg L^{-1}). Levels of clomazone in rivers of the central region of the Rio Grande do Sul province exceeded 1 μg L^{-1} in approximately 50% of the samples analyzed.

Acknowledgements

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Lethal concentration of clomazone, metsulfuron-metil, and quinclorac for silver catfish, *Rhamdia quelen*, fingerlings

Concentração letal do clomazone, metsulfuron-metil e quinclorac para alevinos de jundiá, *Rhamdia quelen*

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ABSTRACT

*The goal of the present work was to determine the lethal concentration (LC50) (96h) of clomazone, metsulfuron-methyl, and quinclorac, herbicides used in rice culture, for the silver catfish, **Rhamdia quelen**. Fingerlings were exposed to different concentrations of the herbicides. The LC50s were 7.32µL L^{-1} for clomazone and 395mg L^{-1} for quinclorac. The LC50 for metsulfuron-methyl was not obtained since all fingerlings survived even at 1200mgL^{-1}. Probably only clomazone can lead to mortality among silver catfish reared in the rice culture system.*

Key words: *quinclorac, clomazone, metsulfuron-menthyl, **Rhamdia quelen**, silver catfish, LC50.*

RESUMO

*O objetivo deste trabalho foi determinar a concentração letal (CL50 96h) de clomazone, metsulfuron-metil e quinclorac, herbicidas utilizados no cultivo de arroz, para alevinos de jundiá, **Rhamdia quelen**. Alevinos foram expostos a diferentes concentrações destes herbicidas. A CL50 foi 7,32µL L^{-1} para clomazone e 395mg L^{-1} para quinclorac. A CL50 para metsulfuron-metil não foi encontrada porque todos os alevinos sobreviveram mesmo à concentração de 1200mg L^{-1}. Provavelmente apenas o clomazone pode provocar alguma mortalidade em jundiás mantidos em rizipiscicultura.*

Palavras-chave: *quinclorac, clomazone, metsulfuron-metil, jundiá, **Rhamdia quelen**, CL50.*

INTRODUCTION

Fish rearing as an integrated and concurrent activity with rice plantation is profitable in areas of limited land resources, as a self sustainable source of fish protein and chances of additional incomes and employments (JAMU & COSTA-PIERCE, 1995). Presently rice cultivation is highly automated and uses fertilizers and pesticides. Extensive irrigation facilities have been constructed to increase the area of rice plantations and subsequently the yields. The concurrent fish-rice culture seems to have been affected adversely by many of the recent changes in the agronomic, social, and economic structure of rice cultivation (FERNANDO, 1993). Although herbicide contamination has been a major problem in the natural water systems, there have been comparatively fewer problems with herbicides in aquaculture ponds. However, unless special care is taken when herbicides are used on agricultural lands surrounding ponds or in application of pesticides in aquaculture management, fish mortality and/or contamination will occur (SEIM et al., 1997). Clomazone (isoxazolidinone), quinclorac (quinoline), and metsulfuron-methyl (sulfonylurea) are post emergence herbicides widely used in rice fields in South Brazil, with activity against Poaceae (JONSSON et al., 1998).

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The sulfonylurea, quinolines and isoxazolidinone herbicides are the most potent herbicides known today, and those are readily soluble in water (FAHL et al., 1995; WARE, 2003). Aquatic contamination with these products may occur in areas of crops with consequent impact on the aquatic fauna (JONSSON & MAIA, 1999). Little attention has been paid to the possible occurrence of a short-term sub-lethal toxicity of herbicides to fish (SAGLIO et al., 2001).

The physicochemical properties of the herbicides differ among the different available compounds, being therefore difficult to generalize about their fate in the environment. The chemical characteristics of quinclorac are: water solubility = 0.065 mg/L, constant of Henry's law (K_H) <3.72 x 10^{-2} Pa m^3/mol, coefficient of partition octane-water ($logK_{ow}$) = -1.15 at pH 7.0 and constant of acidic ionization (pKa) = 4.34; for clomazone, water solubility of 1100mg L^{-1}, K_H = 4.19 x 10^{-3} Pa m^3 mol^{-1}, log K_{ow} = 2.54, K_{oc} between 150 to 562 cm^3 g^{-1}, half-life in water < 30 days and in the soil between 30 to 135 days; for metsulfuron-methyl K_H = 3.3 x 10^{-7} Pa m^3 mol^{-1}, log K_{ow} = -1.7, pK_a = 3.3, K_{oc} = 35 cm^3 g^{-1} and half-life in the soil = 30 days (BARCELÓ & HENION, 2003).

The choice of the silver catfish, ***Rhamdia quelen*** (Quoy and Gaimard, 1824, family ***Heptateridae***, order Siluriformes) for these tests with herbicides was based on the regional ecological importance and the possibility of its use in rice fields. This species is distributed from Southern Mexico to Central Argentina, and its husbandry is spreading out across Southern Brazil. The interest in the culture of this species is increasing because it presents a good growth rate, is an omnivorous fish, has high fertilization and hatching rate, and is well accepted by consumers (GOMES et al., 2000). Therefore, it is a species suitable for rice-fish culture. The objective of the present work was to establish the lethal concentrations (LC50) of clomazone, metsulfuron-methyl, and quinclorac for silver catfish fingerlings.

MATERIALS AND METHODS

Silver catfish (***Rhamdia quelen***) fingerlings were obtained from a commercial fish culture near Santa Maria, Rio Grande do Sul State, Brazil, and transported to the Fish Physiology Laboratory of the Universidade Federal of Santa Maria, Santa Maria, Brazil. The fingerlings (1.42 to 3.57g, 5.1 to 7.0cm) were placed in boxes (44L) at a stocking density of 10 fish/box, and the water temperature was 21 to 22°C. Dissolved oxygen levels were always above 6.8mg L^{-1}, maximum level of non-ionized ammonia was 0.007mg L^{-1}, and water hardness was 20mg L^{-1} $CaCO_3$. The photoperiod was 12h light/12h dark, with luminosity of 0.6lux (measured with a LI-COR photometer model LI-185B), for dark environments reduce stress effects in this species (PIAIA et al., 1999).

The fish were exposed in triplicate for 96h to each herbicide: clomazone (2-(2-chlorophenyl)methyl-4,4-dimethyl-3-isoxazolidinone) (Gamit) 1, 5, 10, 20, and 50µL L^{-1}; quinclorac (3-7dichloroquinoline-8-quinoline carboxylic acid) (Facet) 100, 200, 300, 390, and 400mg L^{-1}; and metsulfuron-methyl (methyl 2-[(4-metoxi-6-methyl-1,3,5-triazine-2-il)amino]carbonyl]amino] sulfonyl]benzoate-sulfonylurea) (Ally) 200, 400, 600, 800, and 1200mg L^{-1}. Herbicides were added to the water only at the beginning of the experiment. Water was not changed throughout the experiment, but feces and pellets residues were removed daily by suction. Fish were fed to satiation three times a day (08:30, 12:00, and 17:30h) with commercial food (42% CP, Supra, Brazil). The swimming and feeding behaviors (normal or abnormal) were checked. One box without herbicide was used as control.

Water pH was measured electrometrically, dissolved oxygen with an oxygen meter (YSIR, Y5512) and water hardness by the EDTA titrimetric method. Total ammonia nitrogen (NH_3, NH_4^+) was determined by nesslerization (GREENBERG et al., 1976), and non-ionized ammonia nitrogen (N-NH_3) was calculated as described by PIPER et al. (1982). All parameters were analyzed everyday. Mortality was determined at 12, 24, 48, 72, and 96h after exposure to the herbicide. The mean lethal concentration (LC50) for 96 hours was calculated according to CHIPPARI-GOMES et al. (1999). The security index for each herbicide was calculated according to RESGALLA Jr. et al. (2002): LC50/recommended level. The correlation between herbicides concentration and water pH and alkalinity was calculated with the aid of the Slide Write Plus Software (Advanced Graphics Software, Inc.). Comparisons among water pH or alkalinity of the different treatments were made by one-way analysis of variance and Tukey test. Analysis was performed using the software Statistica (version 5.1), and the minimum significance level was set at $P < 0.05$.

RESULTS

There was a significant trend to decrease water pH ($y=8.166-0.005x$, $r^2=0.709$, where y= pH (units) and x= quinclorac concentration in mg/L) and alkalinity ($y=41.5-0.094x$, $r^2=0.920$, where y= alkalinity (mg L^{-1} $CaCO_3$) and x= quinclorac concentration in mg L^{-1}) with

increasing quinclorac concentration (Figure 1). Metsulfuron-methyl at concentrations of 200, 800, and 1200mg L^{-1} also reduced water pH relative to control values, but there was no significant correlation of this parameter with metsulfuron-methyl concentration. Water alkalinity was not altered by this herbicide. Clomazone did not significantly change water pH and alkalinity compared to control values (Table 1). Fingerlings exposed to 1.0, 5.0, and 10.0µL L^{-1} clomazone produced 3.3, 23, and 57% mortality within 96h respectively, and at 20 and 50µL L^{-1} mortality was total in less than 24h. The LC50 for clomazone was 7.32µL L^{-1} (confidence interval: 5.68 - 9.03µL L^{-1}), equation y=2.26+3.16x, where x= probits and y=log clomazone concentration (µL L^{-1}) (Figure 2a). All fingerlings exposed to 400mg L^{-1} quinclorac died within 48h, but for those exposed to 390mg L^{-1} or less there was no mortality. For quinclorac, the LC50 was 395.0mg L^{-1} (confidence interval: 394.0 – 395.9mg L^{-1}) (Figure 2b). For metsulfuron-methyl, the LC50 was not obtained since all fingerlings survived even at the concentration of 1200mg L^{-1}. There was no mortality in the control group. The security indexes for clomazone and quinclorac are 10.46 and 526.7 respectively.

Fingerlings exposed to the highest doses of clomazone (20 and 50µL L^{-1}) and quinclorac (390 and 400mg L^{-1}) did not feed, but those maintained with metsulfuron-methyl showed normal feeding behavior. Swimming activity was normal at the lower clomazone doses (1 and 5µL L^{-1}), but higher concentrations provoked erratic swimming. All quinclorac concentrations induced loss of equilibrium and lethargic behavior. Fingerlings exposed to all metsulfuron-methyl concentrations showed burst swimming reactions.

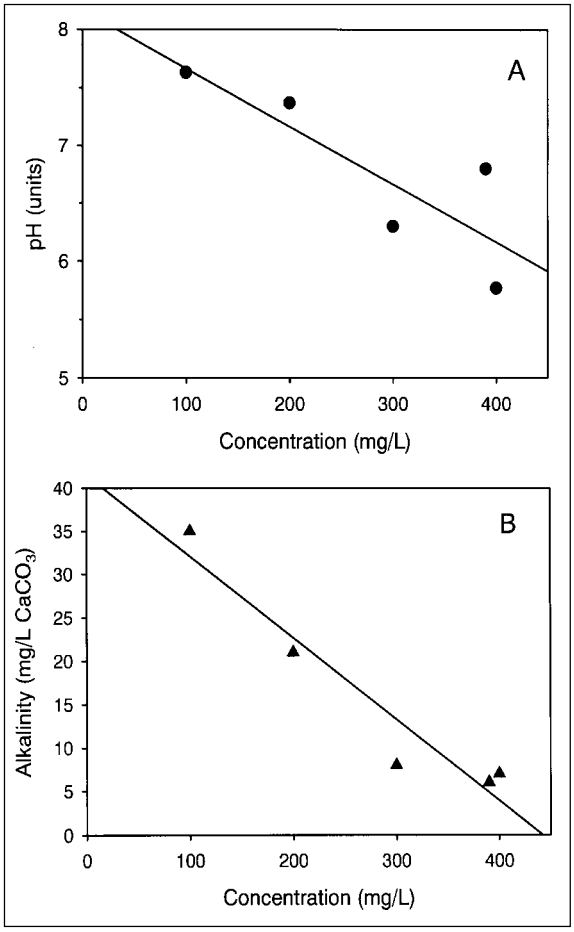

Figure 1 – Relationship among pH (A), alkalinity (B), and quinclorac concentration of the water used in the experiments.

DISCUSSION

Dissolved oxygen and non-ionized ammonia concentrations were maintained at optimum levels for fish culture as described by BOYD (1998). Water pH and alkalinity decreased with the addition of quinclorac and metsulfuron-methyl, but these levels can be considered acceptable since silver catfish fingerlings exhibit 100% survival between 4.0-9.0 pH range (96h) (ZAIONS & BALDISSEROTTO, 2000). Decrease of water pH with the addition of quinclorac and metsulfuron-methyl was expected since both herbicides have acid characteristics (VENCILL et al. 2002).

Table 1 – Water pH and alkalinity in the different treatments.

Treatment	pH (units)	alkalinity (mg/L aCO_3)
Control	7.73 ± 0.06	54 ± 3
Clomazone 1µL/L	7.90 ± 0.07	43 ± 2
Clomazone 5µL/L	7.85 ± 0.09	44 ± 2
Clomazone 10µL/L	7.88 ± 0.06	37 ± 5
Clomazone 20µL/L	8.07 ± 0.03	39 ± 3
Clomazone 50µL/L	8.07 ± 0.03	39 ± 3
Quinclorac 100mg/L	7.63 ± 0.03	35 ± 1*
Quinclorac 200mg/L	7.37 ± 0.03	21 ± 1*
Quinclorac 300mg/L	6.30 ± 0.15*	8 ± 0*
Quinclorac 390mg/L	6.80 ± 0.06*	6± 0*
Quinclorac 400mg/L	5.77 ± 0.06*	7 ± 3*
Metsulfuron-methyl 200mg/L	6.7 ± 0.01*	41 ± 7
Metsulfuron-methyl 400mg/L	7.50 ± 0.01	47 ± 0
Metsulfuron-methyl 600mg/L	7.40 ± 0.06	49 ± 2
Metsulfuron-methyl 800mg/L	7.30 ± 0.06*	58 ± 0
Metsulfuron-methyl 1200mg/L	6.89 ± 0.03*	36 ± 0

* significantly different from control value ($P < 0.05$) in the same column

Silver catfish seems to be more sensitive to clomazone than other species since its LC50 is 7.32µL L^{-1}, while for rainbow trout and bluegill 96h-LC50 values are 19 and 34µL L^{-1} respectively (VENCILL et al., 2002). In addition, for ***Hyphessobrycon scholzei*** fingerlings the 96h-LC50 value is 27.29µL L^{-1} (JONSSON & MAIA, 1998). The LC50 of metsulfuron-methyl for silver catfish was not obtained because there was no fingerling mortality even at the highest concentration tested (1200mg L^{-1}), which was 8 times higher than the LC50 value reported by VENCILL et al. (2002) for rainbow trout (150mg L^{-1}).

Observations on the behavior can be interesting for assessing sublethal effects of contaminants in fish because these endpoints are accessible for many species and stages of development. It is of interest to characterize behaviors that can be routinely used as indicators of acute changes in the environmental chemical composition (SAGLIO et al., 2001). The herbicides can affect various types of behavior in fish, directly as well as indirectly by altering the chemical perception of natural substances of eco-ethological importance (SAGLIO & TRIJASSE, 1998). At higher clomazone concentrations (20 and 50µL L^{-1}) and for all quinclorac levels tested, silver catfish showed altered behavior (equilibrium loss and lethargy). This change of behavior was also observed in juvenile goldfish (***Carassius auratus***) exposed to the herbicides atrazine and diuron (0.005mg/L) (SAGLIO & TRIJASSE, 1998). Convulsions occurred in silver catfish after 30min of exposure to clomazone at 20 and 50mL L^{-1}, a phenomenon which has also been reported in young Nile tilapia (***Oreochromis niloticus***) after exposure to malathion (40mg L^{-1}) for 80min. Later on, all tilapias showed reduced opercular ventilatory movements and posture loss, and the individuals in which the ventilatory movements stopped did not recover (LOPES et al., 1989). Silver catfish exposed to metsulfuron-methyl showed only burst swimming reactions. This change of swimming activity was also observed in goldfish exposed to 0.001, 0.01, 1, and 10mg L^{-1} nicosulfuron (sulfonylurea) (SAGLIO et al., 2001).

In Southern Brazil the herbicides clomazone, metsulfuron-methyl, and quinclorac are sprayed in the rice culture fields (pre-germinated system), at the recommended levels of about 0.7µL L^{-1}, 0.003mg L^{-1}, and 0.75mg/L respectively (VENCILL et al., 2002). The security indexes of clomazone and quinclorac for common carp (***Cyprinus carpio***) are 13.94 and 8.87 respectively, which demonstrated that the difference between LC50 and the recommended concentration in the rice field is very close, indicating a potential toxicity of these herbicides in the fish-rice system for this species (RESGALLA Jr. et al., 2002). For silver catfish, the security index for clomazone (10.46) is similar to that of common carp, but is much higher for quinclorac (526.7). Probably only clomazone can induce some mortality in silver catfish maintained in a rice culture system since the lower dose used in this experiment (1.0µL L^{-1}) resulted in mortality, and this is very close to the dose applied to the rice field. On the other hand, metsulfuron-methyl has low toxicity for common carp because its security index is 7878 (RESGALLA Jr. et al., 2002). It was not possible to calculate the LC50 for metsulfuron-methyl to silver catfish because even the highest concentration tested did not produce any mortality. However, even considering the highest concentration as the LC50, the security index will be 400,000. Therefore, metsulfuron-methyl is very safe for silver catfish.

However, herbicides can affect the phytoplankton by reducing the supply of dissolved

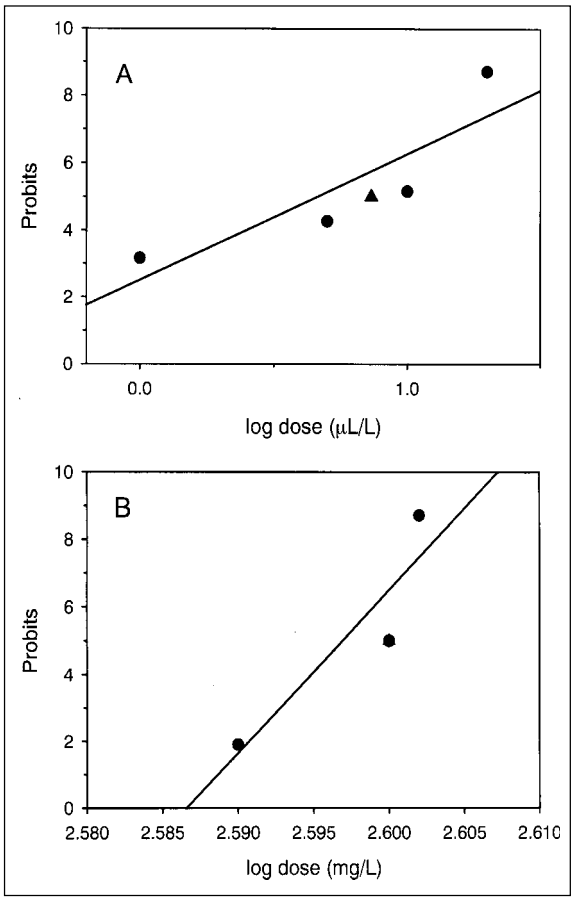

Figure 2 – Mortality of silver catfish exposed for 96h to clomazone (A) and quinclorac (B). Triangles indicate lethal concentration, and circles indicate the tested doses.

oxygen and the removal of nitrogenous compounds in the water (PERSCHBACHER et al., 2002). In the present study the LC50 experiments were carried out at optimal water quality, a fact that certainly does not occur in the rice field. Additional experiments using water from rice field must be done to determine whether these herbicides can alter growth of silver catfish.

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Artigo

POLUIÇÃO DAS ÁGUAS POR HERBICIDAS UTILIZADOS NO CULTIVO DO ARROZ IRRIGADO NA REGIÃO CENTRAL DO ESTADO DO RIO GRANDE DO SUL, BRASIL: PREDIÇÃO TEÓRICA E MONITORAMENTO

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POLLUTION OF WATER BY HERBICIDES USED IN THE IRRIGATED RICE CULTIVATION IN THE CENTRAL AREA OF RIO GRANDE DO SUL STATE, BRAZIL: THEORETICAL PREDICTION AND MONITORING. This article presents an evaluation of the pollution of river water by herbicides used in the culture of irrigated rice in Rio Grande do Sul State, Brazil. Firstly, a theoretical evaluation was made using the approaches suggested by EPA-USA, the "Groundwater Ubiquity Score" index and the Goss method to estimate the pollution possibilities. Afterwards, a monitoring program was established for the rivers of the area from 2001 to 2003 to investigate the presence of herbicide residues. The results indicate that the herbicides clomazone and propanil are the ones with larger presence and frequency in the analyzed samples. The theoretical forecast was confirmed by the results of the monitoring program.

Keywords: herbicides; water pollution; irrigated rice fields.

INTRODUÇÃO

Desde o início de seu desenvolvimento, a produção agrícola está diretamente relacionada com a aplicação de pesticidas para controlar as pestes que atacam os produtos agrícolas, prejudicando as colheitas. A aplicação de pesticidas gera, comumente, grandes problemas: os pesticidas muitas vezes são tóxicos, podendo ser cancerígenos, mutagênicos, teratogênicos e mimetizadores de hormônios[1]; são aplicados em grande quantidade, em áreas bastante extensas e, geralmente, possuem grande persistência no meio ambiente[2], além de gerar sérios problemas de qualidade das águas superficiais e subterrâneas[3]. O efeito e a magnitude decorrentes do uso de pesticidas no ambiente dependem basicamente dos processos de transferência e transformações que ocorrem em cada compartimento do sistema solo-água-planta-atmosfera[4,5].

No cultivo de qualquer sistema agrícola, como por ex., no arroz irrigado em sistema de cultivo pré-germinado, o ponto de partida para o desencadeamento de impacto ambiental é a técnica de aplicação do pesticida. As maiores rotas de dispersão de pesticidas para sistemas aquáticos são o escoamento superficial e a drenagem. O tipo de planta e a topografia do terreno têm importância decisiva na maioria desses processos[6]. Assim, a dinâmica dos pesticidas no solo está relacionada com a precipitação pluvial e o manejo da irrigação[7].

No sistema pré-germinado, a drenagem da área irrigada, efetuada após a semeadura, pode desencadear grave problema ambiental, ao mesmo tempo que pode causar perdas dos nutrientes e/ou de herbicidas que estão em suspensão na água de irrigação que é liberada. Com o decorrer do tempo, as vantagens do sistema são suplantadas pelas desvantagens ambientais.

Aproximadamente um terço de todos os compostos orgânicos produzidos têm como destino o meio ambiente, incluindo a água. Cerca de 700 compostos químicos, incluindo mais de 600 compostos orgânicos, muitos dos quais biologicamente ativos, têm sido detectados em amostras de água[2].

O Brasil, desde a década de 70, destaca-se como um dos maiores consumidores mundiais de pesticidas[8]. Porém, o único dado que nos dá uma indicação da escala em que são aplicados no Brasil são os valores de pesticidas em linha de comercialização, a partir dos quais destaca-se a grande utilização de herbicidas[9]. As culturas responsáveis por este elevado consumo são principalmente soja, cana-de-açúcar, milho e arroz[10]. O estado do Rio Grande do Sul (RS) é responsável pela utilização de cerca de 20% dos pesticidas consumidos no país.

No Brasil são cultivados anualmente 1,3 milhões de hectares com arroz irrigado, dos quais cerca de 950 mil (73%) estão no RS[11]. A orizicultura gaúcha contribui com cerca de 50% da produção nacional de arroz[12]. Apesar da grande contribuição do estado do RS na produção de arroz, muito pouco se fez até o momento em relação a estudos de comportamento e destino dos herbicidas no sistema, visando a manutenção da eficiência com menor risco de dano ambiental[5].

Esse trabalho tem por objetivo avaliar o potencial teórico de lixiviação e o risco de poluição das águas por herbicidas utilizados no cultivo do arroz irrigado na região central do RS, usando para esta avaliação os critérios de "screening" da US-EPA; o índice GUS de vulnerabilidade de águas subterrâneas ("Groundwater Ubiquity Score, GUS"); e o método de Goss. Todos esses procedimentos levam em consideração as propriedades físico-químicas dos herbicidas e as propriedades do solo. Objetivou-se ainda comparar esta avaliação teórica com os resultados obtidos das análises de amostras de águas de rios, coletadas na região em estudo durante o período de cultivo do arroz irrigado nas safras agrícolas de 2000/01, 2001/02 e 2002/03.

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PARTE EXPERIMENTAL

Área do estudo

O estudo foi realizado a campo em área de várzea do Departamento de Fitotecnia da Universidade Federal de Santa Maria (UFSM), em Santa Maria (RS); e na Bacia Hidrográfica dos Rios Vacacaí e Vacacaí-Mirim durante o período de cultivo do arroz irrigado nas safras agrícolas de 2000/01, 2001/02 e 2002/03.

As coordenadas geográficas da região utilizada para o estudo são latitude 29º41'24"S, longitude 53º48'42"W e altitude média de 95 m. O solo é classificado como Planossolo Hidromórfico Eutrófico Arênico, pertencente à unidade de mapeamento Vacacaí. Apresenta relevo plano a suavemente ondulado, substrato de sedimentos aluviais recentes. No sistema americano é enquadrado como Albaqualf. Em geral, essa unidade de mapeamento apresenta textura média (15 a 25% de argila), com baixos a médios teores de matéria orgânica, pobres em nutrientes e mal drenados, bastante influenciados pela presença de água, condicionada pelo relevo.

O clima é classificado como subtropical úmido, classe 'Cfa'. A precipitação pluvial média anual normal varia de 1.322 a 1.769 mm. A temperatura varia em média de 14,1 ºC no mês de julho a 24,9 ºC em janeiro. Os valores extremos das médias das temperaturas máximas e mínimas no mês de julho são de 19,8 e 9,3 ºC, e em janeiro, 31,5 e 18,8 ºC, respectivamente.

A radiação média mensal oscila de 199 em julho a 518 cal cm^{-2} dia^{-1} em dezembro. A umidade relativa do ar varia de 83,8 em julho a 72,5% em dezembro e a precipitação pluvial varia de 111 em novembro a 177 mm em outubro. Os valores máximos de evapotranspiração potencial são da ordem de 138 mm no mês de janeiro e os valores mínimos são de 28 mm com ocorrência em junho.

Herbicidas escolhidos para o estudo

Foram escolhidos para o estudo os herbicidas clomazone, quinclorac, bentazone, 2,4-D e propanil, que são os mais empregados na cultura de arroz irrigado na região central do estado, conforme pesquisa feita nas casas agrícolas da região e informações do Departamento de Fitotecnia da UFSM.

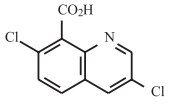
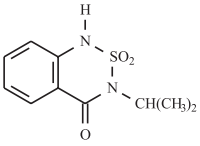
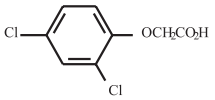
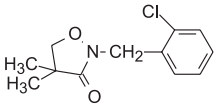
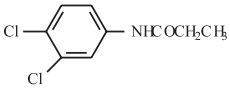
O fator determinante para a escolha desses herbicidas foi o emprego em grande escala no estado do RS, principalmente na região de Santa Maria, onde há grande produção de arroz, além da falta de informações científicas relacionadas com a poluição das águas por herbicidas nessa região.

Na Tabela 1 estão relacionadas algumas propriedades dos herbicidas escolhidos para o estudo[13-15].

Critérios adotados para avaliação

As propriedades físico-químicas dos herbicidas usados nos critérios de avaliação do potencial de risco para ambientes aquáticos foram a constante da lei de Henry (K_H), solubilidade em água, coeficiente de partição octanol-água (K_{ow}), coeficiente de adsorção à matéria orgânica do solo (K_{oc}), constante de ionização ácida (pKa) ou básica (pKb), tempo de meia-vida ($t_{1/2}$), no solo e na água[13,15]. Segundo Barceló e Hennion[13], pesticidas ácidos são os que possuem pKa < 3-4, básicos pKa >10; polares tem valores de log K_{ow} abaixo de 1,5; não polares tem valores de log K_{ow} acima de 4, entre os dois valores são considerados moderadamente polares e, os com log K_{ow} > 3,0 sofrem bioacumulação. A maioria dos valores do índice de GUS foram calculados por não terem sido encontrados na literatura. Para o cálculo foram utilizados os dados da Tabela 2 e a seguinte equação: GUS=log ($t_{1/2}$ solo)×(4-logK_{OC}) O índice de GUS avalia o potencial de determinado composto ser lixiviado,

Tabela 1. Informações dos herbicidas usados no estudo

Nome comum	Estrutura	Nome IUPAC	Número *CAS*	MM (g mol^{-1})
Quinclorac (QUIN)		3,7-dicloroquinolina-8-ácido carboxílico	84087-01-4	242,1
Bentazone (BENT)		3-isopropil-1*H*-2,1,3-benzotiadiazinona-4-(3*H*)-ona-2,2 dióxido	25057-89-0	240,3
2,4-D		(2,4-diclorofenoxi) ácido acético	94-75-7	221,0
Clomazone (CLOM)		2-[(2-clorobenzil)]-4,4-dimetil-1,2-oxazolidin-3-ona	81777-89-1	239,7
Propanil (PROP)		2-metil-3,4-dicloro acetanilida	709-98-8	218,1

MM= massa molecular

atingindo águas subterrâneas e seu valor serve como ferramenta auxiliar para identificação de pesticidas a serem priorizados nas atividades de monitoramento ambiental *in loco*[16].

O comportamento dos pesticidas no ambiente deve ser diferente entre condições temperadas e tropicais. Segundo Castillo *et al*.[17], alguns dados sugerem que as taxas de degradação devem ser mais altas em países tropicais, devido à temperatura mais elevada e à radiação mais intensa.

Critérios usados para avaliar o potencial de lixiviação

Para avaliar o risco potencial de poluição de águas subterrâneas da região pelos herbicidas em estudo, foram usados os seguintes critérios de "screening": Critérios da EPA e índice GUS[13, 15].

Para avaliar a possibilidade dos herbicidas atingirem as águas superficiais foram utilizados os critérios de "screening" sugeridos pelo método de Goss[15].

Critérios da EPA

Em resumo, os critérios de "screening" sugeridos pela EPA na análise preliminar de riscos de poluição de águas subterrâneas por pesticidas são os seguintes: solubilidade em água > 30 mg L^{-1}; coeficiente de adsorção à matéria orgânica do solo (K_{oc}) < 300-500; constante da Lei de Henry (K_H) < 10^{-2} Pa m^3 mol^{-1}; especiação: negativamente carregado a pH normal do ambiente (pH 5-8); meia-vida no solo > 14-21 d; meia-vida na água > 175 d; condições de campo que favorecem a percolação no solo: pluviosidade anual > 250 mm; aqüífero não confinado e solo poroso.

Índice GUS de vulnerabilidade de águas subterrâneas

O índice GUS é calculado através dos valores de meia-vida do composto no solo e do coeficiente de adsorção à matéria orgânica do solo ([GUS = (log $t_{1/2}$ no solo).(4 - log K_{oc})]), não levando em consideração outras propriedades, como por ex., solubilidade em água. As faixas de classificação dos compostos de acordo com sua tendência à lixiviação são GUS < 1,8 – não sofre lixiviação; 1,8 < GUS < 2,8 – faixa de transição e GUS > 2,8 – provável lixiviação.

Método de Goss

Os critérios propostos por esse método para a avaliação do potencial de poluição de águas superficiais por pesticidas, são:
- *alto potencial de transporte associado ao sedimento* (APTAS): meia-vida no solo ≥ 40 d e K_{oc} = 1000 ou meia-vida no solo ≥ 40 d e K_{oc} ≥ 500 e solubilidade em água ≤ 0,5 mg L^{-1};
- *baixo potencial de transporte associado ao sedimento* (BPTAS)*:* meia-vida no solo < 1 d ou meia-vida no solo ≤ 40 d, K_{oc} ≤ 500 e solubilidade em água ≥ 0,5 mg L^{-1} ou meia-vida no solo ≤ 2 d e K_{oc} ≤ 500 ou meia-vida no solo ≤ 4 d e K_{oc} ≤ 900 e solubilidade em água ≥ 0,5 mg L^{-1} ou meia-vida no solo ≤ 40 d e K_{oc} ≤ 900 e solubilidade em água ≥ 2 mg L^{-1};
- *alto potencial de transporte dissolvido em água* (APTDA)*:* meia-vida no solo > 35 d, K_{oc} < 1.000.000 e solubilidade em água ≥ 1 mg L^{-1} ou K_{oc} ≤ 700 e solubilidade em água entre 10 e 100 mg L^{-1};
- *maixo potencial de transporte dissolvido em água* (BPTDA): K_{oc} ≥ 1.000.000 ou meia-vida no solo ≤ 1 d e K_{oc} ≤ 100 ou meia-vida no solo < 35 d e solubilidade em água < 0,5 mg L^{-1} e
- as substâncias que não se enquadram em nenhum dos critérios acima são consideradas como tendo potencial médio para poluírem águas superficiais.

A Tabela 2 relaciona as propriedades físico-químicas dos pesticidas que são utilizadas para avaliar seu potenciail de poluição para as águas[13-15].

Pode-se verificar que na Tabela 2 as propriedades físico-químicas entre os cinco herbicidas estudados são bem diferentes entre si, dessa forma não se podem fazer generalizações sobre os destinos e impactos desses herbicidas no ambiente. Entretanto, é possível avaliar teoricamente, através das propriedades físico-químicas dos herbicidas, se apresentam algum risco potencial de poluição das águas. Para isso, emprega-se, geralmente, alguns critérios de "screening", como por ex. os adotados pela EPA, o índice de GUS e o método de Goss[15].

Amostragem

Após realizada a avaliação teórica preliminar foram coletadas amostras de água de rios na região produtora de arroz nos afluentes dos Rios Vacacaí-Mirim (80 amostras) e Vacacaí (60 amostras), nas safras 2001/02 e 2002/03, para poder se comparar os dados obtidos pelo método de Goss na avaliação teórica. O intervalo entre as coletas foi, em ambas as safras, quinzenal para a Bacia do Vacacaí e semanal para a Bacia do Vacacaí-Mirim, sempre na época de maior atividade de aplicação dos herbicidas. Efetuou-se sempre uma coleta anterior a esse período e uma posterior.

A área estudada pertence à Bacia Hidrográfica dos Rios Vacacaí e Vacacaí-Mirim que está localizada na Depressão Central do Rio Grande do Sul. A área limita ao leste no município de Cachoeira do Sul, distante aproximadamente 180 km de Porto Alegre pela BR-290, ao norte por Santa Maria e Restinga Seca; ao oeste por São Gabriel e ao sul por Caçapava do Sul. Nesta área de abrangência, optou-se pela amostragem em seis locais da Bacia do Rio Vacacaí: Passo do Verde; Passo da Lagoa; Passo do Rocha; Rio São Sepé; Rio Santa Bárbara e Restinga Seca.

Para a Bacia do Rio Vacacaí-Mirim, uma extensão de 40 km foi avaliada, desde o Distrito de Arroio Grande, no município de Santa Maria, até o município de Restinga Seca, onde predomina o cultivo de arroz irrigado em lavouras de pequeno porte. Nesta Bacia, foram selecionados cinco pontos para amostragem. Foi estabelecido um ponto denominado "branco", por supostamente não receber contribuição de águas de lavoura de arroz irrigado. Este

Tabela 2. Propriedades físico-químicas dos herbicidas que permitem avaliar o potencial de risco para ambientes aquáticos

Herbicida	Solubilidade em água (mg L^{-1})	K_{oc} (cm^3 g^{-1})	Log K_{ow}	PV (mPa)(20 °C)	pKa	K_H (Pa m^3 mol^{-1})	$t_{1/2}$ solo (d)	$t_{1/2}$ água (d)
Quinclorac	0,065 (20 °C)	36	-1,15 pH 7,0	< 0,01	4,34	< $3,72.10^{-2}$		
Bentazone	570 (20 °C)	34	5,8 pH 5,0	0,17	3,2-3,3	$7,4.10^{-5}$	14	
2,4-D	311 (25 °C)	60	2,6-2,8	$1,86.10^{-2}$	2,73	$1,32.10^{-5}$	< 7	7,5
Clomazone	1100 (25 °C)	150-562	2,54	19,2		$4,19.10^{-3}$	30-135	> 30
Propanil	130 (25 °C)	239-800	3,3	0,026		$3,6.10^{-3}$	2	1-2

d= dias, PV= pressão de vapor

ponto localiza-se próximo à nascente do Rio Três Barras.

A amostragem da água nos rios foi realizada em três pontos: uma no centro do leito e as demais nas proximidades das margens direita e esquerda, utilizando-se uma garrafa com capacidade para 2 L contendo orifícios da metade da garrafa até a extremidade superior. A garrafa coletora foi acoplada a um suporte com peso, permitindo coletar água desde a superfície até o fundo do rio. As amostras de água de lavoura foram coletadas diretamente em frasco de vidro âmbar, 10 cm abaixo da superfície da lâmina de água. Após coletadas, as amostras foram armazenadas em frasco de vidro âmbar, acidificadas com H_3PO_4 1:1 (v/v) e sob refrigeração transportadas até o laboratório para serem analisadas.

Descrição do procedimento de análise

Todas as amostras foram analisadas conforme descrito por Primel[18] e Zanella *et al*.[19]. Alíquotas de 250 mL de amostra foram acidificadas e pré-concentradas em cartuchos contendo 200 mg de C18. A eluição foi efetuada com 2 vezes 500 µL de metanol. Uma alíquota de 20 µL foi injetada no sistema cromatográfico contendo uma coluna Bondesil C18 (250×4,6 mm i.d; 5 µm) com pré-coluna do mesmo material. A eluição foi efetuada com mistura metanol e água (60:40, v/v), ajustada a pH 4,0 com ácido fosfórico, utilizando uma vazão de 0,8 mL min^{-1}. A detecção foi na região do UV em 220 nm.

RESULTADO E DISCUSSÃO

Águas de superfície

De acordo com os critérios de Goss usados para avaliar se um pesticida ao ser usado na agricultura pode atingir águas de superfície, pode-se dividí-los entre aqueles que podem ser transportados dissolvidos em água e aqueles que são transportados associados ao sedimento em suspensão. Assim, dos herbicidas em estudo o clomazone e o propanil indicam um alto potencial de poluição de águas de superfície (APTDA) porque podem ser transportados dissolvidos em água. O quinclorac apresenta um potencial médio (MPTA) com relação a esse parâmetro, enquanto o bentazone e o 2,4-D indicam um baixo potencial de poluição (BPTDA) de água de superfície no parâmetro solubilidade em água. Quanto ao transporte no sedimento em suspensão, os herbicidas clomazone, 2,4-D e propanil indicam baixo potencial de poluição de águas de superfície (BPTAS), e o bentazone e quinclorac indicam um potencial médio (MPTAS) de poluição dessas águas.

Águas subterrâneas

A Tabela 3 apresenta os resultados da análise do potencial de poluição de águas subterrâneas usando os critérios de "screening" propostos pela EPA. Foram considerados as propriedades apresentadas na Tabela 2 e os critérios citados anteriormente para analisar o risco dos pesticidas usados na região atingirem águas subterrâneas.

Apesar da falta de alguns dados para esta análise, não disponíveis na literatura, pode-se classificar alguns dos herbicidas como compostos que apresentam maior probabilidade de atingir as águas subterrâneas, pois apresentam considerável solubilidade em água, baixa adsorção à matéria orgânica do solo e tempo de meia-vida no solo relativamente alto.

Foram considerados poluentes em potencial somente aqueles princípios ativos para os quais a maioria das propriedades físico-químicas disponíveis indicava uma possibilidade de poluição das águas subterrâneas. Quando as informações disponíveis foram insuficientes para se concluir sobre o potencial de poluição do herbicida, o resultado foi apresentado como inconclusivo.

Segundo Dores *et al*.[15] compostos classificados na faixa de transição e de provável lixiviação de acordo com o índice de GUS requerem investigação adicional, usando-se procedimentos mais detalhados. Compostos classificados como improváveis de sofrerem lixiviação podem, seguramente, ser considerados como não poluentes de águas subterrâneas.

Considerando esta afirmação e os critérios da EPA pode-se dizer que para os herbicidas bentazone, 2,4-D, clomazone e propanil seriam recomendados estudos complementares sobre a possibilidade de poluição de águas subterrâneas na região. Com relação ao quinclorac, por falta de dados sobre diversas de suas propriedades, nada se pode afirmar sobre seu potencial de poluição.

Utilizando-se o índice de GUS, bentazone e clomazone podem ser considerados contaminantes em potencial, propanil como não contaminante, 2,4-D de transição e quinclorac inconclusivo.

Considerando a caracterização físico-hídrica do perfil do solo da área, destaca-se uma zona de maior macroporosidade nas camadas de 30 a 45 cm e de 45 a 60 cm de profundidade, com maiores valores relativos de condutividade hidráulica do perfil quando comparadas com as camadas superficiais. As camadas intermediárias contrastam com a camada arável, que se apresenta com microporosidade mais elevada. As condições texturais do perfil explicam esta modificação, por apresentarem elevados teores de silte nas camadas superficiais (64%) em relação às intermediárias (55 e 38%), contribuindo para os baixos valores de condutividade hidráulica saturada[20]. A possibilidade de lixiviação reduz-se bastante pelo fato deste solo ser mais arenoso na camada de 30 a 60 cm, e ter mais silte e argila na camada superficial. Assim, o movimento da água no perfil é menor na superfície do solo e é por isto que retém a água.

Embora aborde apenas algumas propriedades dos herbicidas sem levar em consideração as particularidades de solo e clima, a análise do potencial de lixiviação, segundo os critérios propostos, pode ser uma ferramenta para a avaliação inicial do potencial de poluição ambiental por herbicidas, pois a conjunção de altas doses, alto potencial de lixiviação e solos com baixa capacidade de

Tabela 3. Resultados da avaliação de risco de poluição das águas subterrâneas com base nos critérios estabelecidos pela EPA

Herbicidas	Solubil.	K_{oc}	$t_{1/2}$ solo	$t_{1/2}$ água	K_H	Resultado
QUIN	N	A		N	A	I
BENT	A	A	A	N	A	PC
2,4-D	A	A	N	N	A	PC
CLOM	A	A	A	N	A	PC
PROP	A	A	N	N	A	PC

N = não atende ao critério; A = atende ao critério como potencialmente perigoso; I = inconclusivo; PC = contaminante em potencial; NC = não contaminante; em branco = dado não disponível; Solubil. = Solubilidade em água.

retenção sugerem uma situação de alto risco do herbicida para o meio ambiente.

Análise de amostra de água de superfície da região em estudo

Pode-se verificar nas Tabelas 4 e 5, onde são demonstrados os resultados obtios para os Rios Vacacaí-Mirim e Vacacaí, respectivamente, que clomazone e propanil são os herbicidas que apresentaram maior percentual de amostras positivas. Isso pode ser devido à grande quantidade de clomazone aplicado em lavouras da região e à alta concentração de propanil que é aplicada na lavoura, como pode ser observado na Tabela 6, que apresenta os valores de resíduos de herbicidas obtidos para 63 amostras de águas coletadas em lavouras na região na safra de 2000-2001. Os resultados indicam que clomazone e propanil são os herbicidas mais freqüentemente detectados na água de lavoura, evidenciando que provavelmente eles sejam os mais utilizados em lavouras da região. Esses resultados confirmam a análise preliminar, onde se utilizou o método de Goss, no qual clomazone e propanil foram classificados como tendo APTDA.

Tabela 4. Número de amostras positivas obtidas para 80 amostras de água do Rio Vacacaí-Mirim e afluentes, coletadas nos anos de 2001-2003

Herbicidas	<LOQ	0,1-0,5 μg L^{-1}	0,5-2,0 μg L^{-1}	> 2,0 μg L^{-1}	% Pos [1]
QUIN	76	0	1	3	5
BENT	70	0	6	4	12,5
2,4-D	75	0	9	1	12,5
CLOM	68	0	6	6	15
PROP	68	0	9	3	15

[1] percentagem de amostras positivas.

Tabela 5. Número de amostras positivas obtidas para 60 amostras de água do Rio Vacacaí e afluentes, coletadas nos anos de 2001-2003

Herbicidas	<LOQ	0,1-0,5 μg L^{-1}	0,5-2,0 μg L^{-1}	> 2,0 μg L^{-1}	% Pos [1]
QUIN	0	0	0	0	0
BENT	54	0	0	6	10
2,4-D	44	0	2	4	10
CLOM	44	0	4	12	27
PROP	49	0	7	4	18

[1] percentagem de amostras positivas.

Tabela 6. Número de amostras positivas obtidas para as 63 amostras de água coletadas em lavouras na safra 2000-2001.

Herbicidas	<LOQ	0,1-0,5 μg L^{-1}	0,5-2,0 μg L^{-1}	> 2,0 μg L^{-1}	% Pos [1]
QUIN	60	0	2	1	5
BENT	60	0	3	0	5
2,4-D	54	0	4	5	14
CLOM	42	0	5	16	33
PROP	52	0	5	6	17

[1] percentagem de amostras positivas.

CONCLUSÃO

Esse trabalho demonstra que a quantidade de herbicidas usados nas lavouras de arroz irrigado influenciam diretamente os níveis de herbicidas que ocorrem nas águas de superfície das proximidades. Para reduzir a quantidade de herbicidas que atingem as águas de superfície são necessários programas de gerenciamento e conscientização para minimizar a quantidade aplicada.

Herbicidas usados na cultura do arroz irrigado têm um efeito prejudicial potencial para a vida aquática, pois a drenagem da água da lavoura de arroz irrigado coincide com a época de reprodução dos peixes. Então, todo sistema de cultivo de arroz que libera água para o meio ambiente precisa ser monitorado com relação à concentração de herbicidas, e planos de gerenciamento, de manejo da cultura e de desempenho para proteger a vida aquática precisam ser implementados.

Considerando a grande importância de se conhecer o nível de herbicidas nas águas, mais estudos são necessários para determinar exatamente os processos de dispersão de herbicidas aplicados na lavoura, tais como volatilização, degradação por microrganismos e luz solar, e adsorção no solo, pois os herbicidas podem ser prejudiciais à saúde humana e ao meio ambiente, demonstrando esses efeitos mesmo em pequenas concentrações.

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Article

Risk Assessment of Surface Water Contamination by Herbicide Residues: Monitoring of Propanil Degradation in Irrigated Rice Field Waters using HPLC-UV and Confirmation by GC-MS

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O estudo avalia a degradação do herbicida propanil e de seu principal produto de degradação, a 3,4-dicloroanilina (3,4-DCA), em água de irrigação de lavouras de arroz irrigado no Estado do Rio Grande do Sul, Brasil. Além disso, estima, também, o risco de contaminação das águas de superfície das proximidades. Após uma etapa de extração em fase sólida, a concentração de ambos os compostos foi determinada por cromatografia líquida de alta eficiência com detecção na região do ultravioleta. A confirmação foi realizada por cromatografia gasosa-espectrometria de massas. A concentração de propanil nas amostras de água variou de 0,1 a 3600 μg L^{-1}. O propanil degradou-se rapidamente em 3,4-DCA, sendo que altas concentrações deste produto foram encontradas, variando entre 1,0 e 567,5 μg L^{-1}. Os tempos de meia-vida para o propanil em água sob condições reais nas safras de 2001, 2002 e 2003 foram 18,2, 12,5 e 12,2 h, respectivamente.

This study evaluates the degradation of the herbicide propanil and of its major degradation product, 3,4-dichloroaniline (3,4-DCA) in water from irrigated rice farming in the State of Rio Grande do Sul, Brazil. It also assesses the contamination risk of surrounding surface waters. After a solid phase extraction step, the concentration of both compounds was determined by high performance liquid chromatography with ultraviolet detection. Confirmation was conducted by gas chromatography-mass spectrometry. Concentrations of propanil in water samples varied from 0.1 to 3600 μg L^{-1}. Propanil was degraded very rapidly to 3,4-DCA and high concentrations of this product were found, varying from 1.0 to 567.5 μg L^{-1} in water. The obtained half-life times for propanil in water under real conditions for the 2001, 2002 and 2003 harvests were 18.2, 12.5 and 12.2 h, respectively.

Keywords: propanil, degradation, rice fields, water analysis, herbicide residues

Introduction

Although agriculture is just one of the countless nonpoint sources of contamination, it is generally targeted as the largest source among all pollutant categories. In Brazil, the degree to which these pollutants contribute to deteriorate water quality has not been quantified. In the United States, however, it is admitted that 50 to 60% of the pollutant load in lakes and rivers comes from agriculture.[1] The rice irrigated culture in Brazil, is notable in the Rio Grande do Sul State, comprising 73% of the cultivated area of this culture. In most of the rice farms, the pesticide applications are made after the irrigation, directly in the water. Depending on the water producer's handling and on the occurrence of rain after pesticide application, there is a risk that residues of these compounds be carried out of the area, contaminating water sources.[2-5]

In the pre-germinated system of irrigated rice cultivation, frequently employed in Brazil, area drainage after sowing set off serious environmental problems, as well as, may cause suspended nutrients and/or pesticides losses in the released irrigation water. This has been

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evidenced in studies carried out by Primel[6] and Machado,[7] in which the occurrence of some herbicides, mainly those that present high persistence, was confirmed in river and irrigation waters. Very little, up to now, has been done in Brazil to study the behavior and destination of herbicides in the system, as an attempt to maintain treatments' efficiency at a lower risk of environmental damage.[8]

Propanil (3,4-dichloropropionanilide) presented in Figure 1A, CAS RN 709-98-8, is one of the most used herbicides in the cultivation of irrigated rice in Brazil. It is a post-emergent selective contact herbicide with short duration used to control the electron transport inhibition of photosynthesis in herbs of wide leaves.[9] According to Barceló *et al.*[10] propanil was also one of the most used in Ebre Delta wetland area (Tarragosa, Spain) and, according to Coupe *et al.*,[11] it was extensively used in the Mississipi Delta area (USA). Various studies[10,12-15] have verified that the herbicide propanil is degraded quickly into 3,4-dichloroaniline (3,4-DCA), presented in Figure 1B, CAS RN 95-76-1.

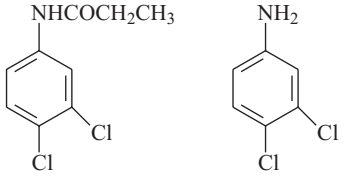

Figure 1. Structure of the herbicide propanil (A) and of the metabolite 3,4-DCA (B).

The meaningful contribution of this study was the development of an analytical method and its application to determine the persistence of propanil herbicide in irrigated rice farm waters. Field experiments carried out tracked propanil degradation and determined its half-life time under field conditions at experimental areas. Therefore, it was possible to assess the contamination risk of adjacent surface waters. The determination of propanil and 3,4-DCA was done by high performance liquid chromatography with ultraviolet detection (HPLC-UV) and gas chromatography-mass spectrometry (GC-MS) was used for confirmation.

Experimental

Instrumentation

An HPLC-UV system Shimadzu (Tokio, Japan) was equipped with an isocratic pump LC-10AD, a UV-visible spectrophotometric detector SPD-10 AV, an integrator C-R6A, an analytical column Bondesil C18 (250 × 4.6 mm i.d.; 5 mm) and a pre-column of the same material (20 × 1 μm), both from Varian (Palo Alto, USA).

The GC-MS system was a Varian model 3800 equipped with an injector model 1079, a capillary column VF 5MS low bleed of 30 m length × 0.25 mm i.d., 0.25 μm film (Varian) and an autosampler model AS8200 with a carrousel for 48 vials, coupled to a mass spectrometer Varian model Saturn 2000, ion trap type, operating with a scan range between 10 to 650 *m/z* and an electron impact (EI) ionization at 70 eV. The scan time was 0.90 s and 1.0 μL injections were made. The data acquisition system was a Saturn GC/MS Workstation, version 5.51.

A vacuum pump Leybold-Heraeus D3 (Germany), a SPE manifold Varian for the simultaneous preconcentration of 20 samples, a pH meter Cole Parmer (Illinois, USA) series 500 and an ultra-sound bath Bandelin Sonorex RK 510 (Berlin, Germany) were also used.

Reagents, solvents and samples

Water used in the preparation of all the solutions was purified in a Milli-Q system (resistivity of 18.2 MΩ cm). Methanol ChromAR HPLC, dichloromethane residue degree (Mallinckrodt, NJ, USA) and phosphoric acid p.a. 85% (Merck, Darmstadt, Germany) were also used. Analytical stock solutions were individually prepared through the dissolution in methanol of the respective solid reference standard of propanil (99.3% of purity) and 3,4-DCA (99.5% purity), both from ChemService (West Chester, PA, USA). After preparation, the solution was stored in amber flask glass at –18 °C.

Description of the field experiment

The experiments were carried out together with the Crop Science Department from the Federal University of Santa Maria (UFSM) in the experimental fields at the campus, located in the central region of Rio Grande do Sul State, Brazil. The experiments were conducted in the agricultural years of 2001, 2002 and 2003 from November to March. The soil was of medium texture, with 22% of clay and 1.9% of organic matter. The experiment was entirely randomized with four repetitions. Fields of 16 m^2 (4 × 4 m) for each repetition were used and the commercial product Stam® 800 GD was applied at a dosage of 3600 g of propanil *per* ha^{-1}.

The pre-germinated system of cultivation was used. For the herbicide application, a precision coastal pulverizer propelled with CO_2 was used, operating at a pressure of 275 kPa and a consumption corresponding to 150 L ha^{-1}. Considering a water layer of 10 cm, the theoretical initial

concentration estimated for the herbicide propanil was of 3600 mg L^{-1}. During the field experiments the water layer was maintained at the same level.

In the 2001 harvest, water sampling was carried out shortly before the herbicide application and on days 1, 2, 7, 14, 21, 28 and 60, after its application. In the following years, water sampling was made on days 1, 2, 3, 5, 7, 10, 14, 21, 28, 35 and 60, after herbicide application.

Water sampling was accomplished in a 1 L amber glass flask at 5 cm under the water surface. The samples were sent to the Laboratory of Analysis of Residues of Pesticides (LARP) of the Chemistry Department at UFSM for chemical analysis.

Description of the analysis procedure

All samples were analyzed as described by Primel[6] and Zanella *et al.*[16] 250 mL aliquots, previously filtered in 47 mm diameter nylon membranes and 0.45 μm porosity, were acidified to pH 3.0 with phosphoric acid and pre-concentrated in a cartridge containing 500 mg of C18. The elution was carried out with two 500 μL methanol aliquots. A volume of 20 μL was injected in the HPLC-UV system. As mobile phase a mixture (24:30:46; v/v) of methanol, acetonitrile and water was used, adjusted to pH 3.0 with phosphoric acid, at a flow-rate of 1.0 mL min^{-1}. The detection was performed by UV at 220 nm.

The confirmation was carried out by GC-MS under the following conditions: carrier gas: helium at a constant pressure of 14 psi, resulting in an initial flow-rate of 1.2 mL min^{-1}; injected volume: 1 μL (splitless); injector temperature of 250 °C; temperature program of the column oven: 45 °C, held for 1.5 min, then a gradient of 10 °C min^{-1} up to 260 °C, maintained for 4 more min; mass spectrometer operating with the following temperatures: transfer line: 290 °C, manifold: 80 °C and ion trap: 240 °C. For the GC-MS analyses, the solvent used for elution in the SPE step was evaporated using a N_2 current and the remaining residue was dissolved in dichloromethane. The GC-MS acquisition data was made in the MS mode, with ion monitoring from the full scan spectrum of the compounds in study. For propanil and 3,4-DCA, the monitored ions were *m/z* 217, 161, 126, 90 and 57; and 161, 126 and 90, respectively.

Results and Discussion

Reversed-phase HPLC with UV detection after a solid-phase extraction step proved to be a good choice for propanil and 3,4-DCA determination, allowing to evaluate these compounds concentration variation in agricultural waters. The HPLC-UV chromatographic separation profile of 3,4-DCA and propanil from a standard containing 1.0 mg L^{-1} each compound is demonstrated in Figure 2, in which these compounds presented retention time (t_R) values of 8.8 and 14.4 min, respectively.

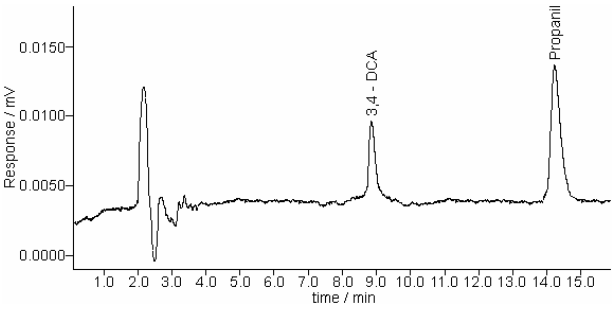

Figure 2. HPLC-UV chromatogram of 3,4-DCA and propanil from a standard at a concentration of 1.0 mg L^{-1} each.

The experimental results of 2001, 2002 and 2003 harvests are presented in Table 1. Propanil herbicide presented residues up to the seventh day after application. The sudden reduction of the concentration of propanil to 2.3 mg L^{-1} on the seventh day or to 0.1 mg L^{-1} on the fifth day, respectively in 2001 and 2003, is due to its fast hydrolysis. Available data indicate that propanil does not persist for a long time in the atmosphere, being metabolized in the matrix soil/water. However, propanil and its metabolite 3,4-DCA can constitute a risk for surface waters and for human health.[17] Observational water studies carried out in the US[18] showed that 3% of 1560 analyzed samples contained propanil concentration up to 2 μg L^{-1} and in 50% of the samples, 3,4-DCA was detected up to 8.9 μg L^{-1}.

Table 1. Results obtained in the three harvests in the study of the propanil and 3,4-DCA

Days after application	Propanil/(μg L^{-1})			3,4-DCA/(μg L^{-1})		
	2001	2002	2003	2001	2002	2003
1	3600	3445	1630	112.3	104.5	195.1
2	1244	2285	283	352.5	265.8	380
3	n.c.	4.0	129	411.4	567.5	316.5
5	n.c.	21.5	0.1	n.c.	166.8	96.7
7	2.3	50.1	n.d.	65.2	12.0	37.9
10	n.c.	0.1	n.d.	n.c.	5.2	3.5
14	n.d.	n.d.	n.d.	1.6	1.0	n.d.

n.c. = not collected; n.d. = not detected

For propanil herbicide, results indicate that the concentration decreases quickly during the first days, in the 2001 harvest. 3,4-DCA concentration increases until the 3rd day and then starts to decrease. 3,4-DCA is detected up until the second week after propanil application as can

be observed in Table 1. On the 14th day after its application, 3,4-DCA presence was still detected at a concentration of 1.6 μg L^{-1}.

In the 2002 harvest, propanil concentration decreases until the 3rd day, increasing in the samples collected on the 5th and 7th days and then decreases again. Herbicide residues were found up until the 10th day after application. 3,4-DCA was detected until the 14th day, as may be observed in Table 1, in which its concentration reached 1.0 μg L^{-1}.

In the 2003 harvest, propanil concentration remains high (129 μg L^{-1}) until the 3rd day after application. No propanil herbicide residues are found after this, whereas 3,4-DCA presence is detected until the 10th day after the herbicide application, as may be observed in Table 1, in which its concentration was 3.5 μg L^{-1}.

Propanil and 3,4-DCA confirmation, as accomplished by GC-MS, and their chromatographic profiles are presented in Figure 3, in which they showed t_R values of 14.2 and 19.6 min, respectively. Propanil and 3,4-DCA mass spectra monitoring ion *m/z* 126 are presented in Figures 4 and 5, respectively. The main fragments identified, their relative intensities and *m/z* relation are shown in Table 2.

Spectra and chromatograms obtained by sample injection were compared to those of standard analytical solutions for these compounds. This ion selection allowed a larger selectivity in the identification of propanil and 3,4-DCA, isolating the signal of these compounds from the signals of impurities present in the samples.

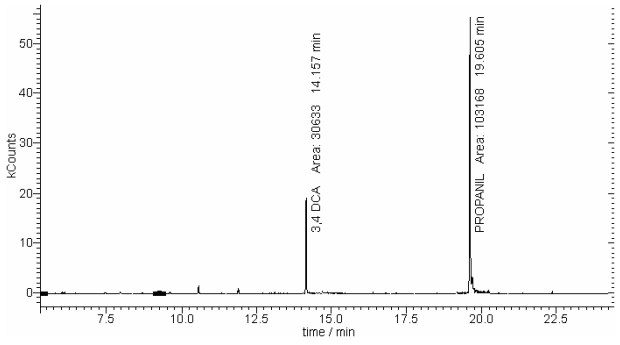

Figure 3. GC-MS chromatogram of 3,4-DCA and propanil.

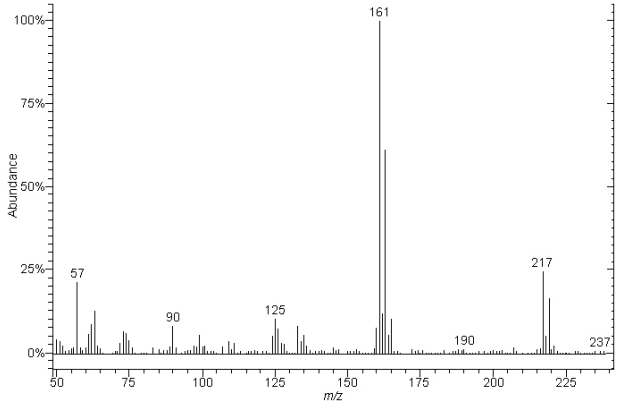

Figure 4. Mass spectra obtained for propanil by GC-MS.

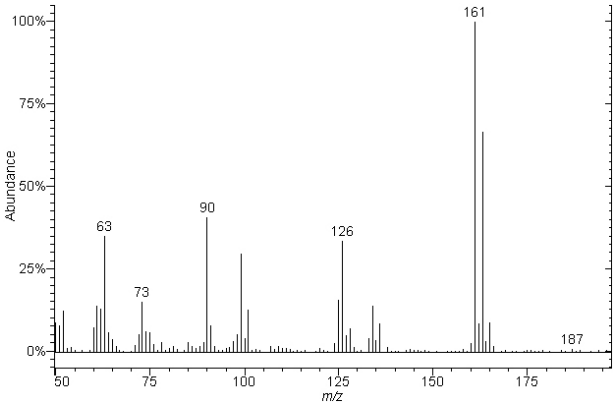

Figure 5. Mass spectra obtained for 3,4-DCA by GC-MS.

Table 2. Ion fragments obtained for propanil and 3,4-DCA by GC-MS operating in the positive mode

Compound	Molecular mass	*m/z* (relative intensity) [characteristic ions]
3,4-DCA	162	161 (100%) $[M-H]^+$ 126 (30%) $[M-H-Cl]^+$ 90 (40%) $[M-H-Cl_2]^+$
Propanil	218	217 (25%) $[M-H]^+$ 161 (100%) $[M-H-COCH_2CH_3]^+$ 125 (12,5%) $[M-H-COCH_2CH_3Cl]^+$ 90 (12,5%) $[M-H-PhN]^+$ 57 (25%) $[M-H-PhCl_2NH_2]^+$

Determination of the half-life time

ln C/Co = - kt equation can be used to determine the order of a reaction if the reagent concentration as a time function is known. One way of characterizing the systems is by the determination of the half-life time ($t_{1/2}$) of the reagent. Then, if we plot ln C as a function of t, we will obtain a straight line, whose angular coefficient (rate constant) is -k. Also this constant -k is detemined by the nature of reagents for a given temperature and is a numerical value characteristic of each reaction.

In order to calculate propanil half-life time in irrigation waters, we used $t_{1/2}$ = ln 2/k equation, in which k is the slope of the straight line. The mean half-life time for propanil was 18.2 h for the 2001 harvest, 12.5 h for 2002 and 12.2 h for 2003. These values are in accordance with propanil $t_{1/2}$ values in water for 1–2 days reported by other authors.[9,10] According to Barceló *et al.*[10] propanil degradation is directly dependent on the environmental

conditions and on the pesticide application rate. An increase of the sun irradiation time was observed in the last two years of this study, suggesting that this could be the main cause for the observed $t_{1/2}$ reduction.

Conclusions

According to the results obtained, it can be recommended that irrigation waters should be maintained for at least 10 days, after propanil herbicide application, before release into the environment, thereby avoiding watercourse contamination. It was also possible to observe in water samples from experimental irrigated rice farms, where herbicide was applied, that propanil was quickly degraded into 3,4-DCA, its main degradation product. These results made the determination of propanil half-life time in irrigation water possible. The average time were 18.2, 12.5 and 12.2 h, respectively, for 2001, 2002 and 2003 harvests.

GC-MS analysis allowed propanil and 3,4-DCA identification, thereby confirming the results obtained by HPLC-UV.

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Short Communication

Effects of four rice herbicides on some metabolic and toxicology parameters of teleost fish (*Leporinus obtusidens*)

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Abstract

Effects of different herbicides on acetylcholinesterase (AChE), catalase and TBARS formation in teleost fish (*Leporinus obtusidens*) were studied. Fish were exposed during 30 days at concentrations of herbicides used in rice field. AChE activity in the brain decreased significantly after exposure to the herbicides clomazone and quinclorac. However, AChE activity increased significantly in muscle tissue after exposure to clomazone, propanil and metsulfuron methyl. Fish exposed to quinclorac, propanil and metsulfuron methyl showed TBARS decreased levels in brain and muscle tissues. However, TBARS and catalase activity increased in liver tissue after clomazone and propanil exposure. This study pointed out long-term effects on AChE activity, oxidative stress and antioxidant enzyme catalase in tissues of *L. obtusidens* after exposure to environmentally relevant concentrations of rice field herbicides. These parameters have been used to monitor fish toxicity in rice field system.

Keywords: Herbicides; *Leporinus obtusidens*; AChE; TBARS; Catalase

1. Introduction

Clomazone (isooxazolidinone), quinclorac (quinoline), propanil (dichloropropionanilide) and metsulforon methyl (sulfonylurea) are herbicides extensively used in agriculture, especially in paddy rice fields in Southern Brazil (Rodrigues and Almeida, 1998; Jonsson et al., 1998). Clomazone is highly effective, but causes groundwater contamination due to its water solubility (1100 mg l^{-1}) and long half-life dissipation averaging from 28 to 84 days (Colby et al., 1989; Zanella et al., 2002). Water solubility of quinclorac is 0.065 mg l^{-1} and its half-life in water is 21 days. Propanil has a water solubility of 130 mg l^{-1} and it is rapidly degraded in water by sunlight with a half-life of 12 h (Rodrigues and Almeida, 1998). Metsulfuron methyl has a water solubility of 9.5 mg l^{-1} and its half-life in the soil is 30 days (Barceló and Hennion, 2002; Ware, 2003).

Recently, there have been a great number of studies considering changes induced by environmental contamination in aquatic organisms (Sancho et al., 2000; Lionetto et al., 2003; Sayeed et al., 2003). Herbicides and pesticides may produce a disruption of the ecological balance causing damage to non-target organisms, such as fish (Oruç and Üner, 1999; Bretaudt et al., 2000). Acetylcholinesterase (AChE; EC 3.1.1.7) activity is frequently used as carbamate and organophosphate toxicity indicators (Chuiko, 2000; Aguiar et al., 2004). However, brain and muscle AChE activity was inhibited by herbicides of other classes, such as isooxazolidinone clomazone (Miron et al., 2005) and the pesticide endosulfan (Dutta and Arends, 2003).

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The inhibition of acetylcholinesterase activity affects animals due to the central role of this enzyme regulating the proper levels of the neurotransmitter acethylcholine in the central nervous system, neuromuscular junction and the sympathetic synapses (Sancho et al., 2000). Disturbances in AChE activity can also affect locomotion and equilibrium in exposed organisms (Saglio and Trijasse, 1998; Bretaudt et al., 2000; Miron et al., 2005).

Many environmental pollutants have lipid peroxidation (LPO) inducing effects on fish (Schlenk et al., 1997; Üner et al., 2005). Contaminants as herbicides and pesticides may induce the formation of reactive oxygen species (ROS), and these substances are highly reactive causing damage to lipids, proteins, carbohydrates and nucleic acids (Fraga et al., 1996; Sevgiler et al., 2004). Variations in the activities of antioxidant enzymes have been proposed as indicators of pollutant mediated oxidative stress (Ahmad et al., 2000; Sayeed et al., 2003). If the antioxidant system does not properly remove potent oxidants such as H_2O_2, superoxide (O_2^-) and hydroxyl radicals (HO), they can lead to oxidative stress (Sevgiler et al., 2004).

Leporinus obtusidens (piava) was chosen for this study, since the effect of herbicide exposure at rice field condition has scarcely been studied on this fish species. Besides, it was also chosen as a model organism due to its sensitivity in detecting potential effects of chemicals. *L. obtusidens* is a native freshwater fish of Southern Brazil with good potential for cultivation (Andrian et al., 1994; Glusczak et al., 2006). The aim of the present study was to verify the relationship between concentrations of herbicides used in rice field and some parameters of toxicity in tissues of *L. obtusidens*.

2. Materials and methods

2.1. Chemicals

Herbicides were obtained commercially as follows: clomazone (2-(2-chlorophenyl)methyl-4,4-dimethyl-3-isooxazolidinone) (Gamit; 50% purity), quinclorac (3,7-dichloroquinoline-8-carboxylic acid) (Facet; 50% purity), propanil (3′,4′-Dichloropropananilide) (Milenia; 36% purity) and metsulfuron methyl (methyl2-[[[(4-metoxi-6-methyl-1,3,5-triazine-2-)amino]carbonil]amino] sulfonyl-benzoate-sulfonylurea) (Ally; 50% purity). The sources of herbicides were FMC (EUA), BASF, MILENIA and DUPONT do Brasil, respectively. Acethylthiocholine, 5,5′dithio-bis(2-nitrobenzoic acid) (DTNB), bovine serum albumin, Triton X-100, hydrogen peroxide (H_2O_2), malondialdehyde (MDA), 2-thiobarbituric acid (TBA) and sodium dodecyl sulfate (SDS) were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Fish

L. obtusidens of both sexes were obtained from the fish farm of Santa Maria Federal University (UFSM) in RS, Brazil. Fish (weight, 10.0 ± 1.0 g; length, 8.0 ± 1.0 cm) were acclimated in tanks (250 l) for 15 days under laboratory conditions. They were kept in continuously aerated water in a static system and with a natural photoperiod (12 h light – 12 h dark). Water parameters were measured every day and were as follow: temperature 22 ± 2.0 °C, pH 6.5 ± 0.2 units, dissolved oxygen 6.2 ± 1.0 mg l^{-1}, non-ionized ammonia 0.7 ± 0.01 μg l^{-1}, nitrite 0.05 ± 0.01 mg l^{-1}, alkalinity 12 ± 1.3 mg l^{-1} $CaCO_3$ and hardness 35 ± 1.5 mg l^{-1} $CaCO_3$. During acclimation, fish were fed once a day with commercial fish pellets (42% crude protein, Supra, Brazil).

2.3. Experimental design

After acclimation, fish were transferred to ponds, and were exposed for 30 days (triplicate). The herbicides used were clomazone (0.5 mg l^{-1}), quinclorac (0.375 mg l^{-1}), propanil (3.6 mg l^{-1}) and metsulfuron methyl (0.002 mg l^{-1}). Herbicides were added to the water only at the beginning of the experiment. During the experimental period fish were fed every day with commercial ration (44% crude protein) (Purina Brazil). Three ponds without herbicide were used as a control, and these ponds were sampled at each time.

2.4. AChE assay

Tissue samples (brain and muscle) were weighed and homogenized in a Potter-Elvejhem glass/Teflon homogenizer with 150 mM NaCl. Homogenates were centrifuged for 15 min at 3000*g* at 5 °C and the supernatant was used as the enzyme source. AChE (EC 3.1.1.7) activity was measured as described by Ellman et al. (1961) and modified by Miron et al. (2005). Aliquots of supernatant (50–100 μl for brain and muscle, respectively) were incubated at 25 °C for 2 min with 0.1 M phosphate buffer, pH 7.5, 1 mM DTNB as chromogen. After 2 min, the reaction was initiated by the addition of acethylthiocholine (0.08 M) as substrate for the reaction mixture. The final volume was 2.0 ml. Absorbances were determined at 412 nm during 2 min. Enzyme activity was expressed as μmol of acethylthiocholine (ASCh) hydrolyzed min^{-1} mg^{-1} of protein.

2.5. Catalase assay

Catalase (EC 1.11.1.6) activity was assayed by ultraviolet spectrophotometry (Nelson and Kiesow, 1972). Samples of liver were homogenized in a Potter-Elvejhem glass/Teflon homogenizer with 20 mM potassium phosphate buffer, pH 7.4 (with 0.1% Triton X-100 and 150 mM NaCl) (1:20 dilution), centrifuged at 10000*g* for 10 min at 4 °C. Briefly, the assay mixture consisted of 2.0 ml potassium phosphate buffer (50 mM, pH 7.0), 0.05 ml H_2O_2 (0.3 M) and 0.05 ml homogenate. Change of H_2O_2 absorbance in 60 s was measured at 240 nm. Catalase activity was calculated in terms of Δ min^{-1} mg^{-1} protein.

2.6. Lipid peroxidation levels (TBARS)

Peroxides produced can be quantified by a TBARS assay. This is performed by a malondialdehyde (MDA) reaction with 2-thiobarbituric acid (TBA), which is optically measured. Liver, muscle and brain homogenates (100–400 μl) were added to 8.1% sodium dodecyl sulfate (SDS) and 2.5 M acetic acid (pH 3.4). Afterwards, 0.8% thiobarbituric acid was added to adjust to a final volume of 2.0 ml. The reaction mixture was placed in a microcentrifuge tube and incubated for 90 min at 95 °C. After cooling, it was centrifuged at 5000*g* for 10 min and optical density at 532 nm was determined. TBARS levels are expressed as nmols MDA mg^{-1} of protein according to Janero (1990) and Ohkawa et al. (1979). Protein levels were estimated by the method of Bradford (1976) using bovine serum albumin as standard.

2.7. Statistical procedures

Statistical analyses were performed using a one-way analysis of variance (ANOVA). Means were compared by Tukey test and expressed as mean ± standard deviation ($n = 8$). Differences were considered to be significant at a probability level of $P < 0.05$ between treatments and control. All statistical analyses were carried out using the Statisitica 5.1 software for windows.

3. Results and discussion

AChE activity after exposure to the herbicides clomazone and quinclorac decreased significantly ($P < 0.05$) in brain (Fig. 1). However, propanil and metsulfuron-methyl did not alter brain AChe activity. Muscle tissue showed AChE activity increased until 65% ($P < 0.05$) after exposure to the herbicides clomazone, propanil and metsulfuron methyl, respectively (Fig. 1), however, quinclorac did not alter muscle AChE activity. AChE activity in the brain of fish exposed to clomazone and quinclorac was lower than that in the control group (reduction of activity ranging from 16.6% to 31%, respectively). Recent data from our laboratory have showed that the herbicide clomazone is a potent brain AChE inhibitor (83%) of teleost fish *Rhamdia quelen* (Miron et al., 2005). AChE activity in brain of *Anguilla anguilla* after molinate exposure decreased significantly ranging from 16% to 31% as time of exposure increased (Sancho et al., 2000). The AChE activity is extremely important for many physiologic functions, such as predator evasion, prey location and orientation toward food. When AChE activity decreases, as in this study, AChE is not broken and accumulates within synapses, which therefore cannot function in a normal way (Fernández-Vega et al., 2002; Dutta and Arends, 2003). The results of the present study showed that in unexposed fish, brain AChE activity was two-fold higher than that in muscle tissue. Higher brain AChE activity compared to that of muscle was also observed in channel catfish (*Ictalurus punctatus*) (Straus and Chambers, 1995) and silver catfish (*R. quelen*) exposed to different herbicides (Miron et al., 2005). Muscle AChE activation observed in the present study after exposure to clomazone, propanil and metsulfuron methyl could be due to an accumulation of the substrate acethylthiocholine in the brain, causing overstimulation of the receptors. Thus, activaction or inhibition of AChE in these tissues can influence the process of cholinergic neurotransmission and promote undesirable effects. Under the test conditions used, the herbicides tested at rice field condition changed brain and muscle AChE activity and can be used to monitor herbicide toxicity.

In the present study, we observed that herbicide exposure at rice field concentration changes TBARS levels in tissues of *L. obtusidens*. Fish exposed to quinclorac, propanil and metsulfuron methyl herbicides decreased TBARS levels ($P < 0.05$) in brain and muscle. However, liver tissue showed increased TBARS levels after clomazone and propanil exposure (Fig. 2). The results concerning TBARS levels may indicate a compensatory response of the fish in order to survive after herbicide induced oxidative stress. Some authors also observed elevated levels of TBARS induced by aquatic contaminants (Li et al., 2003; Üner et al., 2006). According to Li et al. (2003), TBARS levels increased in the liver of *Carassius auratus* exposed to 3, 4-dichloroaniline (0.4 mg l^{-1}) for 15 days. The level of lipid peroxidation may differ among fish species. Elasmobranchs produce higher levels of peroxides than seawater teleosts, which present a higher level than that of freshwater fish.

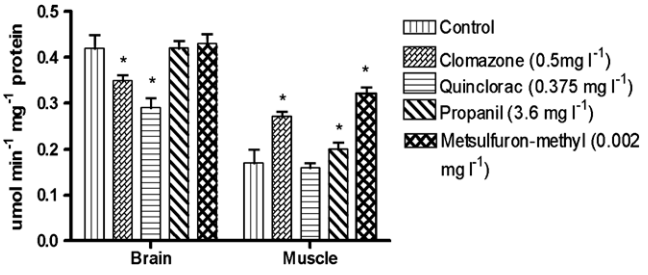

Fig. 1. AChE activity in brain and muscle of *Leporinus obtusidens* exposed to different herbicides (30 days). Values are means ± SD ($n = 8$). Data are expressed as μmol min^{-1} mg^{-1} protein. *Indicates difference between groups and control ($P < 0.05$).

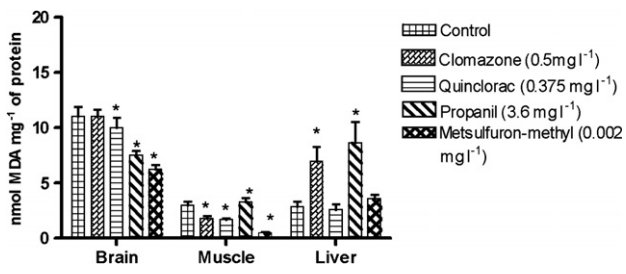

Fig. 2. TBARS levels (nmol MDA mg^{-1} protein) in brain, liver and muscle of *Leporinus obtusidens* exposed to different herbicides (30 days). Data represent the mean ± SD ($n = 8$). *Indicates difference between groups and control ($P < 0.05$).

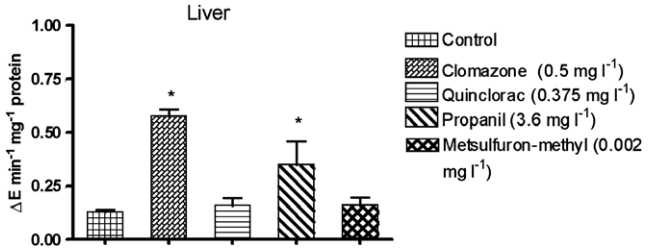

Fig. 3. Catalase activity (ΔE min^{-1} mg^{-1} protein) in liver of *Leporinus obtusidens* exposed to different herbicides (30 days). Data are reported as mean ± SD ($n = 8$). *Indicates significant difference between groups and control ($P < 0.05$).

Differences are also attributed to the variation in antioxidant mechanisms of fish species (Ahmad et al., 2000). The results of the present investigation clearly indicated that the exposure of fish to sublethal concentrations of these herbicides causes significant changes in TBARS production in all investigated tissues. However, the effect varied depending on the tissue considered and the herbicide tested.

This study showed an increase of the catalase activity in the liver of *L. obtusidens* after exposure to the herbicides clomazone and propanil (Fig. 3). However, according to previous experiments in our laboratory (Crestani et al., 2007), a reduction in catalase activity was observed in the liver of silver catfish exposed to clomazone (0.5 or 1.0 mg l^{-1}) after 12, 24 and 96 h. Oxidative stress generated by water containing herbicide may suppress antioxidant defense enzyme activities, due to their oxidative damage and a loss of compensatory mechanisms. Thus, results concerning catalase activity suggested a possible oxidative liver damage, and catalase increase probably helping to detoxify herbicides.

The present study showed that concentrations and class herbicides used in agriculture may cause changes in toxicology parameters of *L. obtusidens*. The evaluation of these parameters can be considered to monitor herbicide toxicity in fish when exposed to contaminated water.

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RICE HERBICIDE MONITORING IN TWO BRAZILIAN RIVERS DURING THE RICE GROWING SEASON

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ABSTRACT: Irrigated rice production can involve environmental contamination with pesticides due to the proximity of the fields to rivers and to management problems. During three years (2000 to 2003) the rice herbicides clomazone, propanil and quinclorac were quantified in water during the rice growing season, in the Vacacaí and Vacacaí-Mirim Rivers, located in Rio Grande do Sul (RS) State, Brazil. Water samples were taken at several locations in each river, selected by their importance in terms of rice drainage area. The samples were analyzed by HPLC-UV. At least one herbicide was detected in 41% of the samples from the Vacacaí River and 33% from the Vacacaí-Mirim River. The most frequent herbicide in both rivers and in each year was clomazone. The amount of herbicides in the river water was dependent on the rainfall regime. River water contamination by rice herbicides is probably caused by the rice water management used in the fields. The maintenance of flooded areas makes herbicides prone to contaminate the environment. To reduce the environmental contamination risk it is necessary to adopt measures to avoid overflow of flooded rice fields, keeping paddy water in the field for time enough to reduce the herbicide concentration before its release and enhancing the quality of the levees to reduce the probability of paddy rice overflow.
Key words*:* clomazone, environmental impact, pesticides, propanil, quinclorac

MONITORAMENTO DE HERBICIDAS EM DOIS RIOS BRASILEIROS DURANTE O PERÍODO DE CULTIVO DO ARROZ

RESUMO: No cultivo de arroz irrigado a possibilidade de contaminação dos mananciais hídricos é ampliada pelas características peculiares das áreas e do sistema de produção. Um estudo de monitoramento foi conduzido durante três anos (2000 a 2003), nos rios Vacacaí e Vacacaí-Mirim, localizados no Estado do Rio Grande do Sul, Brasil, buscando quantificar os herbicidas clomazone, propanil e quinclorac durante o período de cultivo do arroz. As amostras de água foram coletadas em vários locais em cada rio. Os locais de coleta foram selecionados pela importância em termos da captação da água de drenagem. As amostras foram analisadas por HPLC-UV. Herbicidas foram detectados nas águas dos rios durante no período de cultivo do arroz. Foi detectada a presença de pelo menos um herbicida em 41% das amostras no rio Vacacaí e 33% das amostras no rio Vacacaí-Mirim. O herbicida clomazone, foi detectado com maior freqüência nos dois rios. A quantidade de herbicida nas águas dos rios foi dependente do regime de chuva. A contaminação das águas dos rios pelos herbicidas utilizados no arroz provavelemente é decorrente do manejo de água adotado na região. A manutenção de áreas inundadas propicia a contaminação do ambiente por herbicidas. Para reduzir o risco de contaminação ambiental faz-se necessário à adoção de medidas que evitem a saída e liberação da água com resíduo das áreas de cultivo, mantendo-a na lavoura durante o tempo suficente para a redução da concentração do herbicida. A probabilidade de extravasamento pode ser reduzida com a melhor construção das taipas-ronda.
Palavras-chave: clomazone, impacto ambiental, pesticida, propanil, quinclorac

INTRODUCTION

Agrochemical use in agriculture leads to increasing crop yield and profits. However, their excessive use or misuse can cause environmental contamination of surface and ground water that can occur by drift, runoff, drainage and leaching (Cerejeira et al., 2003). Several surface water monitoring programmes have been carried out to quantify the degree of contamination by pesticides (Kammerbauer & Moncada, 1998; Huber et al., 2000; Kolpin et al., 2000; Bouman et al., 2002; Cerejeira et al., 2003; Martínez et al., 2003).

Rice crop conducted under flooded conditions is pointed out as being an activity of high pollution potential (FEPAM, 2004). The factors that contribute to this claim are the large amount of water used to maintain the flood (Machado et al., 2006), the usual proximity of the fields to surface water bodies, the predominant shallow aquifer in these areas, and the intentional and unintentional release of water from the field.

The rice production in Brazil demands intense agrochemical use, mainly herbicides, insecticides and fertilizers (Noldin et al., 2001). Among the rice herbicides registered in Brazil, three mostly used are clomazone, propanil and quinclorac. Clomazone is moderately mobile in sandy soils and its half-life in soils is approximately 24 hours (Vencill, 2002). Studies show that the clomazone concentration in soil solution is dependent on the amount of carbon and water in the soil (Lee et al., 2004) and its persistence in rice field is 28 days in the Rio Grande do Sul State (RS) conditions (Machado et al., 2003). Propanil is weakly adsorbed by the soil, is moderately mobile in sandy soils and of low mobility in clayey soils, with half-life of 1 to 3 days (Vencill, 2002). Studies on the persistence of propanil in irrigated rice conditions showed that its dissipation occurs within 24 hours and that the amount of dissipated propanil corresponds to the concentration of 3,4-dichloroaniline (DCA), indicating biological degradation of propanil to DCA (Deul et al., 1977). Quinclorac has variable mobility depending on soil type and organic matter and it can persist in the soil for one year affecting susceptible crops in rotation programs (Vencill, 2002). Conversely, studies under the conditions of the RS state demonstrated that quinclorac persists in detectable concentrations for 21 days in rice paddy water (Machado et al., 2003).

Therefore, the characteristics of rice fields, the climate conditions and the use of pesticides contribute to the enhanced risk of surface water contamination, justifying the need to quantify their degree of occurrence, to implement measures to prevent it. Thus, a three-year monitoring in the Vacacaí and Vacacaí-Mirim river basins was carried out aiming to quantify the presence of clomazone, propanil and quinclorac during the rice growing season and correlate them with the rainfall regime.

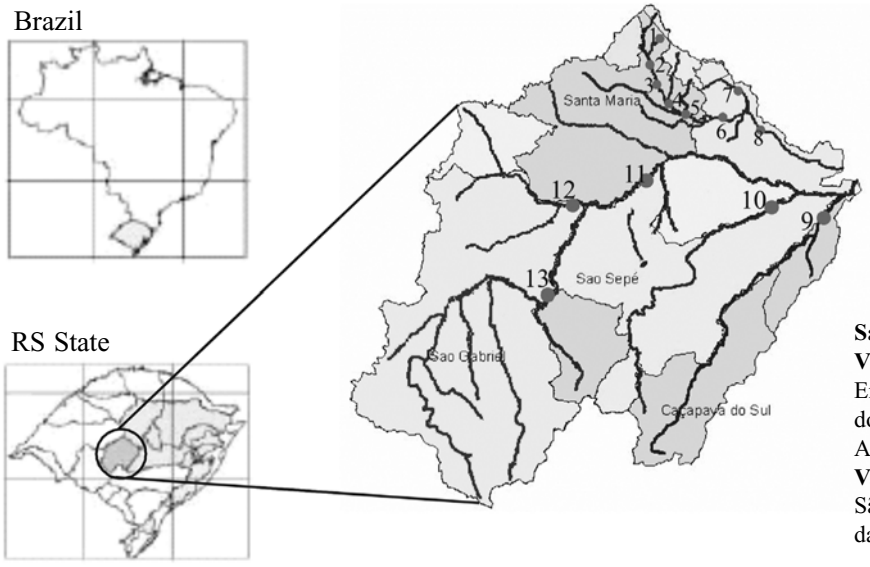
MATERIAL AND METHODS

Chemicals

Stock solutions of clomazone (99.6%), propanil (99.3%) and quinclorac (99%) were prepared individually in methanol and stored at -18 °C. Herbicide water solubilities (mg L^{-1}) are 1100 (25 C); 130 (25 C) and 0.065 (20 C) for clomazone, propanil and quinclorac, respectively.

Study Area

The basins of the Vacacaí and Vacacaí-Mirim Rivers are located in the Depressão Central region of the Rio Grande do Sul (RS) State in southern Brazil (Figure 1). The eastern limit of the basins is the city of Cachoeira do Sul, 180 km far from the state capital Porto Alegre. The northern limits are the cities of Santa Maria and Restinga Seca; the western limit is São Gabriel and the southern, Caçapava do Sul (STE, 1998). The two basins have a total drainage area of



Sampling locations identification
Vacacaí-Mirim River: Três Barras [1]; Envernadinha [2]; Arroio do Meio [3]; Ponte dos Bizzi [4]; RS-287 [5]; Arroio do Só [6]; Arroio da Divisa [7] and Restinga Seca [8]; **Vacacaí River:** Rio Santa Barbara [9]; Rio São Sepê [10]; Passo do Verde [11]; Passo da Lagoa [12] and Passo do Rocha [13].

Figure 1 - Sampling locations in the Vacacaí and Vacacaí-Mirim Rivers, Rio Grande do Sul State (RS), Brazil.

11,136 km^2 and their cities have a total population of about 372,549 inhabitants (Rio Grande do Sul, 2002). In the river basins, irrigated rice is cultivated on about 85,000 ha. During the study 50 to 60% of the rice areas were treated with a tank mix of clomazone and propanil in post emergence, at rates of 350 and 1800 g ha^{-1}, respectively. Quinclorac was normally used at the rate of 375 g ha^{-1} and mostly in the area of Rio Vacacaí-Mirim basin, on 10 to 15 % of the rice area.

The Vacacaí-Mirim river basin is located between the cities of Santa Maria and Restinga Seca, with total length of 40 km, and total drainage area of 1,136 km^2 (Rio Grande do Sul, 2002). In this area, rice is produced, predominantly in small farms (<50 ha), and cultivated in predominantly flat areas, in shallow soils with low natural drainage capacity.

Sampling and sample treatment

The monitoring was conducted during three years in the rice growing seasons (November to February, 2000/01, 2001/02 and 2002/03). In the Vacacaí basin, the monitoring was less detailed with less number of sampling points, with the objective of identifying the most critical locations. Conversely, in the Vacacaí-Mirim basin, the monitoring was more comprehensive, with a greater number of sampling points and done more frequent, with the objective of investigating the seasonal variation of herbicide concentration.

The Vacacaí River - In the first rice growing season (2000/01) 15 points along the river were sampled (Figure 1). These locations were previously selected by STE (STE, 1998). Water samples were taken at two different times: one in December 2000 and another in January 2001 (data not shown). These data were only exploratory. Based on the results, seven locations were selected according to their importance regarding rice water discharge; these points were sampled in the following two years (Figure 1). The location named Restinga Seca, located in the Vacacaí-Mirim basin was sampled together with the Vacacaí River sampling locations. In the second rice growing season (2001/02), the sampling interval was 15 days, during the period between 12/02/00 and 01/27/01, which had greater intensity of herbicide application on the rice fields. An additional sampling was taken in the second half of February, to verify the persistence of the herbicides in the environment. In the third rice growing season (2002/03), the sampling period was a little late as compared with the previous years, 12/19/02 to 01/19/03, due to the delay of rice planting caused by excessive rainfall. Two additional sampling times were included, one in the first half of November 2002 and the other in the first half of February 2003.

The Vacacaí-Mirim River - In the first rice growing season (2000/01) water was collected from eight locations along the river (Figure 1). Water samples were taken every 4 days in the period between 11/26 and 12/29 and every 10 days from this date to 01/27. In the second rice growing season (2001/02) five locations were sampled (Três Barras, Arroio do Meio, RS-287, Arroio do Só and Restinga Seca), with a seven-day interval between 11/28/01 and 01/27/02. In the third rice growing season (2002/03) the same methodology as in the previous year was adopted, with samples between 12/19/02 and 02/03/03. Two additional samples were taken in the second half of February 2002 and 2003, also aiming to evaluate the herbicide persistence in the environment. In this river, the location called Três Barras was used as reference to the other locations, because apparently there was no rice farm upstream. However, some small vegetables, tobacco and corn farms were observed upstream.

At each sampling site a combined sample was obtained from sub-samples taken at three positions in the river profile: one in the center of the river and two close to the borders (1/4 of the river length from the margin) using a 2-liter PET bottle, with holes from the middle to the top of the bottle. The bottle was attached to a weight to collect water along the river profile in depth. The combined sample was dispensed in a clean glass bottle, closed and placed in a box with ice for transporting to the laboratory, for analysis. The glass bottles were washed with the cleaning solution Extran® and previously to each sampling, rinsed with river water.

Extraction and analysis

An aliquot of 250 mL was taken from each sample, acidified and filtered in C18 (200 mg) cartridge previously conditioned for herbicide extraction. The herbicides were eluted with 2 × 0.5 mL methanol (HPLC grade) and captured for future concentration. The solvent was evaporated to dryness using liquid nitrogen and the residues were re-suspended in 0.5 mL methanol (Zanella et al., 2002). The analysis was performed with a HPLC-UV using a C-18 column for separation and, methanol and water as mobile phase (Schlett, 1991; Font et al., 1993; Balinova, 1993).

Quality control and assurance

The average limit of detection and quantitation for each herbicide was respectively: 0.1 and 0.3 μg L^{-1} for clomazone; 0.072 and 0.22 μg L^{-1} for propanil and 0.03 and 0.09 μg L^{-1} for quinclorac. The R^2 for the calibration curves for each herbicide was always above 0.999. The herbicide recovery after fortification in four concentrations was between 88.9 and 107.5%.

RESULTS AND DISCUSSION

Effect of years

On average, at all sampling locations the percentage of contaminated samples varied between the rivers and among rice growing years (Table 1). From the total samples, 38, 20 and 40% of them were contaminated with at least one herbicide in the first, second and third rice growing season, respectively. The differences among the years are related to the rainfall regime of each year. In Santa Maria, during December and January, the normal rainfall is 278.6 mm, however in 2000/01 and 2002/03 the levels were 464 and 411 mm, respectively (data not shown). This excess of rainfall could have caused rice fields to overflow, allowing greater amount of herbicides to reach the river. In addition, in 2001/02, a year with rainfall levels lower than the normal 189.6 mm, only 20% of the samples were contaminated with herbicides.

In a rainy period, the time available for herbicide application is reduced, so the probability of environmental contamination increases because the herbicide concentration is greater and can easily be carried by water. The amount of pesticides reaching the surface and ground water is dependent of the herbicide characteristics, such as water solubility, partitioning and soil persistence, as well as land topography and rainfall regime (Martínez et al., 2003).

Comparing the rivers

Comparing the rivers, the Vacacaí River was the most contaminated one. On the average of all years, 41% of the samples of this river were contaminated with herbicide, while in the Vacacaí-Mirim River, 33% of the samples were contaminated. In the Vacacaí River, average concentrations of 4.5 and 3.7 μg L^{-1} were observed for clomazone and propanil respectively. In 2002/2003, the maximum concentration was 8.9 μg L^{-1} and 11 μg L^{-1} for clomazone and propanil, respectively. The greater herbicide contamination in the Vacacaí River can be explained by the fact that this river has a larger drainage area and larger rice acreage than the Vacacaí-Mirim.

The guidelines for water quality in Brazil do not include the maximum allowed concentration for clomazone, propanil or quinclorac (CONAMA, 1986; Rio Grande do Sul., 1989). Limits for these products are not established in the USEPA legislation (USEPA, 2002) or in the Canadian legislation (CCME, 2002),

Table 1 - Surface water contamination by the herbicides clomazone, propanil and quinclorac in the Vacacaí and Vacacaí-Mirim Rivers, Rio Grande do Sul State (RS), Brazil.

				Concentration μg L^{-1}		
Years	Herbicides	Total number of samples	% CS[1]	Minimum	Average	Maximum
	---------- Vacacaí River ----------					
2001/02	Clomazone	36	30	1.32	4.09	7.72
	Propanil	36	17	1.08	1.76	3.94
	Quinclorac	36	0	--	--	--
	At least 1[2]	36	42			
2002/03	Clomazone	30	20	1.50	4.97	8.85
	Propanil	30	20	0.72	5.59	11.0
	Quinclorac	30	0	--	--	--
	At least 1	30	40			
	---------- Vacacaí-Mirim River ----------					
2000/01	Clomazone	104	27	0.41	1.34	5.62
	Propanil	104	2	0.80	0.86	0.92
	Quinclorac	104	13	0.48	1.57	6.60
	At least 1	104	38			
2001/02	Clomazone	45	11	1.24	2.38	4.82
	Propanil	45	7	1.31	4.98	7.34
	Quinclorac	45	9	1.87	2.79	3.81
	At least 1	45	20			
2002/03	Clomazone	45	20	0.62	2.17	5.10
	Propanil	45	24	0.58	5.62	12.9
	Quinclorac	45	0	--	--	--
	At least 1	45	40			

[1]Contaminated samples. [2]Samples with the presence of at least one herbicide.

though these agencies legislations include a vast number of organic substances. Conversely, the European Union, based on the water quality threshold established by the EU Drinking Water Directive, sets the maximum admissible concentration (MAC) of 0.1 μg L^{-1} for an individual pesticide and of 0.5 μg L^{-1} for total pesticide concentration in any sample of drinking water, except for aldrin, dieldrin, heptachlor and heptachlor epoxide, which are each limited to 0.03 μg L^{-1} maximum level (Hamilton et al., 2003).

Comparing herbicides

Clomazone was detected with more frequency in both rivers, followed by propanil and quinclorac, with frequencies of 21.6, 14 and 4.4% of the samples, respectively. The greater frequency of clomazone can be explained by the high utilization of this product in the region and by the relatively high herbicide persistence in water (Quayle, 2003). These characteristics allow the maintenance of high concentrations of this herbicide in the rice field enhancing the possibility of environmental contamination. Results from an herbicide persistence experiment in rice fields conducted in the Rio Grande do Sul state showed that clomazone was the most persistent when compared with propanil and quinclorac; clomazone was detected for up to 28 days after the herbicide application (Machado et al., 2003). Based on their results, these authors suggested the need for water to be kept in the field for 28 days after herbicide application to avoid environmental contamination. Clomazone is more persistent than propanil, because it has a half-life in the soil of approximately three weeks (Zanella et al., 2000) while propanil is fast metabolized to DCA in the rice fields, with a half-life of approximately one day (Perera et al., 1999).

Quinclorac was not detected in the Vacacaí River, probably because it is used over a smaller acreage as compared to propanil and clomazone; these two herbicides are used alone or in mixture to control grasses in rice. Contrary to our results, in a study carried out in the state of Santa Catarina, Deschamps et al. (2003) found that quinclorac was the most frequent herbicide, in part due to the highest use of quinclorac in that area.

Sampling locations

At the location called Três Barras, used as reference, quinclorac was detected in only 0.9% of the samples collected early in the growing season of 2000/01, and in the other locations quinclorac was detected in 3.8 to 7.7% of the samples (Table 2). In the following year pesticides were not detected in Três Barras, however in the other locations herbicides were detected in 2.2 to 6.7% of the samples. In 2002/03, at Três Barras, propanil and clomazone were detected in 2.2 and 6.7% of the samples, 1 and 3 samples, respectively, which probably came from a 2 ha rice field in which the mixture was applied in the last half of December. At Vacacaí location a steady pesticide detection was observed along the river, with similar percentage of contaminated samples among the sampling locations. There was no trend of increase or decrease of herbicide occurrence along the river.

Seasonal variation

The seasonal variation of water contamination (Figure 2) shows that the concentration of pesticides varied with the growing season and rainfall. In 2000/01, approximately 75% of the contaminated samples occurred in December. However, in 2001/02 more than 65% of the contaminated samples were detected in the

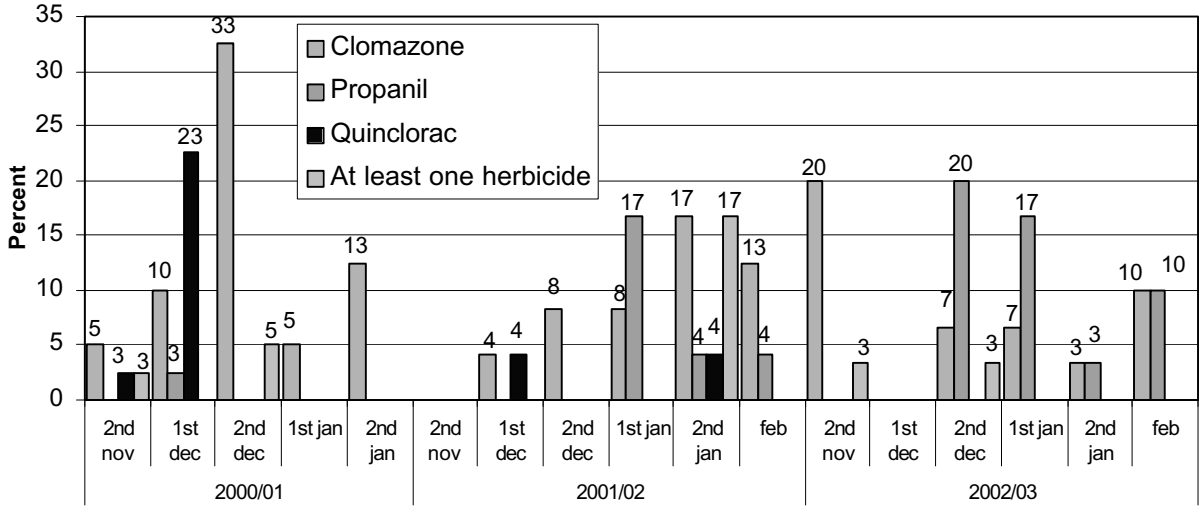


Figure 2 - Percent of samples with herbicide contamination in half (1st and 2nd) of each month (Nov = November, Dec = December, Jan = January, Feb = February), during the studied period (Average of rivers). Rio Grande do Sul, Brazil.

Table 2 - Percentage of samples with residues of clomazone, propanil and quinclorac at each sampling location in the Vacacaí and Vacacaí-Mirim Rivers, Rio Grande do Sul State (RS), Brazil.

Sampling locations	2000/01				2001/02				2002/03			
	Herbicides[1]				Herbicides				Herbicides			
	C	P	Q	T1	C	P	Q	T1	C	P	Q	T1
	Vacacaí River											
	%											
Passo do Verde	--	--	--	--	5.6	2.8	0	8.3	10	3.3	0	13.3
Passo da Lagoa	--	--	--	--	2.8	2.8	0	5.6	3.3	3.3	0	6.7
Passo do Rocha	--	--	--	--	8.3	2.8	0	8.3	3.3	0	0	3.3
Rio São Sepé	--	--	--	--	5.6	2.8	0	5.6	0	6.7	0	6.7
Rio Santa Bárbara	--	--	--	--	2.8	2.8	0	5.6	3.3	3.3	0	6.7
Restinga Seca	--	--	--	--	5.6	2.8	0	8.3	0	3.3	0	3.3
Total	--	--	--	--				42				40
	Vacacaí-Mirim River											
	%											
Três Barras	0	0	0.9	0.9	0	0	0	0	6.7	2.2	0	6.7
Envernadinha	3.8	0	2.9	5.8	NS	NS	NS	NS	NS	NS	NS	NS
Arroio do Meio	2.9	0	0.9	3.8	2.2	0	2.2	4.4	2.2	8.8	0	8.8
Ponte do Bizzi	3.8	0.9	1.9	5.8	NS	NS	NS	NS	NS	NS	NS	NS
RS - 287	3.8	0	0.9	4.8	2.2	4.4	4.4	6.7	4.4	4.4	0	8.8
Arroio do Só	4.8	0	0.9	5.8	2.2	0	2.2	2.2	4.4	6.7	0	11.1
Arroio da Divisa	3.8	0	1.9	3.8	NS	NS	NS	NS	NS	NS	NS	NS
Restinga Seca	3.8	0.9	2.9	7.7	4.4	2.2	0	6.7	2.2	2.2	0	4.4
total				38				20				40

[1]C: clomazone, P: Propanil, Q: Quinclorac and T1: Samples with presence of at least one herbicide. --Samples used just to select the locations to be used in the following years. NS Locations not sampled.

last half of January. This fact can be explained by the low rainfall intensity during December, which allowed the herbicides to stay in the rice field for a longer time. In contrast, the occurrence of 101.3 mm in the last half of January, promoted large amounts of runoff and allowed more herbicides to reach the river.

In late December 2002, a large amount of propanil was detected with concentration of up to 12 μg L^{-1} in three locations of the river, with maximum of 12.9 μg L^{-1}. A severe rainfall of 108.4 mm between December 20th and 24th can explain this event. In this period producers observed that due to the high intensity of rainfall there was overload of the levees destroying many of them, allowing a large amount of water to reach the river.

The seasonal variation found in this experiment is similar to other found in the literature. The surface water pollution by pesticides is dependent on the agricultural activity, with high concentrations during the crop season (Martínez et al., 2003). The amount of pesticides transported to the surface water bodies depends on several factors, including not only soil characteristics, topography, time of application, agricultural practices and chemical properties of each pesticide (Leonard, 1990), but also on the rate of application, its chemical characteristics and the environmental conditions during application (Huber et al., 2000).

CONCLUSIONS

Clomazone, propanil and quinclorac were detected in both rivers during all rice growing seasons. Their concentrations were dependent on the rainfall regime.

The Vacacaí river was the most contaminated, because it has larger drainage and larger rice acreage area than the Vacacaí-Mirim; 41% and 33% of the samples were contaminated in the Vacacaí River and Vacacaí-Mirim River, respectively. Clomazone was the most frequent, followed by propanil and quinclorac.

Actions need to be taken to avoid or reduce the herbicide transport to the rivers. In order to accomplish this, it is necessary to avoid or reduce water runoff from the fields for a period of at least the length of the herbicide persistence. This can be achieved by improving water management and enhancing levees quality to reduce the overflow of the fields in case of excessive rainfall.

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Article

Study of the Degradation of the Herbicide Clomazone in Distilled and in Irrigated Rice Field Waters using HPLC-DAD and GC-MS

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Este estudo avaliou a degradação do herbicida clomazone em água destilada e de campos de arroz irrigado, através de irradiação UV e sob condições naturais. Após a etapa de pré-concentração por extração em fase sólida (SPE), a concentração remanescente foi determinada por cromatografia líquida de alta eficiência com detecção por arranjo de diodos (HPLC-DAD) e a identificação dos produtos de degradação foi efetuada por cromatografia gasosa com espectrometria de massas (GC-MS). Sob irradiação UV, o clomazone foi degradado mais rapidamente na água destilada que na água de superfície. Na água de irrigação, sob luz solar, o clomazone apresentou tempo de meia-vida médio de 3,2 dias em três safras consecutivas, e após a aplicação a concentração na água permaneceu acima de 0,1 μg L^{-1} por cerca de 20 dias. Diversos subprodutos, tais como 2-clorobenzaldeído e 2-clorobenzeno metanol, foram identificados por GC-MS, evidenciando que a concentração dos intermediários aumentou no início e então eles também sofreram degradação.

This study evaluated the degradation of the herbicide clomazone in distilled water and from irrigated rice fields, through UV irradiation and under natural conditions. After a solid phase extraction (SPE) as preconcentration step, the remained concentration of clomazone was determined by high performance liquid chromatography with diode array detection (HPLC-DAD) and the identification of the degradation products was achieved by gas chromatography-mass spectrometry (GC-MS). Under UV irradiation, the clomazone was degraded faster in distilled water than in surface water. In irrigated rice water, under sunlight irradiation, clomazone presented a half-life time average of 3.2 days in three consecutive harvests, and after application the concentration in water remained higher than 0.1 μg L^{-1} for 20 days. Several by-products, like 2-chlorobenzaldehyde and 2-chlorobenzene methanol, were identified by GC-MS, which evidenced that the concentration of intermediates at the begining increase and then they also undergo degradation.

Keywords: clomazone, degradation, HPLC-DAD, GC-MS, water

Introduction

Environmental water pollution by pesticides is a problem with widespread ecological consequences.[1] Herbicides are potential contaminants of environmental water because they are directly applied to the soil or irrigating water. Thus, they can be leached to the surface water and transported into the groundwater.[2,3] According to specialized literature,[2,4] a pesticide can pollute the aquatic environmental if its solubility in water is higher than 30 mg L^{-1}; its K_{oc}, organic carbon partition coefficient is less than 300-500; its K_H, Henry's Law constant is less than 10^{-2} Pa m^3 mol^{-1}, its soil half-life is longer than 2-3 weeks and its water half-life is longer than 25 weeks.

Clomazone, CAS number 81777-89-1, [2-[(2-chlorophenyl) methyl]-4,4-dimethyl-3-isoxazolidinone] is widely used against species of annual broad leaf weeds and grass. Clomazone is currently used in the cultivation of soybeans, cotton, rice, sugar cane, corn, tobacco and a variety of other vegetable crops.[5] It is stable at room temperatures for at least 2 years and it is also stable at 50 ºC for at least 3 months. Half-life time ($t_{1/2}$) with

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sunlight is > 30 days in an aqueous solution and 30-135 days in soil. It is highly soluble in water (1100 mg L^{-1}) and has a K_{oc} of 150-562 mL g^{-1} and a K_H value of 4.19×10^{-3} Pa m^3 mol^{-1}.[6] These properties of clomazone indicate its potential for aquatic environmental pollution. In this study we selected clomazone because it is an important herbicide that has been detected in the majority of the water samples collected from rivers located close to irrigated rice fields in the South of Brazil.[3]

The major sources of pesticide pollution are wastewater from agricultural industries and pesticide formulating or manufacturing plants, and their removal from the aquatic environment has become a very important task and different methods are used including carbon adsorption, ozonization, microbial degradation, photodegradation, etc. Hydrolysis, photolysis, using natural sunlight or xenon arc lamps, aquatic metabolism and field dissipation can also contribute to degradation.[7,8] In practice, the evaluation of degradation products is possible through identification by GC-MS.[9]

Therefore, today, the removal of organic harmful pollutants present in water supplies is investigated through a variety of chemical procedures. Among them, oxidation by several agents besides chlorine, such as ozone, UV radiation and Fenton's reagent, have been extensively and successfully used.[9] The Advanced Oxidation Processes (AOPs), which are constituted by the combination of several oxidants, are characterized by the generation of very reactive and oxidizing free radicals in aqueous solutions, such as the hydroxyl radicals, which have a great destructive power.[10] Many of them are currently employed in the elimination of pesticides,[11,12] however no study has been published about procedures to degrade clomazone.

Photochemical reactions are especially important as a technique to remove harmful chemicals from the waste effluents. Photochemical decomposition of pesticides represents an important transformation pathway that can occur in surface waters.[13] Photochemical AOPs are light induced reactions, mainly oxidations that rely on the generation of hydroxyl radicals through combination with added oxidants (e.g. H_2O_2, Fe^{3+}).

For the determination of herbicides in water samples the high performance liquid chromatography with diode array detection (HPLC-DAD), after a solid phase extraction (SPE) as preconcentration step, is frequently employed.[14]

The objectives of this work were *i*) to compare the kinetic degradation of clomazone in distilled and surface water by using a high-pressure mercury lamp; *ii*) to investigate the pH influence on photodegradation; *iii*) to identify the photodegradation products and *iv*) to follow the degradation in the field under natural conditions. The concentration of clomazone was determined by HPLC-DAD as described in Zanella *et al.*[5] and the identification of the photodegradation products was obtained by GC-MS.

Experimental

Instrumentation

The HPLC-DAD analyses were performed with a Varian (Palo Alto, USA) 9002 pump, a Rheodyne (Cotati, CA, USA) 7125 six-port valve with 20 µL loop, and a Varian ProStar 335 diode array detector. The separation was performed on a 250 × 4.6 mm i.d., 5 µm particle, Bondesil C18 analytical column from Varian, operated at room temperature. The mobile phase was methanol-water 65:35 (v/v) adjusted to pH 4.0 with phosphoric acid. It was prepared from separated measured volumes of methanol and water and was degassed for 15 min in an ultrasonic bath before use. The flow-rate was set at 1.0 mL min^{-1} and quantification was performed by detection at 220 nm.

A Varian 3800 CX gas chromatograph with autosampler 8400 and Saturn 2000 mass spectrometer detector equipped with a CP-Sil 8CB-MS column of 30 m length, 0.25 mm i.d. and 0.25 µm film thickness was used to identify the products of transformation. The following chromatographic conditions were used: injector temperature of 250 °C, column oven temperature program of 45 °C, held for 1.5 min, then a gradient of 10 °C min^{-1} up to 260 °C, maintained for more 4 min. Helium was used as a carrier gas at a constant pressure of 10 psi, resulting in a flow-rate of 1 ml min^{-1}. The temperatures of the ion source and the interface were set at 250 and 290 °C, respectively. The MS was operated in electron impact mode with a potential ionization of 70 eV and the spectra were obtained at a scan range from *m/z* 50 to 650 (full scan mode). The scan time was 0.90 seconds and 1.0 µL injections were made.

Chemicals

Clomazone standard (99.8%) was obtained from Dr. Ehrenstorfer (Augsburg, Germany) and a commercial formulation Gamit® 500 CE, which contained 500 g L^{-1} of clomazone, was obtained on the market. Methanol of HPLC grade was from Mallinckrodt (Phillipsburg, NJ, USA). Phosphoric acid of analytical grade was from Merck (Darmstadt, Germany). Water was purified with a Milli-Q water purification system (Millipore, Bedford, MA, USA). The physicochemical characteristics of selected surface waters were: pH 6.5; oxygen chemical demand 15.4 mg L^{-1} of O_2; turbidimetry 22 UT; real color 10 mg L^{-1} of Pt; total

alkalinity 8.7 mg L^{-1} of $CaCO_3$; total solids 110 mg L^{-1}. The extraction tubes were Bakerbond SPE octadecyl C18 (3 mL, 200 mg) from J. T. Baker (Phillipsburg, NJ, USA).

Photodegradation experiments and SPE preconcentration step

The employed homemade reactor apparatus consisted of a tank reactor with a capacity of 2 L and equipped with magnetic stirring, a system for measuring and controlling the reaction temperature, and a high-pressure mercury lamp of 125 W. All photodegradation experiments were carried out at room temperature (25 °C) in the reactor containing 1.5 L of water spiked at 5 mg L^{-1} of clomazone level using a commercial formulation. Different pH values (3.0 and 6.0) and different water matrices (distilled and surface water) were tested.

After the treatment, an aliquot of the water sample (50 mL) was removed from the reactor and preconcentrated in a solid phase extraction (SPE) system. The SPE column was conditioned by the consecutive passage of 3 mL methanol, 3 mL Milli-Q water, 3 mL Milli-Q water at pH 3.0 (adjusted with phosphoric acid 1:1, v/v). The samples were passed through the SPE column under vacuum at 5 mL min^{-1}. Immediately after that, the column was washed with 3 mL Milli-Q water, the eluate was discarded, and the adsorbent bed was dried under vacuum for 2 min. After drying, the analyte was eluted with 1 mL (2 × 500 μL) methanol and analyzed by HPLC-DAD and GC-MS.

Study of the degradation of herbicides in irrigated rice farming water

The samples of farming water were collected from four experimental fields, with 4 × 4 m each, of rice crops of the Crop Science Department at the Campus of the Federal University of Santa Maria (UFSM). The commercial product Gamit® 500 EC was used for the application of clomazone at 500 g ha^{-1} level, resulting, with a water level of 10 cm, in an initial concentration of 500 μg L^{-1}. The samples were collected on days 1, 2, 3, 5, 7, 10, 14, 21, 28, 35 and 45 after the application of the herbicide in the period between November and March of 2000-2001, 2001-2002 and 2002-2003. The calculation of the degradation was performed using the first-order rate equation:[7]

$$-\ln [C_t]/[C_o] = k \times t \quad (1)$$

where C_t represents the concentration at time t; C_o represents the initial concentration; and k is the degradation constant, obtained by the slope of the straight line. When the concentration is reduced to 50% of the initial amount, the half-life time ($t_{1/2}$) can be determined by:

$$t_{1/2} = 0.693 / k \quad (2)$$

Results and Discussion

Photodegradation of clomazone

For all collected samples, an increase in the degradation of clomazone and of the products were observed in the systems HPLC-DAD and GC-MS, respectively. From the results of the degradation in different water types after UV irradiation (Table 1), it was observed that in distilled water a larger degradation of the herbicide occurs when compared with surface water using an irradiation time up to 120 min. That probably occurs due to the turbidity of the surface water samples, which reduces the penetration of the UV radiation. It was also observed that in distilled water there is not a great influence of the pH on the degradation rate. However, the surface water samples at pH 3.0 present a larger degradation than at pH 6.0. This can be explained by the occurrence, at pH 3.0, of the Photo-Fenton process to some degree, being that the surface water presents a concentration of iron to the order of 1 mg L^{-1}. The dissolved organic substances present in surface water can induce a reduction of the degradation rate. [15]

Table 1. Remaining clomazone concentration (mg L^{-1}) in distilled and surface water at pH 3.0 and 6.0 after different irradiation time*

Irradiation time / (min)	Distilled water		Surface water	
	pH 3.0	pH 6.0	pH 3.0	pH 6.0
0	4.98	4.98	4.95	4.96
5	4.13	3.49	4.34	4.64
10	3.36	1.82	3.80	3.81
15	2.88	1.43	2.95	3.49
30	0.90	0.56	2.00	2.29
45	0.24	0.31	1.22	1.50
60	0.08	0.09	0.53	0.52
90			0.26	0.22
120			0.09	

* Number of replicates, for each degradation experiment, *n*= 3.

Figure 1 shows the HPLC-DAD chromatograms obtained from fortified distilled and surface water samples adjusted to pH 3.0 after exposure to UV radiation for 0, 30 and 60 min.

Table 2 shows the results obtained for the kinetics of the photodegradation of clomazone at different pH values (3.0 and 6.0) for distilled and surface water.

The degradation constant k was obtained from the slope of the curve ln (C) *versus* time (Figure 2). The

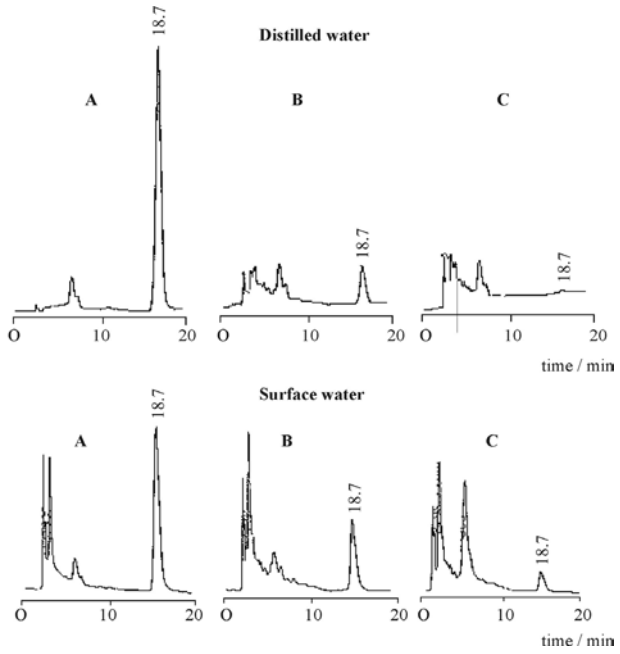

Figure 1. HPLC-DAD chromatograms obtained from fortified (5 mg L^{-1}) distilled and surface water at pH 3.0 after exposure to UV radiation for: A) 0; B) 30 and C) 60 min. t_R clomazone = 18.7 min.

photodegradation for surface water at pH 3.0 was more efficient when compared to the sample at pH 6.0, both presenting kinetics of degradation of the first order. The coefficients r^2 are in general high indicating that the degradation curves fit the data. The results obtained allowed us to conclude that the photodegradation process, proposed for the clomazone herbicide in water samples, is simple and efficient.

Table 2. Kinetics of photodegradation of clomazone in water*

Water samples		Reaction order	k / (min^{-1})	$t_{1/2}$ / (min)
Distilled water	pH 3.0	1	0.0720 ± 0.002	9.7 ± 0.28
	pH 6.0	1	0.0629 ± 0.002	11.0 ± 0.33
Surface water	pH 3.0	1	0.0336 ± 0.0009	20.7 ± 0.57
	pH 6.0	1	0.0358 ± 0.0007	19.4 ± 0.37

* Number of replicates, for each degradation experiment, *n*= 3.

Degradation of herbicides in irrigated rice farming water

Figure 3 shows that after the first week of application the clomazone concentration remains high (198 μg L^{-1} in the 2000-01 harvests; 292 μg L^{-1} in the 2001-02 harvests, and 86 μg L^{-1} in the 2002-03 harvests), residues being found up to the fourth week (1.3; 0.75 and 7.8 μg L^{-1} in the 2000-01; 2001-02 and 2002-03 harvests, respectively). Thus, the results indicate that the water should be maintained in the irrigation fields for at least 28 days, before being released to the environment. This is very important, since studies conducted with fishes had demonstrated short-term effects of exposure to environmentally relevant concentrations of clomazone on AChE activity in brain and muscle tissue.[16,17]

Clomazone presented a half-life time, obtained using equation 2, of 3.1, 3.4 and 3.2 days in the field experiment, in the 2000-01; 2001-02 and 2002-03's harvests, respectively. Analyzing the results obtained from the samples of farming water, it can be concluded that the clomazone herbicide

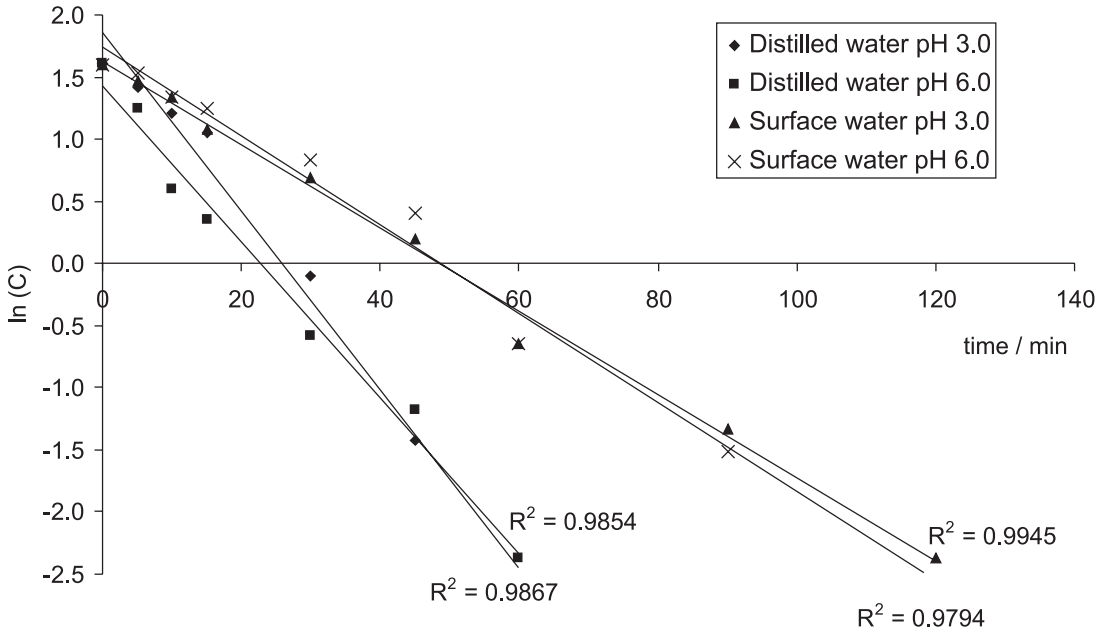

Figure 2. First order rate plots for degradation of water samples, containing 5.0 mg clomazone L^{-1}, irradiated up to 60 min for distilled water, and up to 120 min for surface water.

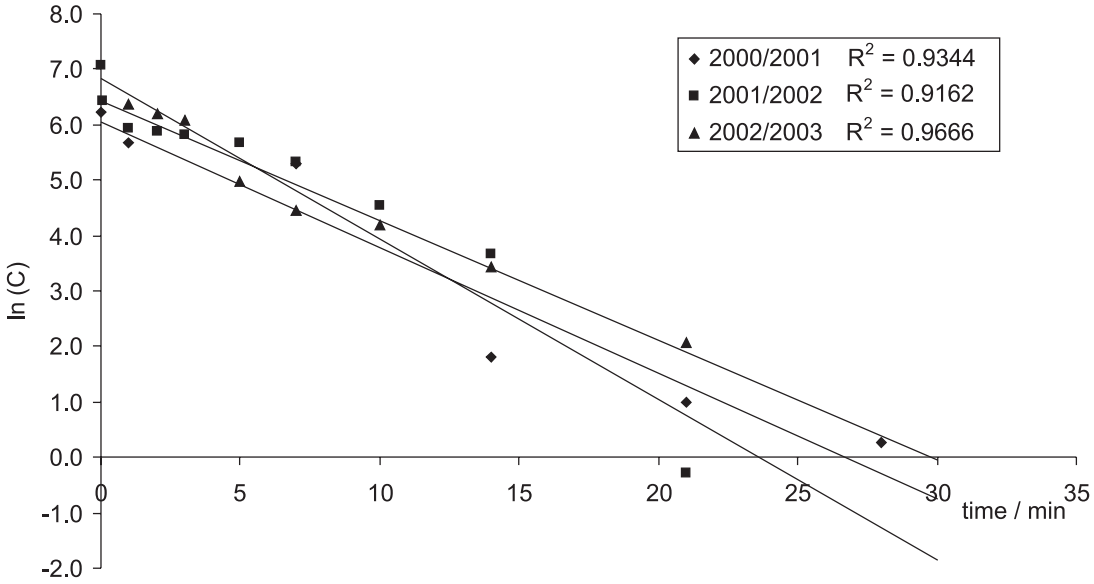

Figure 3. Profile of the degradation of clomazone in irrigated rice farming water. Equations: harvest 2000/2001: y= -0.2062 (±0.0037) x + 6.4501 (±0.1925), harvest 2001/2002: y= -0.1063 (±0.0318) x + 6.7601 (±0.6854) and harvest 2002/2003: y= -0.096 (±0.0070) x + 6.1348 (±0.1159).

is quite persistent, because its half-life time average is 3.2 days.

Degradation products identified by GC-MS

In order to investigate the disappearance of clomazone observed by HPLC-DAD analysis, the SPE extracts of samples of distilled water adjusted to pH 3.0, spiked at 5 mg L^{-1} using a commercial formulation of clomazone and irradiated by UV light at the same conditions of the test were analyzed by GC-MS. The profile of this disappearance is very similar to that obtained by HPLC-DAD, however with GC-MS the total disappearance of the clomazone signal is observed only at 120 min. The GC-MS data confirm the experimental results obtained by HPLC-DAD, that is to say, a fast degradation of the clomazone by UV radiation, presenting a kinetic reaction described by an equation of the first order.

The preconcentration of 50 times allowed the identification of the formed products, especially of the most polar compounds that only produce signals in GC-MS at high concentrations. The use of a silanized liner, a column of low polarity and an SPI injector allowed the observation of some more polar compounds that presented well resolved chromatographic signals. The degradation profiles for the main by-products (2-chlorobenzaldehyde and 2-chlorobenzene methanol), as well as for clomazone, are demonstrated in Figure 4, and both exhibit a regular increase followed by a decrease as expected for the first major degradation products.

Figure 5 presents GC-MS chromatographic signals, data points, spectra, masses and fragmentation suggested for clomazone and identified by-products. The used mass spectrometer, with a scan rate of 5600 *m/z* by second, allowed to obtain enough data points by signal. Each data point represents the average of three acquisitions of the spectra of masses of 50 to 650 *m/z* providing improved spectral quality.

Identification of the compounds

Table 3 presents the data of the detected and confirmed compounds by the GC-MS technique. The evolution, represented by the respective peak area, of clomazone and intermediates formed during the photodegradation treatment indicate a progressive disappearance of clomazone. For the degradation products an initial concentration increase occurred and then they are also degraded.

Clomazone

The fragments of larger intensity are the *m/z* 240 (molecular ion) this only being observed due to a high injected concentration. The ion *m/z* 204 formed by the loss of the atom of chlorine and the *m/z* 125, that according to the suggested fragmentation outline is formed by the breakage of the molecule of clomazone at the carbon bound with nitrogen, and the consequent loss of the $-C_5H_8NO_2$ of *m/z* 114. In comparison with the library NIST98, clomazone (CAS 81777-89-1) presented a spectral fit greater than 85% with the spectrum of the library. As we used a commercial

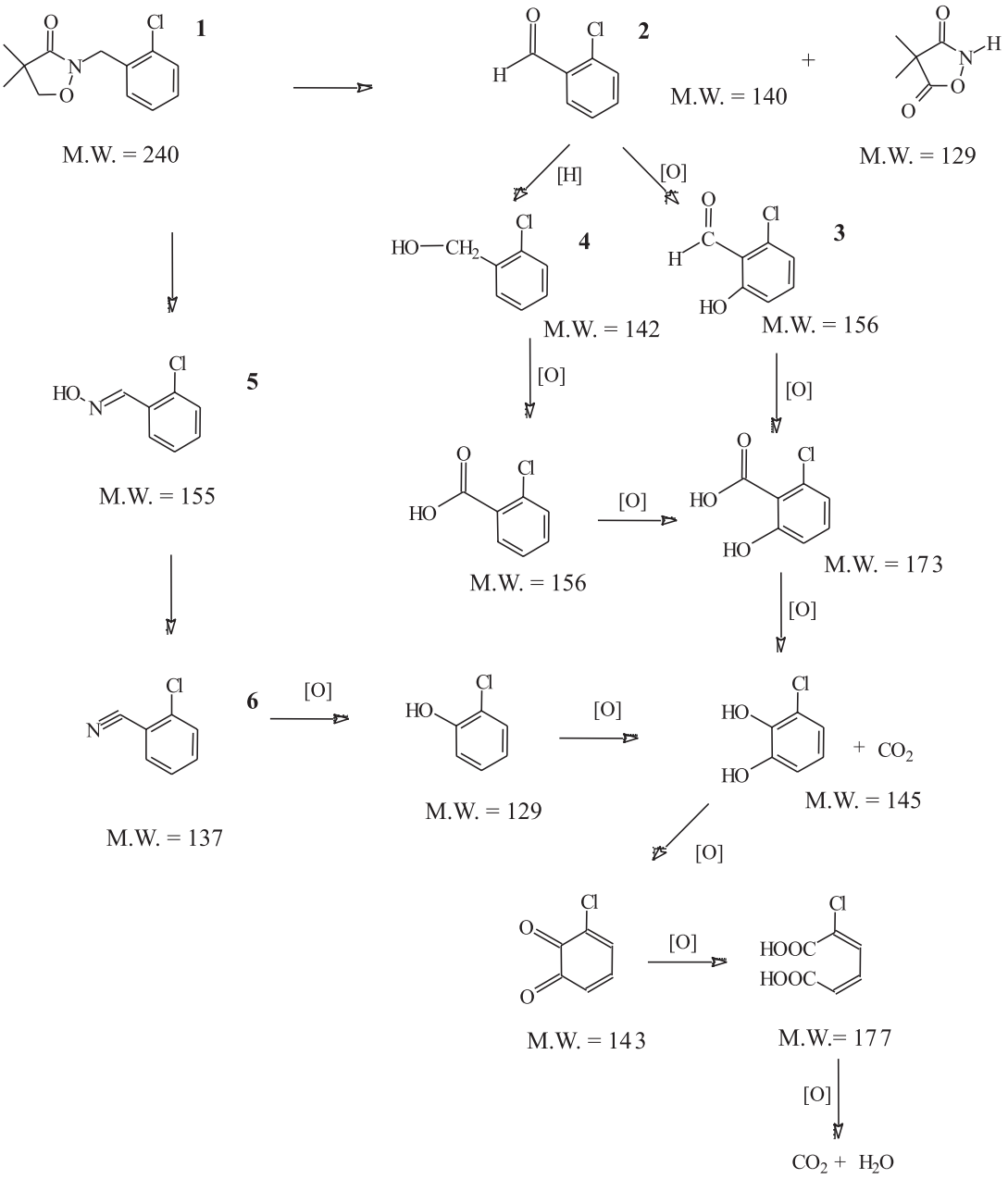

Figure 4. Possible degradation pathways proposed for clomazone. For the compounds **1** to **6** identified by GC-MS the observed molecular weight was shown. For the others, the calculated mass (considering the most common isotope) was shown.

formulation and the preparation of the sample involved an extraction stage in C18 cartridges, we presented the interpretation of the spectrum of clomazone to demonstrate the capacity of the equipment and of the analytical process of producing the characteristic spectrum for the compound in the conditions of the experiment.

2-Chlorobenzaldehyde

It is formed in the degradation process by the cleavage of the C-N bond of the carbon of the methyl group adjacent to the chlorinated aromatic ring, with the consequent liberation of the isoxazolidinone group that was not observed in any of the analyzed extracts. The fragmentation of the 2-chlorobenzaldehyde results the formation of fragments with mass/charge (*m/z*) relations of 139 and 111. The fragment with *m/z* 139 corresponds to the molecular ion and the fragment of *m/z* 111 corresponds to the loss of the -COH group. In comparison with the library NIST98 this compound presented a spectral fit of 90% with the spectrum regarding to 2-chlorobenzaldehyde (CAS 89-98-5).

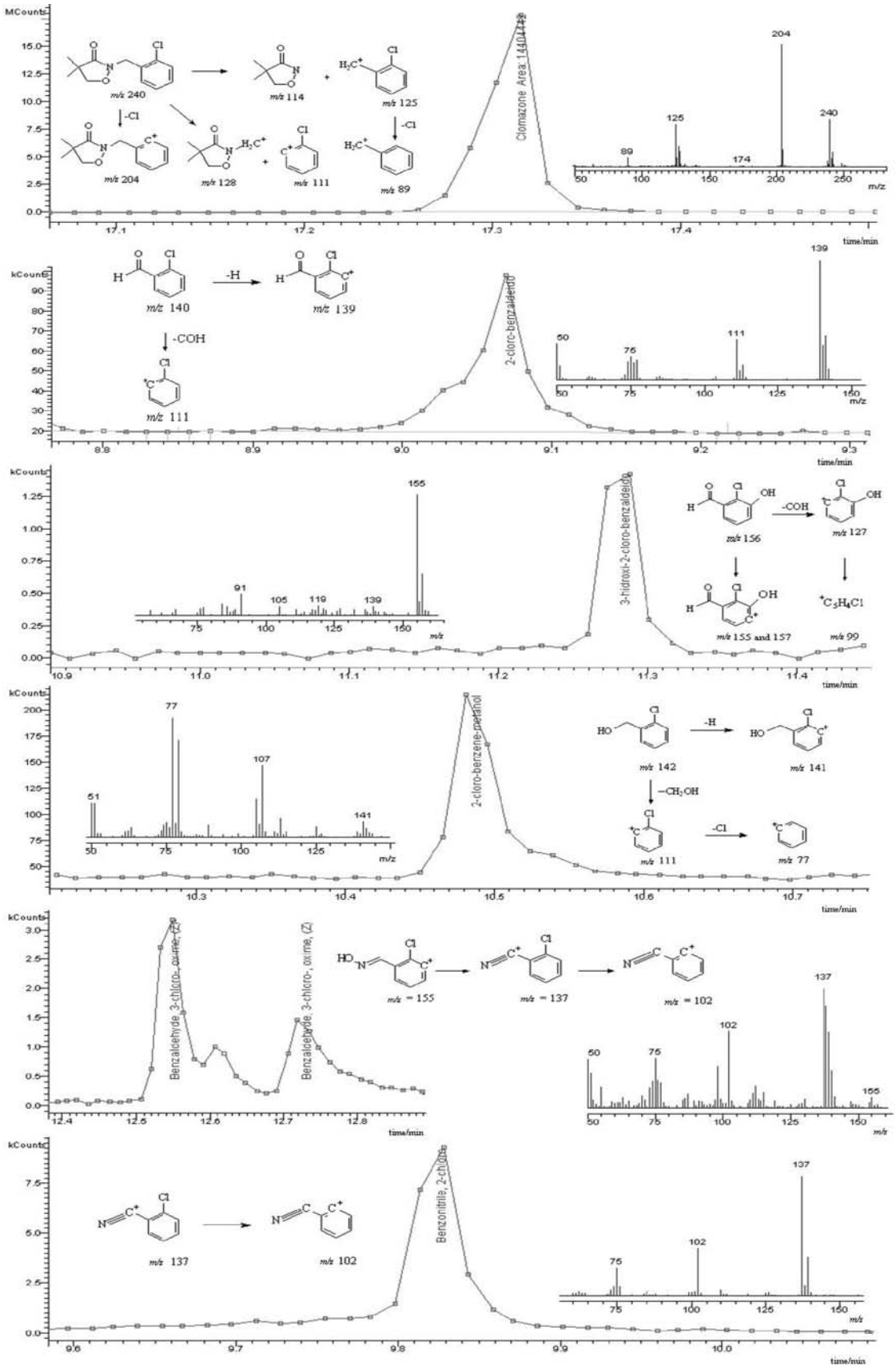

Figure 5. Chromatographic signals, datapoints, spectra, masses and fragmentation suggested for clomazone and identified by-products.

Table 3. Evolution of clomazone and intermediates formed during the photodegradation

MW	239	140	156	142	155	137		
CAS RN	81777-89-1	104-88-1	56962-11-9	17849-38-6	3717-33-7	623-03-0		
t_R /(min)	17.31	8.88	11.28	10.49	9.82	12.72		
m/z fragment	204, 125	139, 111	155, 139, 111, 91	142, 141, 107, 77	155, 137, 102	137, 102, 75		
time /(min)	Peak area of the compounds						Total peak area	Area reduction / %
	1	2	3	5	6	7		
0	48,550.654	64.673	-	208.626	-	-	48,823.953	-
5	47,427.893	187.342	-	433.942	123.097	53.730	49,373.874	0
10	34,821.315	588.836	11.734	529.321	130.992	125.806	35,410.850	20.0
15	34,841.694	635.608	10.802	534.365	79.511	140.729	36,022.469	18.6
30	33,578.809	541.301	6.485	549.604	147.126	218.823	34,676.199	21.7
45	20,924.670	1,507.085	-	347.740	70.077	103.176	22,779.495	48.5
60	7,739.352	1,732.896	-	252.952	97.986	166.072	9,725.200	78.0
90	846.828	852.114	-	39.843	39.389	44.294	1,738.785	96.1
120	101.110	215.278	-	-	18.980	-	316.388	99.3

1 = clomazone; 2 = 2-chloro-benzaldehyde; 3 = 3-hidroxy-2-chloro-benzaldehyde; 5 = 2-chloro-benzene-methanol; 6 = benzaldehyde, 3-chloro-oxime and 7 = benzonitrile-4-chloro.

3-Hydroxy-2-chloro-benzaldehyde

It is formed by the hydroxylation of 2-chloro-benzaldehyde. The main formed fragments presented *m/z* ratios of 155, 157, 127 and 99. The fragments *m/z* 155 and *m/z* 157 correspond to the molecular ion, and the fragment of *m/z* 157 comes with an intensity corresponding to 32.8% of the fragment of *m/z* 155 due to the natural isotopic abundance of the chlorine atom present in the molecule. The fragment of *m/z* 127 is formed by the loss of the group -COH. The fragment of *m/z* 99 is formed by the loss of one more carbon and one oxygen atom. In comparison to the library NIST98 these compounds presented spectral fit greater than 70% with the spectrum regarding 3-hydroxy-2-chloro-benzaldehyde (CAS 56962-10-8). In spite of the small areas in relation to those observed in other compositions, it is important to observe that the formation of this composition indicates the attack by the hydroxyl radical formed by the effect of irradiation with UV light.

2-Chloro-benzene-methanol

It can be formed by the reduction of the 2-chloro-benzaldehyde or by attack of the hydroxyl radical in the moment of the breakage of the C-N bond. In the fragmentation of the 2-chloro-benzene-methanol the fragments of *m/z* 142 and 141 are observed, respectively the molecular ion and the molecular ion less one H atom. The ion with *m/z* 111 is formed by the loss of the -CH2OH, resulting in the protonated fragment formed by the aromatic ring linked to the chlorine. The loss of the atom of chlorine for this fragment generates the ion with *m/z* 77 also observed in the mass spectrum. In comparison with the library NIST98 this compound presented spectral fit greater than 85% with 2-chloro-benzenemethanol (CAS 17849-38-6).

Benzaldehyde, 2-chloro-oxime

Forming the fragments of *m/z* 155, 137 and 102. The fragment *m/z* 155 corresponds to the molecular ion, and the 157 indicates the presence of a chlorine atom. The fragment of *m/z* 137 corresponds to the loss of the hydroxyl by the benzaldehyde, 3-chloro-oxime, also being observed a signal with *m/z* of 138, 139, 140 and 141 formed by the presence of N and Cl in the structure. It is also observed a peak with *m/z* 102 indicating the loss of the chlorine from the previous fragment. Comparing with the library NIST98, this compound presented spectral fit greater than 90% with the benzaldeyhde-3-chloro-oxime (CAS 4006-79-5). In the chromatogram two close signals were observed with t_R between 12.5 and 12.6 min presenting this same characteristic spectrum, which should correspond to the isomer compounds Z and E. In the conditions used in the experiment, the identification by GC-MS doesn't allow to distinguish between the isomeric position, like benzaldehyde, 3-chloro-oxime and benzaldehyde, 2-chloro-oxime, that present the same mass spectrum.

Benzonitrile-2-chloro

Formed by the loss of the OH group by the 2-chloro-oxime-benzaldehyde. The fragmentation formed the ion *m/z* 137 corresponding to the molecular ion and the ion with *m/z* 102 regarding loss of the chlorine. In the

comparison with the library NIST98 this compound presented a spectral fit greater than 95% with the 2-chlorobenzonitrile (CAS 873-32-5).

The acquisition of the data was performed using a scan range from *m/z* 50 to 650 in order to see if it would not form by-products with larger molecular weight than clomazone for mono- or dihydroxylation or aggregate formation[18,19] and to verify other pollutants that could be introduced in the samples in the irradiation processes and extraction.

Using only irradiation the hydroxylation reaction in clomazone does not occur, but rather the breakage of the molecule. Since the products formed in the reaction also disappeared, we believe that the mechanism followed the path of formation of carboxylic acids, which is quite important since this follows the natural path for biological metabolization of organic compounds. A suggestion of the reaction pathway from an environmental perspective is presented in Figure 4.

Conclusion

The obtained information allows a better understanding of the behavior of the herbicide clomazone in distilled and surface waters. The proposed photodegradation process enables an efficient degradation of clomazone in water. The reversed-phase HPLC with DAD detection has proven to be efficient to show the decrease of clomazone during the treatments at the field and in the laboratory. Application of GC-MS for identification and confirmation of clomazone and intermediates formed during the photodegradation treatment increases the reliability of the chromatographic follow up. The evolution of clomazone and intermediates indicate a progressive disappearance of clomazone. For the degradation products an initial increase occurred and then they were degraded. The GC-MS results permit to suggest a degradation pathway for clomazone from an environmental perspective.

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Persistência na água e influência de herbicidas utilizados na lavoura arrozeira sobre a comunidade zooplanctônica de Cladocera, Copepoda e Rotifera

Water persistence and influence of herbicides utilized in rice paddy about zooplankton community of Cladocers Copepods and Rotifers

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RESUMO

Em lavoura de arroz irrigado, é utilizada uma grande quantidade de agroquímicos que, dependendo da sua persistência a campo e toxicidade, podem contaminar corpos d'água e afetar organismos vivos. Com o objetivo de determinar o efeito de concentrações de campo dos herbicidas Clomazone, Quinclorac, Metsulfuron-methyl e Propanil na comunidade zooplanctônica (Cladocera, Copepoda e Rotifera), conduziu-se um experimento em viveiros de aqüicultura, de março a maio de 2005, na estação do outono. Nos dias 1º, 2º, 3º, 7º, 10º, 18º, 31º e 51º após a aplicação dos herbicidas, foram coletadas amostras de água para se determinarem parâmetros físico-químicos da água, concentração dos herbicidas e comunidade zooplanctônica. Os parâmetros médios da qualidade da água foram: oxigênio dissolvido (3,5mg L^{-1}), temperatura (20,1°C), pH (6,0), dureza total (18mg L^{-1} de $CaCO_3$) e alcalinidade total (9mg L^{-1} de $CaCO_3$). A ordem decrescente de persistência dos herbicidas na água foi Clomazone = Quinclorac > Propanil > Metsulfuron-methyl, com média de 31, 31, 10 e 7 dias, respectivamente. Os resultados indicaram que os herbicidas provocaram poucas alterações na densidade de organismos dos grupos Rotifera e Copepoda (Adulto e Nauplio). A densidade do grupo Cladocera permaneceu baixa para todo o período experimental.

Palavras-chave: *agroquímicos, Crustacea, organismos não-alvos, qualidade de água, Sul do Brasil, viveiros de aqüicultura.*

ABSTRACT

In the rice paddy field it is used a large amount of agrochemical that, depending on their field persistence and toxicity, can contaminate water bodies and may affect living organism. With the objective of determining the effect of field concentrations of Clomazone, Quinclorac, Metsulfuron-methyl and Propanil herbicides on zooplankton community (Cladocers, Copepods and Rotifers), it was carried an experiment in aquaculture ponds, during March to May 2005, in autumn season. In the 1st, 2nd, 3rd, 7th, 10th, 18th, 31th and 51th days after the herbicides application, water samples were collected to evaluate the physical chemical water parameters, herbicides concentration and zooplankton community. The water physical chemical parameters means were: dissolved oxygen (3.5mg L^{-1}), temperature (20.1°C), pH (6.0), total hardness (18mg L^{-1} $CaCO_3$) and total alkalinity (9mg L^{-1} $CaCO_3$). The decreasing of herbicides persistence in water was: Clomazone = Quinclorac > Propanil > Metsulfuron-methyl with average of 31, 31, 10 and 7 days, respectively. The results indicated that the herbicides provoke little alteration in density of Rotifers and, Copepods (Adults and Nauplii). The Cladocers group density remained low for the whole experiment period.

Key words: *agrochemicals, Crustacea, non-target organism, water quality, Southern Brazil, aquaculture ponds.*

INTRODUÇÃO

O zooplâncton é constituído por importantes organismos do ecossistema aquático, que ocupam uma posição central da cadeia alimentar, já que transferem energia dos produtores primários para os organismos de níveis tróficos mais elevados, como os peixes. A estrutura de sua comunidade, biomassa e produção influenciam toda a estrutura do ciclo alimentar em ecossistema de água doce (MILLS & FORNEY, 1988).

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Entre muitas substâncias químicas tóxicas, os agroquímicos afetam o zooplâncton em nível individual, populacional e na comunidade (GOODRICH & LEACH, 1990), constituindo-se em fonte de contaminação e, uma vez na água, espalham-se por todo o meio líquido, sendo difícil conter sua dispersão e ação tóxica sobre outros organismos.

Atualmente, os agroquímicos usados na lavoura arrozeira são bastante diversificados, muitos deles com níveis de toxicidade muito baixos e com degradação rápida no ambiente. Todavia, existem também aqueles produtos que oferecem altos riscos ambientais e que se encontram disponíveis no mercado, apesar de haver todo um processo oficial de liberação/ comercialização.

Nos Estados do Rio Grande do Sul (RS) e de Santa Catarina (SC), lavouras cultivadas com arroz irrigado são apontadas como grandes contaminantes ambientais, liberando no ambiente agroquímicos que podem chegar aos mananciais hídricos (MACHADO, 2003). Segundo KOLPIN et al. (1998) e HUBER et al. (2000), herbicidas persistentes e com grande mobilidade têm sido detectados em águas de superfície e subterrâneas, representando riscos para o ambiente, e prejudicando a qualidade da água. Inúmeros estudos indicam que estressores naturais podem alterar a sensibilidade da comunidade zooplanctônica a agroquímicos, fazendo com que espécies de zooplâncton, em ambiente natural, sejam mais sensíveis do que quando cultivadas em condições de laboratório (HANAZATO, 2001).

Clomazone, Quinclorac, Metsulfuron-methyl e Propanil são herbicidas recomendados para o controle de plantas daninhas em arroz irrigado no Sul do Brasil (SOSBAI, 2005). Na maioria das lavouras, as aplicações são seguidas de inundação da área e, em alguns casos, os herbicidas são aplicados diretamente na água de irrigação. Dependendo do manejo da água adotado e da precipitação pluvial após a aplicação, existe o risco de que resíduos desses produtos sejam carreados para fora da área (SUDO et al., 2002).

Embora se observe o uso corrente de herbicidas na lavoura arrozeira e haja possibilidade de contaminação de áreas adjacentes a esta, as pesquisas de monitoramento de herbicidas na água de irrigação são recentes no Brasil. Em vista disso, conduziu-se um experimento com o objetivo de determinar a persistência na água e a influência de Clomazone, Quinclorac, Metsulfuron-methyl e Propanil na comunidade zooplanctônica de água doce.

MATERIAL E MÉTODOS

O trabalho foi realizado durante 51 dias (31/ 03/2005 a 20/05/2005), em viveiros de aqüicultura localizados em área de várzea do Departamento de Fitotecnia da UFSM, em Santa Maria (RS). O delineamento experimental foi inteiramente casualizado com quatro repetições. Foram escavadas unidades experimentais (viveiros de aqüicultura) com capacidade de 22,5m^3 (5,0 x 3,0 x 1,5m), aplicando-se os herbicidas: Clomazone [2-[(2-clorobenzil)]-4,4-dimetil-1,2-oxazolidin-3-ona] (0,5mg L^{-1}), Quinclorac [3,7-dicloroquinolina-8-ácido carboxílico] (0,375mg L^{-1}), Metsulfuron-methyl (metil2-[(4-metoxi-6-metil-1,3,5-triazina-2-il)amino]carbonil]amino] sulfonil]benzoatosulfoniluréia) (0,002mg L^{-1}) e Propanil [2-metil-3,4-dicloro acetanilida] (3,6mg L^{-1}), totalizando 4 tratamentos mais o tratamento controle (sem herbicida).

As coletas de água para análise foram realizadas no 1º, 3º, 7º, 10º, 18º, 31º e 51º dia após a aplicação dos herbicidas e as amostras armazenadas em frascos de vidro âmbar de 1 litro. As amostras foram analisadas no Laboratório de Análises de Resíduos de Pesticidas (LARP) da UFSM, segundo descrito por ZANELLA et al. (2000), empregando-se cromatografia líquida de alta performance com detecção no ultravioleta (HPLC-UV). A persistência dos herbicidas em água foi definida como o período em dias entre a aplicação dos herbicidas e a última coleta com concentração quantificável.

Os parâmetros físico-químicos da água determinados foram: pH (pHmetro Schott Handylab 1), temperatura e oxigênio dissolvido (Oxímetro Oakton) e alcalinidade e dureza total (APHA, 1992).

O zooplâncton foi amostrado no 1º, 2º, 3º, 7º, 10º, 18º, 31º e 51º dia após a aplicação dos herbicidas, utilizando-se uma rede coletora de plâncton (25µm) no horário compreendido entre 4h30min e 6h30min, sendo as amostras fixadas em formol (4%). Posteriormente, com o auxílio de uma pipeta volumétrica, retiraram-se de cada amostra subamostras que foram separadas em placa de Bogorov para análise quali-quantitativa dos grupos zooplanctônicos, empregando-se o uso de microscópio esteroscópio.

Os resultados da densidade de cada grupo do zooplâncton (Cladocera, Copepoda e Rotifera) foram submetidos à análise de variância (ANOVA) e as médias foram comparadas pelo teste de Tukey ($P < 0,05$). Para verificar as associações existentes entre os parâmetros físico-químicos da água (grupo I) e a comunidade zooplanctônica (grupo II), procedeu-se à análise de correlação canônica (CRUZ & REGAZZI, 1994), utilizando-se para isso a covariância entre os dois grupos.

RESULTADOS E DISCUSSÃO

As concentrações mais altas dos herbicidas na água ocorreram nos primeiros dias após a aplicação,

decrescendo com o tempo de amostragem, com variação entre os herbicidas analisados (Tabela 1), que demonstram consistência e concordância com dados já reportados (CAPRI et al., 1999; MACHADO et al., 2003). Os herbicidas Clomazone e Quinclorac foram mais persistentes na água, sendo detectados até 31 dias após a aplicação, corroborando os resultados obtidos por outros pesquisadores (CUMMING et al., 2002). No Brasil, foi detectado Clomazone na água de irrigação até 32 dias após à aplicação, em concentrações na ordem de 0,6mg L^{-1} (NOLDIN et al., 1997), sendo também o herbicida mais freqüentemente encontrado em águas de rios (MARCHEZAN et al., 2003).

A concentração de Quinclorac manteve-se alta até o 7º dia (102mg L^{-1}), sendo detectada até o 31º dia (Tabela 1). Em condições de campo, Quinclorac persistiu até 31 dias, ao contrário dos resultados encontrados nos testes de laboratório (CROSBY, 2003). O Propanil apresentou a menor persistência (Tabela 1). A redução da concentração do produto de 3.600 para 10mg L^{-1}, no 7º dia, deve-se, provavelmente a sua degradação rápida em meio aquoso.

Os parâmetros físico-químicos da água (Tabela 2) demonstraram que os níveis de oxigênio dissolvido variaram entre 2,4 a 4,6mg L^{-1}, considerados baixos para organismos aquáticos. A temperatura da água variou de 16,4 a 23,9 ºC e o pH de 5,5 a 6,1. Na última amostragem, o aumento do pH deve-se provavelmente à redução na fixação de CO_2 da água pelo fitoplâncton, provocado pela menor temperatura. Já a dureza total variou de 14 a 20mg L^{-1} de $CaCO_3$ e a alcalinidade total entre 5 e 14mg L^{-1} de $CaCO_3$.

Os pares canônicos foram significativos apenas para a primeira ordem ($r = 0,74$) ($P < 0,004$) (Tabela 3). Os grupos considerados (parâmetros físico-químicos e grupos zooplanctônicos) não são independentes e as associações intergrupos foram estabelecidas da seguinte forma: quando a água está com temperatura mais elevada e com menores valores de pH, há uma tendência na redução da densidade do grupo Nauplio. Estes resultados estão de acordo com RIETZLER (1995), que, estudando a dinâmica de populações do grupo Copepoda na Represa do Lobo (Itirapina, Brotas - São Paulo), observou alta mortalidade de Nauplio com a elevação da temperatura da água.

Para Cladocera (Tabela 4), não houve diferença significativa entre os tratamentos até o 7º dia. Entretanto, a partir do 10º dia, em geral, o tratamento com Quinclorac apresentou aumento na densidade ($P < 0,05$) em relação ao controle. Já para Clomazone, a partir do 18º dia houve uma tendência ao aumento de organismos deste grupo. Na comparação entre os dias amostrados, Metsulfuron-methyl e Propanil apresentaram baixa densidade de organismos, assim como no tratamento controle. Dessa forma, não é possível afirmar que os herbicidas em estudo afetaram ou não o grupo Cladocera, pois este se manteve em baixas densidades durante maior parte do período amostrado. A baixa população de Cladocera pode ser atribuída ao fato deste grupo gerar formas de estágios latentes duradouros (POLLARD et al., 2003).

Quanto à Rotifera (Tabela 4), não se verificou diferença significativa entre os tratamentos nas três primeiras amostragens. Por outro lado, com a aplicação de Propanil e Metsulfuron-methyl, houve redução da densidade na amostragem do 7º dia em relação ao controle. Para Clomazone e Quinclorac, no 7º e no 10º dia, não foram observadas diferenças em relação ao controle. Já no 18º dia, ocorreu um aumento significativo na densidade deste grupo, com posterior redução a partir do 31º dia.

Comparando os dias amostrais, houve recuperação de Rotifera a partir do 7º dia para os herbicidas Clomazone e Quinclorac, assim como no controle. Propanil e Metsulfuron-methyl, mantiveram-

Tabela 1 - Concentração e persistência dos herbicidas aplicados nos viveiros de aqüicultura.

Herbicida	Concentração (μg L^{-1})[2]							Persistência (dias)[4]
	Inicial[1]	1º	3º	7º	10º	18º	Final[3]	
Clomazone	500	376	322	127	70,8	47	5,6	31
Quinclorac	375	204	121	102	60,7	13	6,4	31
Metsulfuron-methyl	2	1,35	1,27	1,0	nd[5]	nd	1,0	7
Propanil	3.600	1.644	317	10	0,5	nd	0,5	10

[1]Concentração teórica, em μg L^{-1}, na água.
[2]O limite de quantificação do método analítico após a etapa de pré-concentração foi de 0,1μg L^{-1}. para Clomazone e Propanil, e de 0,5μg L^{-1} para o Quinclorac e o Metsulfuron-methyl.
[3]Concentração encontrada na última coleta que ainda apresentou resíduo quantificável de herbicida.
[4]Data da última coleta em que foi quantificado o herbicida.
[5]Não detectável.

Tabela 2 - Média dos parâmetros físico-químicos da água nos viveiros de aqüicultura durante o experimento.

Amostragem	Parâmetros avaliados[2]				
(dia)	OD[1]	T(ºC)	pH	Dureza total[1]	Alcalinidade total[1]
1º	2,7	21,5	5,9	20	8
2º	4,1	23,9	6,1	20	8
3º	3,8	21,1	5,5	18	7
7º	3,4	20,7	6,0	19	8
10º	2,4	21,6	5,8	14	7
18º	4,6	18,4	6,1	14	5
31º	4,3	16,4	6,0	18	14
51º	2,9	17,1	6,5	19	11
Média	3,5	20,1	6,0	18	9,0

[1]Oxigênio dissolvido (mg L^{-1}), dureza total (mg L^{-1} de $CaCO_3$), alcalinidade total (mg L^{-1} de $CaCO_3$).
[2]Média de valores entre os tratamentos.

se, de uma forma geral, com pequenas alterações na densidade, ao longo dos dias.

Os resultados do presente experimento mostram que Rotifera foi menos sensível a agroquímicos, assim como verificado por HAVENS & HANAZATO (1993). Concordando com isto, NEVES et al. (2003) afirmam que o grupo Rotifera, por ser constituído de organismos pequenos e apresentar ciclo curto de vida, possui ampla tolerância à variabilidade de fatores ambientais.

Quanto à Copepoda Adulto (Tabela 4), pode-se verificar que até o 31º dia não foram observadas diferenças dos tratamentos com herbicidas em relação ao controle. Já no 51º dia, nos tratamentos com Clomazone e Propanil, ocorreram densidades significativamente maiores quando comparadas ao controle. Analisando o comportamento do grupo entre as amostragens, verificou-se que para Propanil ocorreu um aumento significativo de organismos no 2º dia e uma queda acentuada a partir do 3º dia, permanecendo assim até a amostragem no 31º dia. Não houve diferenças na densidade deste grupo para Quinclorac e Metsulfuron-methyl. O tratamento Clomazone seguiu os mesmos padrões de densidade de organismos do controle.

Copepoda Adulto, comparado aos demais, foi o grupo que apresentou as mais altas densidades de organismos nos três primeiros dias iniciais de

Tabela 3 - Média, desvio padrão e correlações canônicas entre os parâmetros físico-químicos da água (Grupo I) e os organismos zooplanctônicos (Grupo II).

		Pares canônicos	
Grupos I e II	x ± DS[1]	1º	2º
Oxigênio (mg L^{-1})	3,53 ± 0,87	0,1901	0,3912
Temperatura (°C)	20,12 ± 2,45	0,7241	-0,6679
pH	5,97 ± 0,36	-0,6485	-0,4554
Dureza (mg L^{-1} $CaCO_3$)	17,65 ± 5,89	-0,1113	0,1568
Alcalinidade (mg L^{-1} $CaCO_3$)	8,49 ± 3,76	0,0801	-0,4108
Cladocera[2]	0,53 ± 0,77	-0,2433	0,1536
Copepoda Adulto[2]	4,01 ± 3,29	0,3177	-0,6640
Copepoda Nauplio[2]	2,10 ± 2,41	-0,9810	-0,1604
Rotifera[1]	3,00 ± 3,06	-0,2613	0,0959
r (ρ)[3]	-	0,7488	0,5137
Significância (α)	-	0,0041	0,5451
x^2	-	40,68	11,88

[1]x ± DS. = média ± desvio padrão.
[2]Organismos L^{-1}.
[3]Coeficiente de correlação.

Tabela 4 - Densidade populacional (organismos L^{-1}) de Cladocera, Rotifera, Copepoda (Adulto e Nauplio) durante o período de amostragem.

	Amostragens (dias)							
Tratamentos	1º	2º	3º	7º	10º	18º	31º	51º
				Cladocera				
Controle	NS 0 ns	0 ns	0 ns	1 ns	0 b	0 ns	0 bc	0 b
Clomazone	B 0	B 0	B 0	B 0	B 0 b	AB 1	A 2 ab	AB 1 ab
Quinclorac	C 0	C 0	C 0	BC 0	AB 2 a	ABC 1	A 3 a	A 3 a
Metsulfuron	NS 0	0	0	0	0 b	0	0 c	1 b
Propanil	NS 0	0	0	0	0 b	0	1 bc	0 b
Média	0	0	0	0	0	0	1	1
				Rotifera				
Controle	C 2 ns	BC 3 ns	BC 3 ns	A 12a	ABC 5a	C 1b	AB 8ab	C 2 ns
Clomazone	C 1	BC 1	BC 2	AB 5ab	ABC 3ab	A 11a	BC 2c	BC 1
Quinclorac	BC 2	BC1	C 1	AB 6a	AB 5a	A 8a	ABC 3bc	ABC 4
Metsulfuron	B 1	B 1	B 0	B 1bc	B 2ab	B 2b	A 10a	B 1
Propanil	BC 0	ABC 1	C 0	C 0c	ABC 1b	A 4ab	AB 4abc	ABC 3
Média	1	1	1	5	3	5	5	2
				Copepoda Adulto				
Controle	AB 5 ns	A 8ab	AB 3 ns	AB 3 ns	B 1 ns	B 1 ns	AB 3 ns	AB 2b
Clomazone	AB 8	AB 5b	AB 4	B 2	B 2	B 3	B 3	A 11a
Quinclorac	NS 2	2b	2	2	2	1	2	5ab
Metsulfuron	NS 4	5b	6	3	3	1	1	5ab
Propanil	BC 6	A 18a	BC 6	BC 3	C 1	C 1	C 2	AB 9a
Média	5	8	4	3	2	1	2	7
				Copepoda Nauplio				
Controle	NS 1 ns	3 ns	1 ns	2 ns	1 ns	1 ns	3 ns	1c
Clomazone	B 0	B 1	B 0	B 3	B 1	B 3	B 3	A 13a
Quinclorac	B 1	B 2	B 1	B 1	B 0	B 2	AB 3	A 9ab
Metsulfuron	NS 2	1	1	3	1	1	1	5bc
Propanil	AB 2	AB 2	B 1	B 1	B 1	B 0	B 1	A 7ab
Média	1	2	1	2	1	2	2	7

*Na linha, médias não antecedidas da mesma letra maiúscula e, na coluna, médias não seguidas da mesma letra minúscula diferem entre si pelo teste de Tukey em nível de 5% de probabilidade de erro.
ns/NS Teste F não-significativo em nível de 5% de probabilidade.

amostragem, corroborando com CÁCERES & SOLUK (2002), que afirmam que Copepoda apresenta habilidade de colonizar mais rapidamente o novo habitat quando comparado com Cladocera. Estudos realizados por BONACINA & PASTERIA (2001) mostraram que Copepoda e algumas espécies de Rotifera foram pioneiras na colonização de um lago acidificado estéril na Itália.

Para Copepoda Nauplio, até o 31º dia de coleta, sua densidade manteve-se constante, ocorrendo apenas no 51º dia um aumento significativo ($P<0,05$) nas densidades nos tratamentos com Clomazone, Quinclorac e Propanil em relação ao controle. Já no decorrer das coletas, as densidades de organismos deste grupo mostraram-se inalteráveis, verificando-se pouca influência dos tratamentos para com este grupo. Este fato foi confirmado por LAMPERT et al. (1989), que observaram maior grau de tolerância de Copepoda Adulto e Nauplio, em ambiente contaminado pelo herbicida atrazine.

A reduzida densidade de Rotifera no início e final das coletas pode estar relacionada à maior densidade de Copepoda, responsável pela predação de Rotifera. Estudos de GILBERT (1988) ressaltam que Rotifera é mais abundante quando Cladocera encontra-se em menor número de organismos. Entre os grupos, a redução na população não pode ser atribuída exclusivamente aos herbicidas aplicados, pois outros

fatores de ordem biológica podem interagir com os agrotóxicos, tendo conseqüências na população destes grupos (JAK et al., 1996). Segundo GAGNETEN (2002), os herbicidas podem provocar redução na densidade do zooplâncton, em especial na de crustáceos herbívoros (Cladocera e Copepoda), por determinar a diminuição dos recursos alimentares e trocas na estrutura da comunidade algal e, associado a isto, podem apresentar efeitos tóxicos.

Os resultados apresentados permitem, ainda, uma aplicação ampla, podendo servir de subsídios para programas de monitoramento de bacias hidrográficas que recebem aporte de água drenada de lavouras de arroz irrigado, no sentido de adoção de procedimentos que evitem ou minimizem os riscos de contaminação ambiental. Entre esses procedimentos estão a seleção e aplicação de herbicidas que, preferencialmente, apresentem degradação rápida. Além dos herbicidas, outros agroquímicos devem ser testados, uma vez que esses compostos, nas suas diversas transformações, podem gerar também compostos nocivos ao meio ambiente.

CONCLUÇÕES

Pelos resultados obtidos com os herbicidas testados, depreende-se que a água deva ser mantida na lavoura por cerca de 31 dias após a aplicação do produto, como medida inicial de segurança.

Os herbicidas Clomazone, Quinclorac, Metsulfuron-methyl e Propanil provocaram poucas alterações na densidade da comunidade zooplanctônica para os grupos Rotifera e Copepoda (Adulto e Nauplio). A densidade do grupo Cladocera permaneceu baixa ao longo do experimento.

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PERSISTÊNCIA DOS HERBICIDAS IMAZETHAPYR E CLOMAZONE EM LÂMINA DE ÁGUA DO ARROZ IRRIGADO[1]

Imazethapyr and Clomazone Persistence in Rice Paddy Water

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RESUMO - Os herbicidas podem persistir no solo ou ser carreados para fora da área, contaminando mananciais hídricos a jusante da lavoura. Em vista disso, o presente trabalho objetivou estimar a persistência dos herbicidas imazethapyr e clomazone na lâmina de água de arroz irrigado. Para isso, foi realizado um ensaio com diferentes doses e épocas de aplicação da mistura formulada (75 g i.a. ha^{-1} de imazethapyr + 25 g i.a. ha^{-1} de imazapic) e clomazone (1.500 g i.a. ha^{-1}). Para determinação dos produtos na água de irrigação, foram coletadas amostras de água a partir do primeiro dia até 62 dias após a inundação. Os resultados demonstraram que o período de detecção dos herbicidas na água de irrigação foi mais longo para o imazethapyr que para o clomazone. A meia-vida do imazethapyr na lâmina da água variou conforme o tratamento, com valores entre 1,6 e 6,2 dias, e a do clomazone foi de cinco dias.

Palavras-chave: imazethapyr, clomazone, residual na água, *Oryza sativa*.

ABSTRACT - Herbicides can persist in soil and be transported from the application site to the environment. An experiment was conducted to estimate imazethapyr and clomazone persistence in rice paddy water. The treatments included application of the formulated herbicide mixture (imazethapyr 75 g a.i. L^{-1} + imazapic 25 g a.i. L^{-1}) and clomazone (500 g a.i. L^{-1}). Imazethapyr and clomazone concentrations in water were evaluated from the 1st to the 62nd day after flooding. The period of herbicide detection in water was longer for imazethapyr. Imazethapyr half-life in paddy water varied between 1.6 and 6.2 days and clomazone half-life was 5 days.

Keywords: imazethapyr, clomazone, residues in water, *Oryza sativa*.

INTRODUÇÃO

A água é um recurso natural renovável de reservas limitadas e demanda crescente. A agricultura demanda grande volume de água, sendo responsável por 69% da extração anual (FAO, 2003). Além dessa alta demanda, a agricultura ainda oferece riscos de contaminação dos mananciais hídricos superficiais e subterrâneos, devido ao uso de agroquímicos nas lavouras. Nos Estados Unidos, estima-se que de 50 a 60% da carga poluente de lagos e rios provenha de práticas agrícolas (Gburek & Sharpley, 1997).

A lavoura de arroz irrigado é um dos sistemas de produção que mais demandam água. No Rio Grande do Sul, são utilizados

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anualmente cerca de 1 milhão de hectares para o cultivo do arroz, sendo usados em média 5.374 a 6.422 m^3 de água por hectare de arroz, desconsiderando as perdas de água por condução (Machado et al., 2006). Além disso, para assegurar maior produtividade, o uso de agroquímicos tem sido largamente adotado, ocasionando especulações acerca da responsabilidade da lavoura orizícola na contaminação dos mananciais hídricos.

A mistura formulada de imazethapyr e imazapic (75 e 25 g i.a. L^{-1}, respectivamente) é um dos herbicidas mais utilizados na lavoura orizícola gaúcha para controle do arroz-vermelho. As duas moléculas herbicidas pertencem ao grupo químico das imidazolinonas e são caracterizadas pela eficácia em baixas doses, pelo largo espectro de controle de plantas daninhas e pela longa persistência no solo (Shaw & Wixson, 1991; Loux & Reese, 1993). Estudos indicam que a persistência desses herbicidas no solo é influenciada pelo pH (Loux & Reese, 1992), pela umidade (Baughman & Shaw, 1996) e pelo teor de matéria orgânica do solo (Stougaard et al., 1990). Os herbicidas do grupo químico das imidazolinonas apresentam como principais mecanismos de dissipação a degradação microbiana (Goetz et al., 1990) e a decomposição fotolítica, especialmente quando expostos à luz ultravioleta (Mallipudi et al., 1991). Tanto o imazethapyr quanto o imazapic sofrem limitada biodegradação sob condições anaeróbicas (Senseman, 2007).

O herbicida clomazone tem sua atividade influenciada pela matéria orgânica e textura (Loux & Slife, 1989). A meia-vida do clomazone no solo varia de 5 a 117 dias, dependendo do tipo do solo e das condições ambientais (Curran et al., 1992; Kirksey et al., 1996; Mervosh et al., 1995). Senseman (2007) relata que a persistência do clomazone é menor em solos arenosos do que em solos argilosos. A degradação do clomazone é mais rápida em condições anaeróbicas do que em condições aeróbicas (Senseman, 2007); em solo em condições aeróbicas, a meia-vida do herbicida varia de 90 a 276 dias e, em solo anaeróbico, sua meia-vida média é de 60 dias (California, 2003).

Em grande parte das lavouras de arroz, a aplicação dos herbicidas é seguida pela inundação da área e, dependendo do manejo de água adotado e da precipitação pluvial, os herbicidas podem persistir por maior tempo no ambiente e ser transportados para fora da área, contaminando os mananciais hídricos a jusante da lavoura. Por isso, o presente trabalho visou estimar a persistência dos herbicidas imazethapyr e clomazone em lâmina de água da lavoura de arroz irrigado.

MATERIAL E MÉTODOS

O experimento foi conduzido no ano agrícola 2004/05, na área experimental do Departamento de Fitotecnia da UFSM, em área de várzea, onde não havia histórico da aplicação de imazethapyr e clomazone para controle de plantas daninhas. O solo é classificado como Planossolo Hidromórfico eutrófico arênico, com as seguintes características: $pH_{água}$(1:1) = 4,5; P = 6,9 mg dm^{-3}; K = 55 mg dm^{-3}; MO = 1,2%; Ca = 2,5 $cmol_c$ dm^{-3}; Mg = 1,3 $cmol_c$ dm^{-3}; Al = 1,4 $cmol_c$ dm^{-3}; e argila = 17%. O clima é classificado como subtropical úmido, classe 'Cfa'; as temperaturas mínimas, máximas e médias, a insolação e a precipitação verificadas durante o período de ensaio encontram-se na Tabela 1.

O delineamento experimental utilizado foi de blocos ao acaso, contendo quatro tratamentos e cinco repetições, com unidades experimentais medindo 5 x 4 m (20 m^2). Os tratamentos constituíram da aplicação da mistura formulada de imazethapyr e imazapic (75 + 25 g i.a. L^{-1}) ou da aplicação de clomazone (Tabela 2). Nos tratamentos 1 a 3 foi expressa somente a concentração de imazethapyr, pois foi o único analisado na água.

O preparo do solo foi realizado no sistema convencional, consistindo em duas gradagens pesadas e três gradagens leves para nivelamento do terreno. O cultivar IRGA 422 CL foi semeado em linhas espaçadas de 0,17 m, em 28/10/2004, na densidade de 120 kg de sementes ha^{-1}; a emergência do arroz ocorreu aos 12 dias após a semeadura (DAS). Juntamente com a semeadura do arroz, foi realizada a adubação de base, aplicando-se 7, 70 e 105 kg ha^{-1} de N, P_2O_5 e K_2O, respectivamente. Para adubação de cobertura, foram utilizados 120 kg ha^{-1} de N na forma de uréia, aplicando-se a metade da dose no início do perfilhamento (V4) e o restante na iniciação da panícula (R0),

Tabela 1 - Temperaturas mínimas, máximas e médias, insolação e precipitação pluvial, por decêndio, ocorridas durante o período de realização do experimento. Santa Maria-RS, 2006

Mês	Decêndio	Temperatura (°C)			Insolação (h)	Precipitação (mm)
		Máxima	Mínima	Média		
Outubro	01 – 10	25,5	10,9	18,2	10,1	4,4
	11 – 20	24,7	13,2	19,0	7,2	94,3
	21 – 31	26,8	12,3	19,6	8,6	21,0
Novembro	01 – 10	25,9	14,7	20,3	5,2	123,6
	11 – 20	25,7	14,9	20,3	8,5	24,1
	21 – 30	27,7	15,4	21,6	6,2	0,0
Dezembro	01 – 10	30,5	19,7	25,1	6,5	29,0
	11 – 20	30,2	16,7	23,5	9,7	32,8
	21 – 31	30,5	17,0	23,7	10,7	0,4
Janeiro	01 – 10	34,9	21,8	28,3	8,0	14,1
	11 – 20	33,2	19,2	26,2	10,4	35,7
	21 – 31	32,4	18,6	25,5	8,6	0,0

* Dados coletados na Estação Meteorológica da Universidade Federal de Santa Maria/RS/Brasil.

segundo escala de Counce et al. (2000). Juntamente com a segunda aplicação de N em cobertura, foram utilizados 500 g i.a. ha^{-1} do inseticida carbofuran, para controle de larvas do gorgulho-aquático-do-arroz (*Oryzophagus oryzae*).

A aplicação do herbicida em PRE foi efetuada aos 2 DAS, utilizando-se pulverizador costal pressurizado com CO_2 munido de pontas 110 02 do tipo leque, calibrado para aplicar uma vazão de 125 L ha^{-1}. A aplicação em POS foi efetuada 16 dias após a emergência (DAE), quando a maioria das plantas do arroz cultivado se encontrava no estádio V4, ou seja, com quatro folhas formadas, enquanto as plantas de arroz-vermelho se encontravam no estádio V5. Para aplicação em POS, utilizou-se o mesmo pulverizador acima referido, com vazão de 150 L ha^{-1} e adição de 0,5% v v^{-1} de óleo mineral emulsionável. A inundação da área foi realizada um dia após a aplicação do tratamento em POS, com lâmina d'água de 10 cm de altura, aproximadamente. Cada parcela foi separada por taipas, com entrada e saída de água individual, como forma de evitar a contaminação entre os tratamentos, sendo a irrigação mantida durante todo o ciclo da cultura.

Durante o período entre a aplicação dos herbicidas em PRE e a entrada d'água na lavoura ocorreram precipitações, mas a água ficou retida nas parcelas. No entanto, aos 11 dias após a aplicação dos tratamentos em PRE, devido à precipitação de 63 mm, realizou-se coleta d'água, para detecção dos resíduos de herbicidas na água da chuva e posterior drenagem das parcelas. Foram realizadas ainda coletas de água, em cada parcela, no 1º, 2º, 3º, 5º, 7º, 10º, 14º, 21º, 28º, 35º, 42º, 49º, 56º e 62º dias após a inundação do ensaio; o período entre a aplicação dos tratamentos em PRE e a entrada de água foi de 26 dias. Depois de coletadas, as amostras foram armazenadas em frasco de vidro âmbar, acidificadas com H_3PO_4 1:1 (v.v^{-1}) e, sob refrigeração, transportadas para a análise química no Laboratório de Análise de Resíduos de Pesticidas (LARP) do Departamento de Química da UFSM, para análise conforme metodologia descrita por Zanella et al. (2003).

Alíquota de 250 mL de amostra foi acidificada e pré-concentrada em cartuchos contendo 200 mg de C_{18}, sendo a eluição executada por duas vezes com 500 μL de metanol. A detecção e a quantificação dos herbicidas foram

realizadas utilizando-se HPLC-UV, a 220 nm, munidas de uma coluna Bondesil C_{18} (250 × 4,6 mm i.d; 5 μm), com fase móvel constituída de metanol e água (60:40 vv^{-1}), ajustada a pH 4,0 com ácido fosfórico, com vazão de 0,8 mL min^{-1}. O logaritmo natural da concentração restante do imazethapyr [ln (C/Co)] foi calculado e, através da plotagem desse valor com o tempo em horas, foi obtida a constante da taxa de dissipação dos herbicidas na água (k_p). Os valores da meia-vida dos herbicidas foram calculados usando a equação:

$$t_{1/2} = \frac{\ln(2)}{k_p}$$

sendo k_p o valor absoluto da inclinação e a taxa de dissipação dos herbicidas na água. As constantes da taxa de dissipação dos herbicidas foram submetidas à análise de variância, sendo as médias comparadas pelo teste de Tukey ($P \leq 0,05$).

RESULTADOS E DISCUSSÃO

A maior persistência de imazethapyr foi observada com a aplicação de 52,5 g ha^{-1} de herbicida em PRE, seguido da mesma dose em POS, com níveis detectáveis em água até 27 dias após o estabelecimento da lâmina de água na área (Tabela 2). Resultados similares foram encontrados por Marcolin et al. (2003), que verificaram concentração detectável de imazethapyr na lâmina d'água até os 30 dias após sua aplicação. Já a detecção de clomazone foi observada até os 13 dias após a entrada da água – comportamento similar à aplicação somente em PRE de imazethapyr, na dose de 75 g ha^{-1}. Autores como Machado et al. (2003) encontraram persistência de 28 dias do clomazone na lâmina de água.

A concentração dos herbicidas decresceu, tanto para o imazethapyr quanto para o clomazone, em função do tempo (Figura 1A). Esse decréscimo pode ser explicado pela existência de condições climáticas favoráveis à degradação dos herbicidas, como insolação e temperatura (Tabela 1). O clomazone sofre degradação microbiana em solos úmidos e sob altas temperaturas (Colômbia, 2005). Em solo arenoso, a degradação do clomazone é mais rápida, devido à sua disponibilidade na solução do solo. Cumming & Doyle (2002), avaliando quatro tipos diferentes de solo, encontraram maior persistência do clomazone em solo com maior teor de argila. Teores menores de argila e matéria orgânica também contribuem na dissipação do imazethapyr, pois o torna mais disponível na solução do solo (Avila, 2005). Segundo esse autor, maior quantidade de água na solução do solo facilita a diluição do herbicida e sua mobilidade, diminuindo, com isso, sua concentração.

Em contrapartida, a baixa sorção do herbicida ao solo (Senseman, 2007) pode ter facilitado sua lixiviação, proporcionando seu transporte para camadas mais profundas, onde a degradação microbiana não é tão eficiente. Estudos indicam que o imazethapyr, em solos não-revolvidos, move-se na coluna do solo até 30 cm (O'Dell et al., 1992). O imazethapyr é adsorvido em maior quantidade em pH baixo (Che et al., 1992; Gennari et al., 1998), tornando-se menos móvel e mais persistente no solo (Loux & Reese, 1993). A sorção tem, portanto,

Tabela 2 - Efeito do tratamento herbicida no período de detecção (PD) dos herbicidas, constante de dissipação (k) e meia-vida dos herbicidas em água ($t_{½}$), calculados a partir da entrada de água. Santa Maria-RS, 2006

Tratamento	PD		k	$t_{½}$ (dias)
	DAEA[4/]	Total[5/]		
Imazethapyr (52,5[1/] PRE[2/] + 52,5 POS[3/])	27	53	0,1126 b	6,2
Imazethapyr (75,0 PRE)	13	39	0,4450 a	1,6
Imazethapyr (75,0 POS)	20	20	0,1342 b	5,2
Clomazone (1500 PRE)	13	39	0,1376 b	5,0

[1/] Dose expressa em gramas de ingrediente ativo por hectare; [2/] aplicação em pré-emergência; [3/] aplicação em pós-emergência; [4/] período em dias após a entrada de água; [5/] período total, desde a aplicação do herbicida; [6/] Médias não seguidas pela mesma letra diferem pelo teste de Tukey ($p < 0,05$).

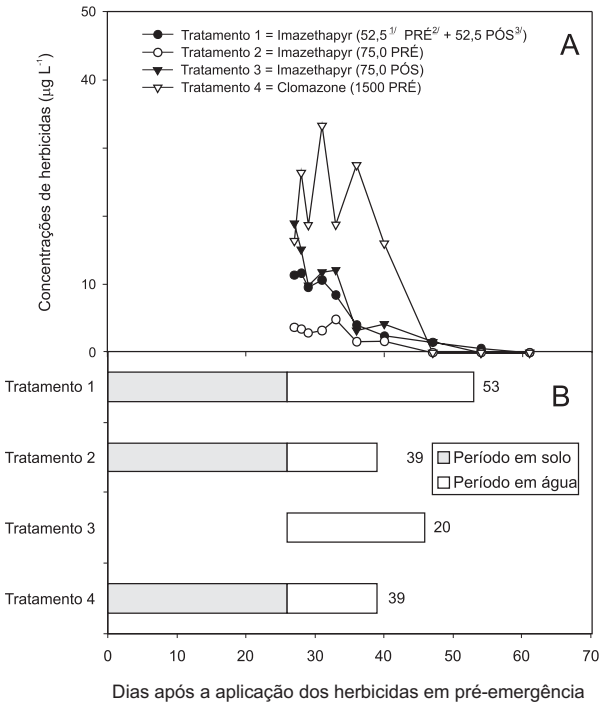

Figura 1 **-** A: concentração dos herbicidas imazethapyr e clomazone na lâmina de água do arroz irrigado do tratamento herbicida. B: período em que o tratamento herbicida ficou em água e em solo. Santa Maria-RS, 2006. 1/ Dose expressa em gramas de ingrediente ativo por hectare; 2/PRE = herbicida aplicado em pré-emergência; 3/ POS = herbicida aplicado em pós-emergência.

impacto na distribuição, biodisponibilidade e persistência de herbicidas no ambiente.

Para o clomazone, a volatilidade é outro fator que contribui em sua dissipação. Além de possuir elevada pressão de vapor, o que proporciona alta volatilidade, a umidade do solo, decorrente da irrigação, pode ter acelerado as perdas do herbicida por volatilização. Thelen et al. (1988) verificaram perdas de clomazone por volatilização com o aumento da umidade do solo. Resultados semelhantes foram encontrados por Cumming & Doyle (2002), que citam as perdas por vapor em local de elevada umidade no solo.

Os fatores anteriormente expostos (precipitações, características do solo e propriedades físico-químicas dos herbicidas) podem ter ocasionado a redução da concentração do imazethapyr e do clomazone encontrada na coleta realizada logo após a entrada d'água no experimento, que ocorreu 26 dias após a aplicação dos herbicidas em PRE. Com a inundação da área, outros fatores influenciaram a degradação dos herbicidas, como hidrólise e degradação anaeróbica, até estes alcançarem sua concentração mínima detectável na água (Tabela 2). O herbicida que apresentou maior meia-vida na água foi o clomazone. Estudos demonstram que, dissolvido em água, tal herbicida não degrada facilmente sob a luz, apresentando meia-vida de 30 dias (Califórnia, 2003). Logo, a decomposição do clomazone na água pode ser explicada pelo fato de o herbicida ser rapidamente degradado em condições anaeróbicas. O Departamento de Pesticidas da Califórnia (2003) relata elevada persistência do clomazone no solo sob condições aeróbicas; contudo, sob condições anaeróbicas, a degradação do clomazone é acelerada.

Quanto ao herbicida imazethapyr, sua aplicação em PRE apresentou a maior meia-vida entre as doses e épocas de sua aplicação. Para o imazethapyr, a fotólise é um dos principais mecanismos de sua dissipação em condições anaeróbicas, já que a degradação microbiana do herbicida, nessas condições, é quase insignificante (Senseman, 2007). A fotólise, por sua vez, é mais eficiente sob intensa insolação – condição satisfeita no período de detecção do herbicida na lâmina de água – devido à ocorrência de poucas precipitações (Tabela 1). Logo, a menor meia-vida do imazethapyr na aplicação somente em POS pode ter decorrido do fato de o herbicida ter tido menor tempo para reações com o solo antes da entrada d'água, o que diminui a adsorção dele ao solo, facilitando sua fotodecomposição em água. Avila (2005) afirma ainda que, quando aplicado em PRE, o herbicida dispõe de mais tempo para a sorção ao solo, diminuindo sua disponibilidade na solução do solo. Assim, segundo o autor, a adsorção ao solo pode afetar a fotodecomposição do imazethapyr aplicado em PRE. Em contrapartida, há suposição de que, após algumas semanas de alagamento, essas reações do herbicida com o solo possam ser desfeitas, em função da elevação do pH a próximo da neutralidade (Snyder & Slaton, 2002), o que disponibilizaria aos poucos as moléculas do herbicida na lâmina d'água. Essa mudança no pH, sob inundação da área, pode ocorrer semanas após a entrada d'água, dependendo do tipo do solo, do nível da matéria orgânica, da população de

microrganismos, de temperatura e de outras propriedades químicas do solo (Snyder & Slaton, 2002).

A meia-vida do imazethapyr na lâmina d'água variou conforme o tratamento, com valores entre 1,6 e 6,2 dias, e a do clomazone foi de cinco dias. Contudo, essa meia-vida refere-se à sua dissipação em água, e em solo a meia-vida pode ser maior. Além do período de 27 e 13 dias de detecção na lâmina de água de irrigação para imazethapyr e clomazone, respectivamente, cabe ressaltar ainda que os herbicidas persistiram por 26 dias no solo, durante o período entre sua aplicação em PRE e a entrada da lâmina d'água na lavoura, totalizando um período de 53 e 39 dias, respectivamente para imazethapyr e clomazone. Nesse período, esses herbicidas podem, potencialmente, ser transportados da lavoura para fora do sistema produtivo, recomendando-se a adoção de práticas de manejo que reduzam essa possibilidade.

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Cladocers, Copepods and Rotifers in rice-fish culture handled with metsulfuron-methyl and azimsulfuron herbicides and carbofuran insecticide

Cladocera, Copepoda e Rotifera em rizipiscicultura tratada com os herbicidas metsulfuron-metílico e azimsulfuron e o inseticida carbofuran

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ABSTRACT

*This study determined the effects of metsulfuron-methyl, azimsulfuron and carbofuran in communities: Cladocers, Copepods and Rotifers that are present in irrigated rice farming with the rice-fish system. The field experiment was conducted in the 2004/05 growing season with eight treatments. The fish species were: **Cyprinus carpio**, **Ctenopharyngodon idella** and **Aristichthys nobilis**, introduced seven days after treatments were applied. Water samples were collected 17 days before and 1st, 3rd, 10th, 18th, 31th, 51th, and 75th days after the agrochemicals were applied for identification and evaluation of the zooplankton. The results indicated that the herbicides did not affect the zooplankton community studied and carbofuran insecticide application provoked negative effects in Cladocers. Copepods and Rotifers were slightly affected by carbofuran.*

Key words: *agrochemicals, non-target organisms, zooplankton communities, rice culture.*

RESUMO

*O presente estudo determinou o efeito de metsulfuron-metílico, azimsulfuron e carbofuran nas comunidades: Cladocera, Copepoda e Rotifera presentes em lavouras de arroz irrigado com o sistema de rizipiscicultura. O experimento foi conduzido durante a safra agrícola 2004/05 com oito tratamentos. As espécies de peixes utilizadas foram: **Cyprinus carpio**, **Ctenopharyngodon idella** e **Aristichthys nobilis**, introduzidas sete dias após a aplicação dos tratamentos. Amostras de água foram coletadas 17 dias antes e no(s) 1º, 3º, 10º, 18º, 31º, 51º e 75º dias após a aplicação dos tratamentos para a identificação e a avaliação de zooplâncton. Os resultados indicam que os herbicidas estudados não afetaram a comunidade zooplanctônica e a aplicação do inseticida carbofuran provocou efeitos negativos em Cladocera. Copepoda e Rotifera foram pouco afetados pelo carbofuran.*

Palavras-chave: *agroquímicos, organismos não-alvos, comunidade zooplanctônica, lavoura arrozeira.*

INTRODUCTION

Zooplankton communities play a key role in aquatic ecosystems by feeding on microalgae and particulate organic matter and serving as the main food to larvae and juvenile fishes. Moreover, these organisms are frequently employed in ecotoxicologic assays since they are one of the most sensitive groups to the effects of toxic chemical products, as well as they are in a central position in the lentic food chain (HANAZATO, 2001; GAGNETEN, 2002).

Once agrochemicals are extensively used in agriculture, their biocide activity increases the probability of negative impacts on non-target organisms, such as the aquatic biota (TREMOLADA et al., 2004; SÁNCHEZ-BAYO & GOKA, 2006). Nevertheless, most of the information about the toxicity of these compounds is based on laboratorial tests, and the combined effect of agrochemical is seldom studied (BARRY & LOGAN, 1998; WENDT-RASH et al., 2003).

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In the Rio Grande do Sul (RS) State, Brazil, irrigated rice cultures are pointed out as the main environmental contaminants, due to the utilization of a great number and quantities of agrochemicals, which are later liberated in the environment and could reach the hydrous source. A system which emerges as an alternative to reduce the agrochemicals application in rice cultures, in the pre-germinated cultivation system, is the rice-fish culture, that is the associated irrigated rice cultivation and fish culture. This system also reduces the use of field machines (MARCHEZAN et al., 2006).

In this system, the polycultive of carps is employed, because different species of carps present different feeding habits and thus do a better field preparation. The common carp ***Cyprinus carpio*** has omnivorous feeding habit consuming seeds, earthworms, insects, small mollusks and so on, and which removes the soil searching for food (LAMMENS et al., 1991). The grass carp ***Ctenopharyngodon idella***, has herbivorous feeding habit (YANG et al., 1990) and the bighead carp ***Aristichthys nobilis*** is zooplanktofagous (CREMER & SMITHERMAN, 1980). However, in some situations, only the fish utilization in the field is not enough to improve the production levels in the rice culture, making necessary the application of herbicides and insecticides (HALWART, 2001).

The carbofuran insecticide (carbamato group) is utilized for the insect control, as for ***Oryzophagus oryzae*** larvae (rice water weevil), which can reduce the rice culture productivity in up to 10% (MARTINS et al., 2004). The azimsulfuron herbicide is used for the control of ***Sagittaria*** sp. (arrow-head), ***Cyperus*** sp. (sedges) and ***Fimbristylis*** sp. (fimbristyles), and metsulfuron-methyl is used for the control of ***Aeschynomene*** sp. (jointvetch) and ***Heteranthera*** sp. (mud plantain) (SOSBAI, 2003), important invader plants in rice culture.

As there are few studies in field focusing on the effect of agrochemical on zooplankton communities, the aim of this study was to determine the effects of metsulfuron-methyl, azimsulfuron and carbofuran on the Cladocers, Copepods (adults and nauplii) and Rotifers communities present in irrigated rice cultures with the system of rice-fish culture system.

MATERIALS AND METHODS

Experiments were carried out at Universidade Federal de Santa Maria (UFSM - Rio Grande do Sul State, Brazil) throughout the harvest time of irrigated rice culture in 2004/2005, during October 2004 to March 2005 in a systematized holm area. The randomized block experimental design was used, in a two-factor way (treatment x days), with four replications. The experimental units (plots) were $48m^2$ (8 x 6m), with refuge (side trench to shelter the fish, measuring 5.3m in length x 0.7m width x 0. m deep), representing about 8% of the total area of plots. The rice was sowed in the pre-germinated culture system in 10/22/2004, and the water lamina was kept about 0.10m in height during all the culture cycle.

The fish species utilized were: 60 % common carp (8.7 ± 0.82g); 20% grass carp (13.2 ± 2.72g) and 20% bighead carp (16.4 ± 0.25g), which were introduced into the plots, in the refuge areas, at the seventh day after the treatments' application, at the settlement density of 20.000 fingerlings ha^{-1}. The fish proportion utilized was those recommended for rice-fish culture in Rio Grande do Sul, State, Brazil, according to species feeding habits (COTRIM, 2000).

Agrochemicals were applied 30 days after the rice seeding at the following concentrations: Ally® (metsulfuron-methyl) – 3.3g ha^{-1} (1.98g i.a. ha^{-1}), Gulliver® (azimsulfuron) - 5g ha^{-1} (5g i.a. ha^{-1}), and Diafuran 50G® (carbofuran) – 1500g ha^{-1} (750g i.a ha^{-1}) (SOSBAI, 2003). The treatments utilized were: [T1] azimsulfuron in rice-fish culture; [T2] metsulfuron-methyl in rice-fish culture; [T3] carbofuran in rice-fish culture; [T4] azimsulfuron, metsulfuron-methyl and carbofuran in rice-fish culture; [T5] azimsulfuron, metsulfuron-methyl and carbofuran in area with rice only; [T6] azimsulfuron and metsulfuron-methyl in rice-fish culture; [T7] control 1 (only rice, without agrochemicals); [T8] control 2 (rice-fish culture without agrochemicals).

While realizing the experiment, water samples of 1L from each plot were collected and conditioned in amber glass flasks, and sent to the Laboratory of Analysis of Pesticide Residues (LARP) at UFSM. Samplings were realized before the agrochemicals' application and among the first and the 75^{th} days after application. The products were analyzed by High Performance Liquid Chromatography with detection at ultraviolet light (HPLC-UV), following the methodology described by ZANELLA et al. (2000).

The water physical-chemical parameters were monitored in the course of the experiment, at the moment of zooplankton samplings, with the measure of pH (pHmeter Schott Handylab 1), total hardness (APHA, 1992), temperature and dissolved oxygen (oxymeter Oakton), total alkalinity (APHA, 1992) and determination of the water transparency (Secchi Disk).

Water samples were collected from the plot, in the refuge areas, in eight distinct periods: 17 days

before the application (17th DBA) and at the 1st, 3rd, 10th, 18th, 31st, 51st, and 75th days after application (DAA) (November 2004 to February 2005). The samplings were carried out from 4h30min to 6h30min am, with a plankton collecting net (25μm mesh), when the samples were fixed in formaldehyde 4%. At the laboratory, the samples were concentrated to 60mL. Subsamples of 1mL were taken with a volumetric pipette and transferred to Bogorov plates for quali-quantitative analysis of the zooplanktonic groups, under a stereoscopy microscopy.

The results of the zooplankton groups' density were submitted to the two-factor (treatment and days) analysis of variance (ANOVA), for the evaluation of the interaction between them. The means were compared by the Tukey test ($P < 0.05$) to determine the differences among treatments. The Analysis of Canonical Correlation (CRUZ & REGAZZI, 1994) was employed to verify the associations between the water physical-chemical parameters (group I) and the zooplankton community (group II).

RESULTS AND DISCUSSION

The mean physical-chemical parameters of the water throughout the experiment were (minimun and maximun): dissolved oxygen (0.6-2.2mg L^{-1}), temperature (17.6-25.7°C), pH (6.4-7.0), total hardness (26-74mg L^{-1} $CaCO_3$), total alkalinity (16-31mg L^{-1} $CaCO_3$) and water transparency (20-50cm).

Canonical pairs were significant only for the first order (canonical correlation equal to 0.83) ($P< 0.01$). The considered groups (water physical-chemical parameters and zooplankton groups) were not independent and the inter-group associations were established in the following way: when the temperature and total hardness (concentration of calcium and magnesium ions) of the water were lower and the water transparency was higher, the Copepods (adults and nauplii) group was found at greater density.

Concerning the application of metsulfuron-methyl and azimsulfuron to the experiment, in the first water sampling performed about 12 hours after the treatment application, the presence of these herbicides was not detected (detection limit - LOD = 0.001mg L^{-1}). However, carbofuran was detected in the rice culture up to 17 days after its application, with a value of 0.013mg L^{-1} (4% of the initial dose), demonstrated that this value meeting under of the maximum contamination level (0.04mg L^{-1}) for drinking water, according to U.S. Environmental Protection Agency (EPA, 2006).

No significant difference among treatments was found for the Cladocers group at the 17th DBA. At the first DAA, there was difference between the treatments T4 and T5 in relation to the control 1 (T7). For the treatment T6, which combined the two herbicides, a density of organisms L^{-1} significantly greater than the control 1 (T7) was observed. Also, at the third DAA, this pattern was kept for the treatments that utilized carbofuran (T3, T4 and T5), with an accentuated decrease in the density of this group. From the 10th DAA to the 75th, no significant differences were obtained among treatments (Table 1). Concerning the sampling days, it was observed a tendency in decreasing the Cladocers community from the 10th DAA.

In the present study, the Cladocers group was the most sensitive to the effect of the carbofuran agrochemical (treatments T3, T4 and T5), when compared to the other zooplanktonic groups, since at the 3rd DAA an accentuated decline of this group's density was observed. Although T4 and T5 presented mixture with the herbicides, the isolated application of metsulfuron-methyl or azimsulfuron, or even their association, did not differ significantly from the control treatment 1 (T7), and did not affect negatively this group.

The present results are in accordance to HANAZATO (1991), who utilized the insecticide carbaryl (carbamato group) in experimental tanks in Japan and found that Cladocers was more sensitive to this agrochemical than Rotifers which presented low sensitivity. RELYEA (2005) studied several agrochemicals, including the effect of the insecticide carbaryl on zooplankton (Cladocers and Copepods) and verified that at the low concentration tested in laboratory (0.51mg L^{-1} of carbaryl) a rapid elimination of Cladocers (***D. pulex, D. ambigua, D. longiremis, Ceriodaphnia* sp.** and ***Scapholebris* sp.**) occurred in two weeks of experiment, contributing for a biodiversity decline. HERBRANDSON et al. (2003) evaluated the effect of carbofuran (concentrations from 0 to 0.16mg L^{-1}) in combination to solids in suspension (from 0 to 10mg L^{-1}) for ***Daphnia magna*** (Cladocers) and verified that at the carbofuran concentration of 0.159mg L^{-1}, without the add of solids in suspension to the water, for a 48h period, 98% of the ***Daphnia*** population were affected, with EC_{50}-48h (effective concentration) of 0.092mg L^{-1}.

For the Rotifers group, a significant decrease in the density was observed for treatments T2 and T6 in relation to control 1 (T7) and T3 at the first DAA. From the third DAA up to the end of samplings (75th DAA), no significant differences were found among treatments (Table 1). For the sampled days in

Table 1 - Population density (organisms L^{-1}) of Cladocers, Rotifers and Copepods (adults and nauplii) from November/2004 to February/2005.

Days	17 DBA	1*	3*	10*	18*	31*	51*	75*
Treatments	----------Cladocers----------							
T1(A+R)	19 ns ^{AB}	25 ab ^{A}	12 a ^{ABC}	2 ns ^{BC}	1 ns ^{C}	1 ns ^{C}	3 ns ^{BC}	1 ns ^{C}
T2(M+R)	23 ^{A}	15 bc ^{ABC}	17 a ^{AB}	1 ^{BCD}	1 ^{D}	1 ^{CD}	1 ^{BCD}	2 ^{ABCD}
T3(C+R)	23 ^{A}	1 dc ^{B}	0 b ^{B}	0 ^{B}	0 ^{B}	1 ^{B}	2 ^{B}	1 ^{B}
T4(A+M+C+R)	22 ^{A}	0 d ^{B}	0 b ^{B}	0 ^{B}	0 ^{B}	0 ^{B}	2 ^{B}	4 ^{AB}
T5(A+M+C)	14 ^{A}	0 d ^{B}	0 b ^{B}	0 ^{B}	0 ^{B}	7 ^{AB}	3 ^{AB}	2 ^{AB}
T6(A+M+R)	36 ^{A}	56 a ^{A}	29 a ^{A}	2 ^{B}	1 ^{B}	1 ^{B}	2 ^{B}	2 ^{B}
T7(Ri)	12 ^{AB}	18 bc ^{A}	15 a ^{AB}	5 ^{AB}	2 ^{AB}	1 ^{B}	1 ^{AB}	1 ^{AB}
T8(R+Ri)	10 ^{AB}	21 ab ^{A}	12 ab ^{AB}	2 ^{AB}	2 ^{B}	0 ^{B}	1 ^{B}	0 ^{B}
Mean	20	17	11	2	1	1	2	2
	----------Rotifers----------							
T1(A+R)	1 ns ^{NS}	10 ab	1 ns	1 ns	1 ns	2 ns	3 ns	2 ns
T2(M+R)	4 ^{NS}	0 b	0	1	2	1	2	2
T3(C+R)	2 ^{B}	21a ^{A}	14 ^{AB}	2 ^{B}	3 ^{AB}	1 ^{B}	5 ^{AB}	3 ^{AB}
T4(A+M+C+R)	2 ^{NS}	8 ab	1	2	4	2	3	2
T5(A+M+C)	1 ^{NS}	4 ab	4	2	2	2	3	1
T6(A+M+R)	2 ^{NS}	1 b	1	1	3	0	4	0
T7(Ri)	^{AB}	21a ^{A}	11 ^{AB}	1 ^{B}	1 ^{AB}	1 ^{AB}	4 ^{AB}	0 ^{B}
T8(R+Ri)	1 ^{NS}	4 ab	1	0	2	2	3	1
Mean	2	9	4	1	2	1	3	1
	----------Adults Copepods----------							
T1(A+R)	12 ns ^{NS}	11 ns	8 ns	2 b	1 ab	1 ab	1 ab	2 ns
T2(M+R)	13 ^{A}	3 ^{AB}	5 ^{AB}	1 ab ^{B}	1 b ^{B}	1 ab ^{AB}	1 b ^{B}	2 ^{AB}
T3(C+R)	17 ^{A}	3 ^{AB}	1 ^{B}	1 ab ^{B}	1 ab ^{B}	1 ab ^{B}	0 b ^{B}	1 ^{B}
T4(A+M+C+R)	13 ^{A}	6 ^{AB}	3 ^{AB}	1 b ^{B}	2 ab ^{AB}	1 ab ^{B}	1 ab ^{AB}	4 ^{AB}
T5(A+M+C)	11 ^{NS}	12	3	4 ab	5 ab	7 ab	11a	3
T6(A+M+R)	14 ^{A}	6 ^{AB}	5 ^{AB}	1 ab ^{B}	1 b ^{B}	0 b ^{B}	1 ab ^{B}	1 ^{B}
T7(Ri)	19 ^{NS}	10	7	13a	12a	12a	8 ab	5
T8(R+Ri)	13 ^{A}	8 ^{AB}	7 ^{AB}	1 ab ^{AB}	3 ab ^{AB}	0 b ^{B}	0 b ^{B}	2 ^{B}
Mean	14	8	10	3	3	3	3	2
	----------Nauplii Copepods----------							
T1(A+R)	31 ns ^{A}	26 ns ^{AB}	10 ns ^{ABC}	6 ns ^{BC}	2 ns ^{C}	2 ns ^{C}	1 ns ^{C}	2 ns ^{C}
T2(M+R)	24 ^{AB}	8 ^{AB}	9 ^{AB}	5 ^{AB}	3 ^{B}	2 ^{B}	1 ^{B}	1 ^{B}
T3(C+R)	44 ^{A}	19 ^{AB}	5 ^{BC}	3 ^{BC}	2 ^{C}	1 ^{C}	1 ^{C}	3 ^{BC}
T4(A+M+C+R)	26 ^{A}	23 ^{AB}	4 ^{BC}	1 ^{C}	1 ^{C}	3 ^{C}	1 ^{C}	3 ^{C}
T5(A+M+C)	20 ^{A}	20 ^{A}	11 ^{AB}	4 ^{AB}	8 ^{AB}	4 ^{AB}	7 ^{AB}	1 ^{B}
T6(A+M+R)	22 ^{A}	12 ^{AB}	7 ^{AB}	2 ^{B}	1 ^{B}	1 ^{B}	0 ^{B}	1 ^{B}
T7(Ri)	17 ^{AB}	20 ^{AB}	25 ^{A}	9 ^{AB}	12 ^{AB}	9 ^{AB}	10 ^{AB}	4 ^{B}
T8(R+Ri)	30 ^{A}	16 ^{AB}	14 ^{ABC}	2 ^{BCD}	1 ^{D}	2 ^{BCD}	1 ^{D}	1 ^{CD}
Mean	27	18	11	4	4	3	3	2

Means followed by distinct minuscule letters at columns and by capital letters at lines differ from each other by the Tukey test ($P < 0.05$).
Ns = F test non-significant at column and NS = F test non-significant at line.
DBA = days before agrochemical application.
*1 to 75 = days after agrochemical application.
(A) Azimsulfuron; (M) Metsulfuron-methyl; (C) Carbofuran; (R) Rice-fish culture; (Ri) = Rice.

treatments T7 and T3, significant differences were found at the 10th DAA, in relation to the first DAA, and only in T3 at 31th DAA.

In general, the Rotifers group was little affected by the agrochemical treatments, conserving low densities along the experiment. These data are in agreement with those from HAVENS & HANAZATO (1993), who observed that Rotifers was the less sensitive zooplankton group in acidified lakes and in the presence of agrochemicals at the environment, presenting a great diversity of species besides being high tolerant taxon. NEVES et al. (2003) stated that

Rotifers possess wide tolerance to the variability of environmental factor due to its small size and short life cycle.

Significant differences were not observed between treatments at the 17th DBA and the 1st and 3rd DAA for the group Adult Copepods. At the 10th DAA there was a significant difference between the treatment T4 and the control treatment 1 (T7). At the 18th DAA the treatments T2 and T6 presented means significantly lesser than the control 1 (T7). At the 31st DAA, control 1 (T7) presented organisms density significantly greater than T6 and T8. At the 51st DAA the treatments T2, T3 and T8 differed from treatment T5. Nevertheless, at the 75th DAA no significant differences were observed in adult Copepods among treatments (Table 1). For the sampled days' analysis, no differences were observed among days in each treatment applied for Adult Copepods.

For the Nauplii Copepods no significant differences were observed among treatments at all sampled days (Table 1). Among the sampled days for each treatment, significant differences were observed for treatments T1, T3, T4 and T8 between the 1st DAA and the 18th DAA.

Little influence of agrochemicals treatments was observed for adult and nauplii Copepods. According to NEVES et al. (2003), the high density of immature forms of Copepods is due to the continuous reproduction of these organisms in tropical regions. The feeding habits of Copepods vary with the life phase at which they are, since adult Copepods can be carnivorous (predators), detritivores and filtrators, whereas nauplia are filtrators, and frequently herbivorous. It is important to point out that within the zooplankton community there is competition for food (bacteria, unicellular algae, among others) and even intra and interspecific predation.

A factor that can determine the proportion between young and adult forms is the intensive predation by invertebrates and vertebrates. At the present study, the fish introduction to the plots seven days after the treatments application did not alter the evaluated zooplankton density (nor increase or decrease), despite the colonization only by the bighead carp, which is zooplanktofagous.

Evidences indicate that natural stressors can modify the sensitivity of the zooplankton community exposed to agrochemicals, so that these organisms are more sensitive to agrochemicals at natural environments than if they are cultivated at controlled conditions in laboratory (LUGO et al., 1998). This could take place because at the natural environment the organisms are exposed to mixtures of chemical compounds, which when combined can cause behavioral, physiological and biochemical changes, death or other adverse effects (HERBRANDSON et al., 2003). In this case, agrochemicals can affect the interactions in the zooplankton community and cause secondary effects to the structure and composition of the biological community (HANAZATO, 2001; VILLARROEL et al., 2003). According to GAGNETEN (2002), the interaction between agrochemicals and biological factors can reduce or increase the consequences of the aquatic environment contamination. PRATT & BARREIRO (1998) comment that agrochemicals can induce an accentuated decrease of the zooplankton community, mainly herbivorous crustaceans (Cladocers and Copepods Calanoides), because they determine reduction of trophic locals and changes in the algae structural communities.

The crescent order of sensitivity to the agrochemical carbofuran was: Nauplii Copepods < Adult Copepods < Rotifers < Cladocers. So, the present study demonstrated that adverse effects occurred to the zooplankton community as consequence of exposition of the aquatic ecosystem organisms to the carbofuran.

CONCLUSION

It can be concluded that the herbicides did not affect the zooplankton community studied and the application of the agrochemical carbofuran cause negative effects to the zooplankton community from the rice farming, in system of rice-fish culture, for the Cladocers group. Copepods (adults and nauplii) and Rotifers are little affected by the application of this agrochemical.

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ACETYLCHOLINESTERASE ENZYME ACTIVITY IN CARP BRAIN AND MUSCLE AFTER ACUTE EXPOSURE TO DIAFURAN

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ABSTRACT: Sublethal adverse effects may result from exposure of aquatic organisms to insecticides at environmentally relevant concentrations. Fingerlings of the common carp (*Cyprinus carpio*, Linnaeus, 1758), grass carp (*Ctenopharyngodon idella*, Valenciennes, 1844), and bighead carp *(Aristichthys nobilis*, Richardson, 1845*)* were exposed to diafuran, an insecticide widely used during rice cultivation in Southern Brazil. The aim of this study was to verify the relationship between the lethal concentration (LC_{50}) of diafuran and the acetylcholinesterase (AChE) activity in brain and muscle tissues of these species as a possible early biomarker of exposure to this insecticide. LC_{50} was determined for fish exposed to diafuran concentrations during 96 h (short term): common carp: control, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg L^{-1}; grass carp: control, 1.0, 2.0, 3.0 and 3.5 mg L^{-1} and, bighead carp: control, 0.5, 1.0, 1.5, 2.0, 3.0 and 4.0 mg L^{-1}, as well as the determination of AChE at concentrations near LC_{50} for these species. LC_{50} values (nominal concentrations) were 1.81 mg L^{-1} for the common carp, 2.71 mg L^{-1} for the grass carp and, 2.37 mg L^{-1} for the bighead carp. All carps exposed to diafuran were lethargic (lower concentrations) or immobile. Diafuran inhibited the acetylcholinesterase activity in brain (~38%) and muscle (~50%) of all species. Muscle of bighead carp under control treatment showed higher specific AChE activity than brain (14.44 against 5.94 μmol min^{-1} g $protein^{-1}$, respectively). Concentrations of diafuran used for rice cropping may affect *Cyprinus carpio*, *Ctenopharyngodon idella* and *Aristichthys nobilis* behaviors and the AChE activities in brain and muscle of these species may be an early biomarker of toxicity of this insecticide.
Key words: *Cyprinus carpio*, *Ctenopharyngodon idella*, *Aristichthys nobilis*, pesticide, lethal concentration

ATIVIDADE DA ENZIMA ACETILCOLINESTERASE EM CÉREBRO E MÚSCULO DE CARPAS APÓS EXPOSIÇÃO AGUDA AO DIAFURAN

RESUMO: Exposição a inseticidas em concentrações elevadas no ambiente podem ocasionar efeitos adversos subletais em organismos aquáticos. Alevinos de carpa húngara (*Cyprinus carpio*, Linnaeus, 1758), carpa capim (*Ctenopharyngodon idella*, Valenciennes, 1844) e carpa cabeça grande *(Aristichthys nobilis*, Richardson, 1845) foram expostos ao diafuran, um inseticida utilizado na cultura do arroz no sul do Brasil. O objetivo deste estudo foi verificar a relação entre concentração letal mediana (CL_{50}) do diafuran e a atividade da enzima acetilcolinesterase (AChE) em cérebro e músculo dessas espécies, como um possível biomarcador inicial da exposição a este inseticida. A CL_{50} foi determinada com peixes expostos a concentrações de diafuran em 96 h: carpa húngara: controle; 0,5; 1,0; 1,5; 2,0; 2,5 e 3,0 mg L^{-1}; carpa capim: controle; 1,0; 2,0; 3,0 e 3,5 mg L^{-1} e carpa cabeça grande: controle; 0,5; 1,0; 1,5; 2,0; 3,0 e 4,0 mg L^{-1}, bem como a determinação da AChE em concentrações próximas da CL_{50} para essas espécies. Valores de CL_{50} (concentrações nominais) foram de 1,81 mg L^{-1} para carpa húngara, 2,71 mg L^{-1} para carpa capim e 2,37 mg L^{-1} para carpa cabeça grande. Todas as carpas expostas ao diafuran estavam letárgicas (menores concentrações) ou imóveis. Diafuran inibiu significativamente a atividade da AChE em cérebro (~38 %) e músculo (~50 %) de todas as espécies estudadas. Atividade da AChE em músculo para carpa cabeça grande foi mais alta que cérebro (14,44

contra 5,94 μmol min^{-1} g $proteína^{-1}$, respectivamente). Este estudo demonstrou que concentrações de diafuran utilizadas na cultura do arroz podem afetar o comportamento de *Cyprinus carpio*, *Ctenopharyngodon idella* e *Aristichthys nobilis*, e a atividade da acetilcolinesterase cerebral e muscular pode ser um biomarcador inicial de toxicidade deste inseticida.
Palavras-chave: *Cyprinus carpio*, *Ctenopharyngodon idella*, *Aristichthys nobilis*, agroquímico, concentração letal

INTRODUCTION

Insecticides are used extensively in agriculture, but their levels in superficial waters generally range far below lethal concentrations for aquatic organisms. However, sublethal adverse effects may result from exposure of aquatic organisms to insecticides at environmentally relevant concentrations (Das & Mukherjee, 2003; Saglio et al., 1996). Diafuran (carbamate) is used in rice fields to control pests and the contamination of water bodies adjacent to rice fields by carbofuran, mainly through run off, is quite possible (Adhikari et al., 2004). Pesticides used in pest control programs seem to produce many physiological and biochemical changes in freshwater organisms by influencing the activities of several enzymes (Sancho et al., 1998).

Acetylcholinesterase (AChE, EC 3.1.1.7) activity is routinely used as a biomarker of the exposure to certain groups of contaminants, such as organophosphate and carbamate insecticides (Grue et al., 1997). Low concentrations of the compounds can inhibit AChE, which leads to an accumulation of acetylcholine at central cholinergic synapses and neuromuscular junctions (Sancho et al., 1997; Varó et al., 2003). The inhibition of the acetylcholinesterase by pesticides can affect locomotion and equilibrium of exposed organisms (Saglio & Trijasse, 1998; Bretaud et al., 2000). In fish, previous studies on carbofuran have focused on the effects of high concentrations on inhibition of AChE activity (Health et al., 1997).

Freshwater aquaculture constitutes one-third of the total fish production in Southern Brazil, with carps being the dominant species. Thus, the aim of this study was to verify the relationship between the lethal concentration (LC_{50}) of diafuran used during rice cropping and the AChE enzyme activity in brain and muscle tissue of the common carp (*Cyprinus carpio*, Linnaeus, 1758), the grass carp (*Ctenopharyngodon idella*, Valenciennes, 1844), and the bighead carp (*Aristichthys nobilis*, Richardson, 1845), as a possible early biomarker of the exposure of these organisms to the insecticide.

MATERIAL AND METHODS

Chemicals

All reagents used in the experiments were of the highest analytical grade Acetylthiocholine, DTNB (5,5'dithio-bis 2 nitrobenzoic acid), bovine serum albumin and Carbofuran (2,3-dihidro-2,2-dimetyl-7-benzofuranil-n-metylcarbamate) were obtained commercially, the last as Diafuran (50% purity).

Exposures

Common carp (5.5 ± 0.5 g and 7.7 ± 2.2 cm), grass carp (11.7 ± 3.3 g and 10.4 ± 3.1 cm), and bighead carp (11.3 ± 3.4 g and 10.2 ± 3.0 cm) fingerlings were obtained from a commercial fish grower near Santa Maria, Rio Grande do Sul State, Brazil. Fish were acclimated to laboratory conditions for 7 days. They were kept in tanks (250 L) and the water was constantly aerated by a static system. Fingerlings were then transferred to continously aerated 40 L boxes and maintained in an air conditioned room. Groups of ten fish/box (three replicates each species) (n = 3) were exposed for 96 h to different diafuran concentrations (dissolved in water) (mg L^{-1}): common carp (0.5, 1.0, 1.5, 2.0, 2.5, and 3.0), grass carp (1.0, 2.0, 3.0, and 3.5), and bighead carp (0.5, 1.0, 1.5, 2.0, 3.0, and 4.0). Control fish were maintained under the same conditions.

Diafuran was added to the water only at the beginning of the experiment and the water quality did not change throughout the experimental period: temperature: 20.5 ± 0.63°C, pH: 8.3 ± 0.02, dissolved oxygen: 4.9 ± 0.17 mg L^{-1}, total alkalinity: 156.5 ± 7.93 mg L^{-1} $CaCO_3$, hardness: 41.4 ± 3.91 mg L^{-1} $CaCO_3$, total ammonia nitrogen: 0.9 ± 0.12 mg L^{-1}, and nitrite: 0.01 ± 0.004 mg L^{-1}. The quantification of waterborne carbofuran was performed by high performance liquid chromatography with photodiode array detection after a solid-phase extraction step as described by Gándara et al. (2002). Measured values were similar to nominal values (~5% variation).

Mortality for each insecticide concentration was recorded (12 h) to estimate LC_{50}. Throughout the experimental period the swimming activity (normal, erratic swimming, lethargy, immobility) of the fish was observed, recorded and compared to the control.

Sampling and enzyme assay

At the end of the exposure period eight surviving fish from each treatment were killed and placed on ice and tissues (brain and muscle) were removed, frozen in liquid nitrogen and then stored at -20°C until AChE assay. For determination of enzyme activity the

lower diafuran concentrations used in the LC_{50} experiment were used (mg L^{-1}): common carp (0.5, 1.0, 1.5, and 2.0), grass carp (1.0, 2.0, and 3.0), and bighead carp (1.0, 1.5, 2.0, and 3.0). All enzyme tests were made in triplicate. Brain and muscle tissues were weighed and homogenized in 150 mM NaCl (15 mL) using a Potter-Elvejhem glass/Teflon homogenizer. The homogenates were centrifuged for 15 min at 3000 g at 5°C and the supernatant was used as the enzyme source. AChE activity was measured as described by Ellman et al. (1961) and modified by Villescas et al. (1981). Aliquots of the supernatant (50-100 mL) (brain and muscle, respectively) were incubated at 25°C for 2 min with 0.1 M phosphate buffer, pH 7.5; 1 mM DTNB as chromogen. After 2 min, the reaction was initiated by the addition of acethylthiocholine (AcSCh) (0.08 M) as substrate for the reaction mixture. The final volume was 2.0 mL. Absorbances were determined at 412 nm during 2 min. Enzyme activity was expressed as mmoL of AcSCh hydrolyzed per min and per gram of protein.

Statistics analysis

Means of LC_{50} for 96 h were calculated using probit analysis as described by Finney (1971). The AChE activity data were analyzed using one-way analysis of variance followed by the Tukey-Kramer test and expressed as mean ± standard error. The differences between treatments and controls were tested ($p < 0.05$).

RESULTS

The 96h-LC_{50} of diafuran were: 1.81 mg L^{-1} for the common carp (confidence interval: 1.67 to 1.96), 2.71 mg L^{-1} for the grass carp (confidence interval: 2.50 to 2.89), and 2.37 mg L^{-1} for the bighead carp (confidence interval: 2.07 to 2.76). The AChE activity in brain and muscle of the unexposed control of common and grass carps was similar, however the muscle of the unexposed control of bighead carp presented higher specific AChE activity than the brain (14.44 against 5.94 mmol/AcSCh/min/ g protein, respectively) (Figure 1).

After diafuran exposure, the AChE activity decreased ($p < 0.05$) for all concentrations in both tissues in relation to the control. Maximum inhibition of the AChE activity for all species was reached when exposed to 1 mg L^{-1} diafuran**.** Maximum percentage AChE activity for 1 mg L^{-1} of diafuran in brain and muscle tissue compared to control was 28.92 and 28.89% for common carp, 30.17 and 55.45% for grass carp, and 55.22 and 64.54% for bighead carp (Figure 1). AChE inhibition was higher in the brain of common and grass carps exposed to 1 and 2 mg L^{-1} of diafuran than in bighead carp (Figure 2A). In addition, the highest AChE inhibition in fish muscle exposed to 1 and 2 mg L^{-1} diafuran was observed for the common carp (Figure 2B).

Swimming activity was normal only for control fish. At the lowest diafuran concentrations fish were lethargic (0.5 mg L^{-1} for common carp, 1.0 and 2.0 mg L^{-1} for grass carp and 0.5 and 1.0 mg L^{-1} for bighead carp), and at higher concentrations they remained immobile in the boxes (1.0, 1.5, 2.0, and 2.5 mg L^{-1} for common carp, 3.0 mg L^{-1} for grass carp and 1.5, 2.0, and 3.0 mg L^{-1} for bighead carp).

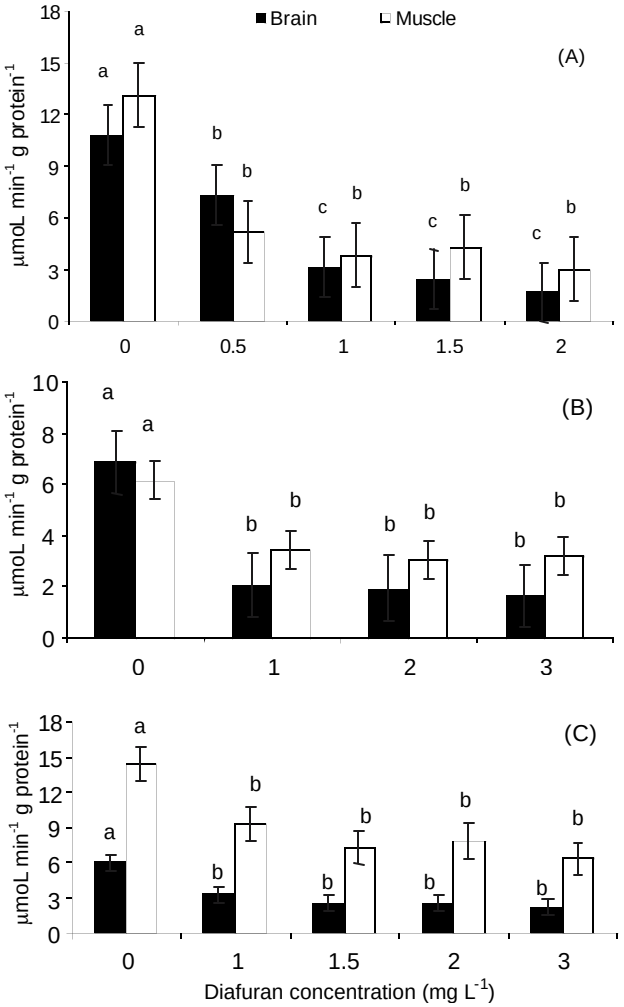

Figure 1 - Effects of 96 h diafuran exposure on AChE activity in brain and muscle of (A) common carp (*Cyprinus carpio*), (B) grass carp (*Ctenopharyngodon idella*) and (C) bighead carp (*Aristichthys nobilis*). AChE activity (mmoL/AcSCh min g protein) is expressed as mean ± SEM. Different low case letters indicate difference of AChE activity among diafuran concentrations in the same tissue ($p < 0.05$).

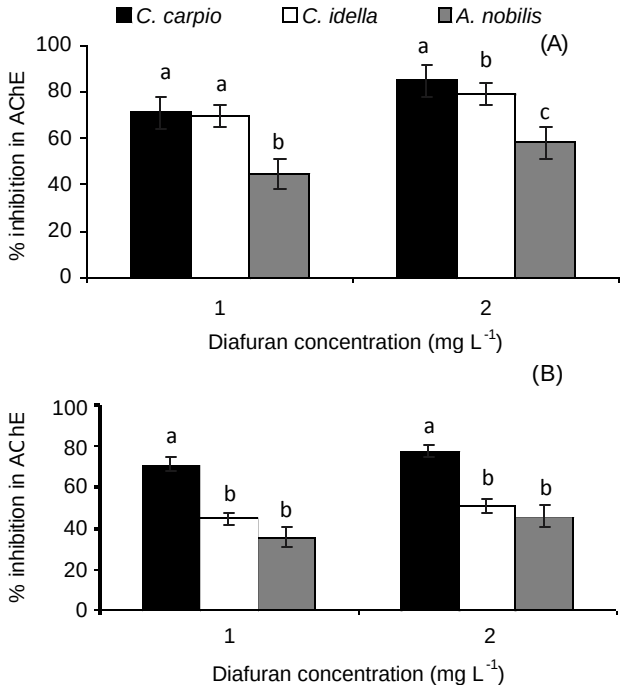

Figure 2 - AChE activity in brain (A) and muscle (B) of common carp (*Cyprinus carpio)*, grass carp (*Ctenopharyngodon idella*) and bighead carp *(Aristichthys nobilis*) after 96 h exposure to different diafuran concentrations. % inhibition is expressed as mean ± SEM *(n = 8)*. Different low case letters indicate difference of AChE activity among species in the same diafuran concentration ($p < 0.05$).

DISCUSSION

Diafuran (50% carbofuran) LC_{50} for the three carps species (common, grass and bighead carp) was 1.81, 2.71 and 2.37 mg L^{-1}, respectively. As the recommended concentration of this insecticide in the rice field is 0.75 mg L^{-1} (SOSB AI, 2003), its use could be harmful for these species. In the field there is a faster decomposition of carbofuran (Plese et al., 2005), but in their study fish were not exposed to optimal water conditions (dissolved oxygen levels, temperature, pH) as in the laboratory. The 96-h LC_{50} for carbofuran (dissolved in water) in common carp larvae was 1.55 mg L^{-1} (2.5 ± 0.5 cm) (Kaur & Dhawan, 1993), similar to the value of the present study. On the other hand, according to the British Crop Protection Council (1991) and Resgalla et al. (2002) the LC_{50} of carbofuran for common carp fingerlings (2-8 cm) was 0.5 and 0.612 mg L^{-1} respectively, indicating that this species is sensible to this insecticide. However, both studies (British Crop Protection Council, 1991; Resgalla et al., 2002) used pure carbofuran (99%) dissolved in acetone and in experimental conditions without aeration. Pure carbofuran is very toxic to fish, and usually fish LC_{50} values are below 1 mg L^{-1} (Trotter et al., 1991).

AChE activity is frequently used as a biomarker of insecticide and pesticide toxicity. The activity of this enzyme is extremely important for many physiological functions, such as prey location, predator evasion and orientation toward food (Miron et al., 2005). When AChE activity decreases, ACh is not broken and accumulates within synapses which therefore cannot function in a normal way (Dutta & Arends, 2003).

In unexposed bighead carp muscle, the AChE-specific activity was two-fold higher than that observed in brain tissue and for common and grass carps the values were similar. Higher muscle AChE activity compared to that of brain was also observed in juvenile goldfish (*Carassius auratus)* (Bretaud et al., 2000). However, in channel catfish (*Ictalurus punctatus*) the AChE-specific activity was higher in the brain than in the muscle (Straus & Chambers, 1995). For all diafuran concentrations, for the three species, there was a decrease in the AChE activity in brain and muscle tissue. In the same way, common carp (6-10 cm) exposed to carbofuran (99%) presented lower brain AChE activity (Dembelé et al., 2000). Three different size groups (fry: 3-4, fingerlings: 6-8 and sub-adults: 10-12cm) of Nile tilapia (*Oreochromis niloticus*) exposed to carbosulfan (carbamate) 1, 4, 8 e 10 μg L^{-1} for 48 h presented lower brain AChE activity with the increasing concentration of carbosulfan (Chandrasekara & Pathiratne, 2007).

Changes in brain and muscle AChE activity observed in common, grass and bighead carps exposed to diafuran probably reflected in movement disturbances, with fish lethargic and immobile in the boxes, help to explain behavoir alterations induced by insecticides. Erratic swimming, convulsions and lethargy were also observed in fathead minnows (*Pimephales promelas*) exposed to carbofuran (0.2 g L^{-1}) (Health et al., 1997) and European eel (*Anguilla anguilla*) (Sancho et al., 1997; Fernández-Vega et al., 2002) after exposure to fenitrothion and thiobencarb, respectively, and silver catfish (*Rhamdia quelen*) fingerlings exposed to 10 mg L^{-1} clomazone for 96 h (Miron et al., 2005).

Cholinesterase inhibition in brain and muscle produce adverse effects in movement because the AChE participates in neuronal and neuromuscular transmissions (Fernández-Vega et al., 1999, 2002). Diafuran provoked high AChE inhibition in brain and muscle for all carps. Common carp exposed to 0.05 mg L^{-1} of carbofuran for 48 h showed 80% inhibition in brain AChE activity (Bertrand et al., 1998), and exposure to 50 μg L^{-1} in goldfish inhibited 23% AChE activity in skeletal muscle (Bretaud et al., 2000). Common carp exposed to 0.1, 0.22 μg L^{-1} carbofuran (99%) showed 27.8 and 75% AChE activity inhibition, respec-

tively, after 24 h (Dembelé et al., 2000). In addition, AChE activity inhibition in brain of Nile tilapia exposed to 10 μg L^{-1} carbofuran (48 h) was 59% (Chandrasekara & Pathiratne, 2007). Brain AChE inhibition was also observed in European eels exposed to diazinon (0.042 mg L^{-1} inhibition higher than 75%) (Cerón et al., 1996), and *Lepomis macrochirus* exposed to endosulfan (0.001 mg L^{-1} for 96 h, inhibition of 16%) (Dutta & Arends, 2003).

Carbofuran elicits acute excessive intoxication by virtue of reversible inhibition (carbamylation) of AChE, which hydrolyses acetylcholine (ACh), a neurohumoral transmitter. The inhibition of AChE consequently leads to excessive ACh accumulation at the synapses and neuromuscular juntions, resulting in overstimulation of ACh receptors, which could ultimately end in death due to respiratory failure (Gupta, 1994). Carbamate insecticides possess inhibitory effects on AChE activity at low concentrations in various freshwater fish species (Silva Filho et al., 2004; Liu et al., 2007)

Different results reported in the literature may depend on data such as chemical formulations of pesticides, their application and degradation rates, absorption estimative, and residues in run off water in the aquatic environment. Additional research is necessary to explain the species-specific differences in the relationship between AChE inhibition and mortality, and the physiologic perturbations associated with AChE inhibition (Fulton & Key, 2001). Although there is some controversy in the literature regarding the extent of the AChE inhibition required to cause death in aquatic animals, most estimatives lie in the 70-85% range. It seems that the extent of AChE inhibition leading to death is dependent upon the species and the type of tissue examined (Bretaud et al, 2000; Fulton & Key, 2001). Therefore, the in vivo response of carp brain and muscle AChE is a promising biomarker to demostrate the presence of anticholinesterase pesticides such as carbofuran in tropical waters at very low concentrations.

CONCLUSIONS

Concentrations of diafuran used during rice cropping may affect *Cyprinus carpio*, *Ctenopharyngodon idella* and *Aristichthys nobilis* behavior and the AChE activity in brain and muscle tissues of these species may be an early biomarker of the toxicity of this insecticide.

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Toxicological and metabolic parameters of the teleost fish (*Leporinus obtusidens*) in response to commercial herbicides containing clomazone and propanil

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ABSTRACT

Pesticides, such as herbicides can affect the metabolic and toxicological parameters on fish. For this reason, an experiment was carried out with the objective of to evaluate the effects of commercial formulations of clomazone and propanil herbicides on acetylcholinesterase (AChE), thiobarbituric acid-reactive substances (TBARS), catalase (CAT) and metabolic parameters in teleost fish (*Leporinus obtusidens*). Fish were exposed during 90 days to field measured concentration of the herbicides clomazone and propanil (376 and 1644 μg/L, respectively) on rice paddy water. Specific AChE activity in the brain and muscle decreased and TBARS levels decreased in brain, muscle and liver tissues. Liver catalase decreased after exposure to both herbicides. Metabolic parameters in the liver and white muscle showed different changes after exposure to both herbicides. In summary, the results showed that clomazone and propanil affects toxicological and metabolic parameters of piavas. These results suggest that environmentally relevant herbicides concentrations are toxic to *Leporinus obtusidens*.

1. Introduction

Different industrial and agricultural activities can contribute to environmental pollution, thus affecting the aquatic systems, which are the end point for these contaminations [1]. Chemical pesticides can increase this environmental contamination, affecting fish health and survival [2]. Recently, there have been a number of studies regarding the effects of environmental contaminants on aquatic organisms [3–5]. The bioavailability of the pesticide depends on its behavior in the environment (soil, water and plant), which varies in accordance with its physical–chemical characteristics.

Clomazone (isoxazolidinone) and propanil (dichloropropionanilide) are herbicides widely used in agriculture, especially in paddy rice fields in Southern Brazil [6]. Clomazone is highly effective, but causes groundwater contamination due to its high water solubility (1100 mg/L) and long half-life dissipation, averaging from 28 to 84 days [7,8]. Propanil is a post emergence herbicide used to control weeds in rice. Water solubility of propanil is 130 mg/L and it is rapidly degraded in water by sunlight, with a half-life of 12 h [6]. The transformation of this herbicide occurs by hydrolysis in water resulting in a 3,4-dicloroanilina (DCA) metabolite that is considered more toxic to organisms than propanil itself [9].

There are several methods used to indicate pesticide effects in fish. The activity of acetylcholinesterase (AChE; EC 3.1.1.7) is widely used as a fast detection method for pesticide toxicity [10,11]. Acetylcholinesterase is an enzyme that catalyses the hydrolysis of acetylcholine into choline and acetate in the synaptic cleft. When inhibition of AChE activity occurs, the neurotransmitter acetylcholine (ACh) is not hydrolyzed in nerve synapses and neuromuscular junctions, causing an abnormal amount of ACh at these sites, which leads to overactivation of muscular tissue [12]. This reduction of AChE can affect locomotion and equilibrium in exposed organisms and may impair feeding, escape reflexes, and reproductive behavior [13–15].

However, the effects of pesticides are not restricted to AChE alterations. In addition, it has been reported that these contaminants may induce the formation of reactive oxygen species (ROS) and alterations in the antioxidant system [16,17]. The catalase (CAT) enzyme is a primary antioxidant defense component that protects fish from oxidative stress by converting hydrogen peroxide to oxygen and water [18]. Variations in antioxidant enzyme activities have been proposed as indicators of pollutant mediated oxidative stress [5,19]. Additionally, ROS can react with biological macromolecules and produce lipid peroxidation (LPO). LPO has been suggested to be one of the molecular mechanisms involved in pesticide-induced toxicity [16,19,20].

Alterations in biochemical and physiological parameters are also observed in fish after exposure to pesticides. Carbohydrate

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and protein metabolism and blood parameters can be used as good indicators to detect the sublethal effects of different pollutants in fish tissues [21]. For this reason, parameters such as glycogen, lactate, glucose and protein were determined.

The piava (*Leporinus obtusidens*) is a native freshwater fish of Southern Brazil with good potential for cultivation [22]. The effect of commercial herbicide formulations used in rice fields has been scarcely studied in fish species. In terms of methodology, little is known about the changes in AChE, catalase activity, thiobarbituric acid-reactive substances (TBARS) and metabolic response to long-term exposure to herbicides in *L. obtusidens*. Thus, the present study aimed to investigate the effects of rice field concentrations of the herbicides clomazone and propanil in *L. obtusidens*, and to determine possible toxicity indicators in fish.

2. Materials and methods

2.1. Chemicals

Commercial herbicides were obtained as follows: clomazone (2-(2-chlorophenyl) methyl-4,4-dimethyl-3-isoxazolidinone) (Gamit; 500 g/L) and propanil (3′,4′-dichloropropananilide) (Stam 360 g/L). The sources of herbicides were: FMC (EUA) and MILENIA (Brazil), respectively. Acethylthiocholine, 5,5'dithio-bis(2-nitrobenzoic acid) (DTNB), bovine serum albumin, Triton X-100, hydrogen peroxide (H_2O_2), malondialdehyde (MDA), 2-thiobarbituric acid (TBA) and sodium dodecyl sulfate (SDS) were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Fish

Leporinus obtusidens of both sexes with an average weight of 10.0 ± 1.0 g and length 8.0 ± 1.0 cm were obtained from fish farm (RS, Brazil). The fish were acclimated in laboratory conditions for 15 days, in tanks (250 L). They were kept in continuously aerated water with a static system and with a natural photoperiod (12 h light/12 h dark). In this period the fish were fed once a day with commercial fish pellets (42%). During acclimation period (15 days), the average of water parameters were as follows: temperature 20.0 ± 1.0 °C, pH 7.4 ± 0.3 units, dissolved oxygen 7.1 ± 0.5 mg/L, nonionized ammonia 0.6 ± 0.04 μg/L, nitrite 0.05 ± 0.02 mg/L, alkalinity 32 ± 1.0 mg/L $CaCO_3$, and hardness 20 ± 2.0 mg/L $CaCO_3$. During experimental period (90 days) the average of water parameters were as follows: temperature 21.5 ± 2.0 °C, pH 6.0 ± 0.2 units, dissolved oxygen 4.17 ± 2.0 mg/L, nonionized ammonia 0.7 ± 0.01 μg/L, nitrite 0.05 ± 0.01 mg/L, alkalinity 10 ± 1.3 mg/L $CaCO_3$, and hardness 16 ± 1.5 mg/L $CaCO_3$. During acclimation period the fish were fed once a day with commercial fish pellets (42% crude protein, Supra, Brazil). After acclimation fish were transferred to field ponds (35 ± 5 m^2 or 10 ± 2 m^3).

2.3. Experimental design

The experimental design used was a randomized block design with three replications (ponds). Fifteen fish per pond (triplicate) were exposed for 90 days to the treatments. We chose 90 days because it is the average time that the rice remain in field at south of Brazil. The experiment was carried out in the paddy field, with the fish trapped in submersed cages, measuring 0.30 m (diameter) × 1.05 m (length). Herbicide concentration in water was monitored from the first day until it was not detected. Herbicide was analyzed by High Pressure Liquid Chromatography (HPLC) using the method described by Zanella et al. [8]. The treatments included the commercial herbicides containing clomazone (376 μg/L) and propanil (1644 μg/L) that correspond to 0.376 and 1.644 mg/L of active ingredient (a.i.). A check (control) without herbicide application was carried out at same time exposure. Herbicides were added to the water at the beginning of the experiment. During the experimental period fish were fed once a day with commercial fish food (42% crude protein) (Purina Brazil). At the end of the exposure period (90 days) all fish were sampled and tissues (brain, muscle and liver) were collected. For the enzymatic and metabolic measurements, eight fish samples were collected of each triplicate for analyze because the fish were very small.

2.4. AChE activity assay

The AChE (EC 3.1.1.7) activity was measured using the method described by Ellman et al. [23] and modified by Miron et al. [15]. Samples (brain and muscle) were weighted and homogenized in a Potter–Elvejhem glass/Teflon homogenizer with 150 mM NaCl. The homogenate was then centrifuged for 15 min at 3000 *g* at 5 °C and the supernatant was used as enzyme source. Aliquots of supernatant (50 and 100 μL) (brain and muscle, respectively) were incubated at 25 °C for 2 min with a solution containing 0.1 M phosphate buffer pH 7.5 and 1 mM DTNB. After the incubation period, the reaction was initiated by the addition of acethylthiocholine (0.08 M) as substrate for the reaction mixture. The final volume was 2.0 mL. Absorbance was measured by spectrophotometry at 412 nm during 2 min. Enzyme activity was expressed as μmol of acethylthiocholine (ASCh) hydrolyzed per minute per milligram of protein.

2.5. Catalase activity assay

Catalase (EC 1.11.1.6) activity was assayed by ultraviolet spectrophotometry Nelson and Kiesow [24]. Samples of liver were homogenized in a Potter–Elvejhem glass/Teflon homogenizer with 20 mM potassium phosphate buffer, pH 7.4 (with 0.1% Triton X-100 and 150 mM NaCl) (1:20 dilution), centrifuged at 10000*g* for 10 min at 4 °C. The assay mixture consisted of 2.0 mL potassium phosphate buffer (50 mM, pH 7.0), 0.05 mL H_2O_2 (0.3 M) and 0.05 mL supernatant. Change of H_2O_2 absorbance in 60 s was measured at 240 nm. Catalase activity was expressed in terms of μmol/min/mg protein.

2.6. TBARS determination

Lipid peroxidation was estimated by a TBARS assay, performed by a malondialdehyde (MDA) reaction with 2-thiobarbituric acid (TBA), which was optically measured. Liver, muscle and brain homogenates (100–400 μL) were added to 8.1% sodium dodecyl sulfate (SDS), 2.5 M acetic acid (pH 3.4), 0.8% thiobarbituric acid were added to adjust to a final volume of 2.0 mL. The reaction mixture was placed in a micro-centrifuge tube and incubated for 90 min at 95 °C. After cooling, it was centrifuged at 5.000 *g* for 10 min and optical density was measured by spectrophotometer at 532 nm. TBARS levels were expressed as nmols MDA per mg of protein according to Ohkawa et al. [25]. Protein levels were estimated by the method described by Bradford [26], using bovine serum albumin as standard.

2.7. Metabolic parameters

Liver and muscle glycogen (μmol glucosyl-glucose) were estimated according to Bidinotto et al. [27]. The tissues were weighed (25 and 100 mg, respectively) and was add KOH and ethanol (1 and 3 mL, respectively), for hydrolysis and precipitation of glycogen. For protein determination, the tissues were heated with KOH at 100 °C and centrifuged at 10.000 *g* for 10 min. Supernatant (20 μl) was used to estimate the protein level according method

described by Lowry et al. [28]. For lactate and glucose (sugar soluble) determination, tissue samples were homogenized by adding 10% trichloroacetic acid (1:20 dilution) using a motor-driven Teflon pestle and centrifuged at 5.000 *g* for 10 min for flocculation of the proteins. The deproteinated supernatant (20–50 μl) was used for lactate determination using the method described by Harrower and Brown [29] and sugar soluble was measured according to Park and Johnson [30].

2.8. Statistical procedures

The dada were submitted to a one-way analysis of variance (ANOVA) and treatment means were compared to the Dunnett test ($p \leqslant 0.05$). For the enzymatic and metabolic measurements, eight fish samples were collected of each triplicate, thus the "*n*" number was 24. Data ($n = 24$) were expressed as means ± standard deviation.

4. Results

Acetylcholinesterase as well as catalase activity in all tissues and herbicides tested for a period of 90 days showed a significant decrease (Figs. 1 and 2). TBARS levels were significantly decreased in the brain, white muscle and liver tissues after exposure to both herbicides (Fig. 3). Glycogen levels in the liver were increased after exposure to both clomazone and propanil. Lactate levels did not show any alterations in the hepatic tissue after exposure to herbicides. Liver protein increase in groups exposure to herbicides when compared to control values. However, the herbicide exposure did not cause alterations in liver glucose levels (Table 1). The muscle tissue showed a significant decrease of glycogen and lactate after clomazone and propanil exposure. Glucose levels were significantly decreased in the muscle when the fish were exposed to clomazone, but exposure to propanil did not alter this parameter. Muscle protein levels did not show alterations in herbicides exposure groups when compared to control levels (Table 1). In the plasma, lactate showed increased levels for both herbicides. On the other hand, glucose levels in the plasma were altered only after exposure to commercial formulation containing propanil. Plasma protein levels remain near to control values after both herbicides exposure (Table 1). Clomazone and propanil residues were monitored in water of rice field system to verify herbicide active ingredient presence in water. These results were shown in Fig. 4.

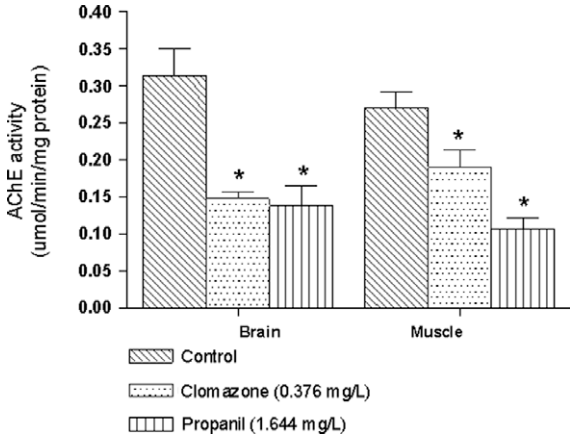

Fig. 1. Acetylcholinesterase (AChE) activity in brain and muscle of *Leporinus obtusidens* after 90 days exposed to the commercial herbicides containing clomazone and propanil at rice paddy field concentrations, 0.376 mg a.i./L and 1.644 mg a.i./L, respectively. Values are means ± SD ($n = 24$), *significant difference between groups and control values ($P \leqslant 0.05$).

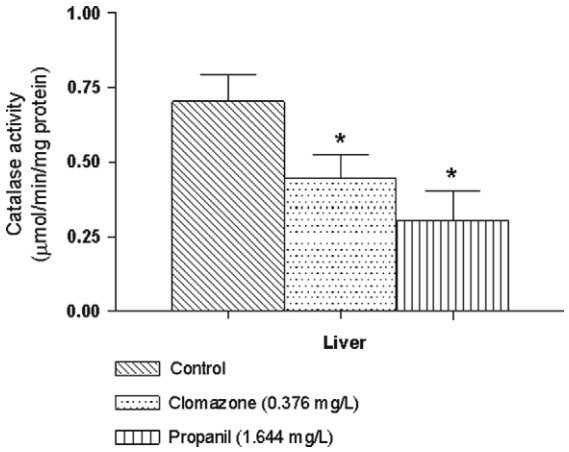

Fig. 2. Liver catalase (CAT) activity in *Leporinus obtusidens* after 90 days exposed to commercial herbicides containing clomazone and propanil at rice paddy field concentrations, 376 μg a.i./L and 1644 μg a.i./L, respectively. Data are reported as means ± SD ($n = 24$). *Significant difference between groups and control values ($P \leqslant 0.05$).

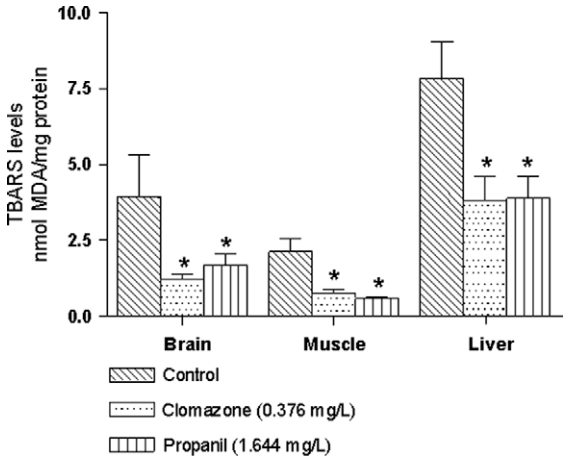

Fig. 3. TBARS levels (nmol MDA/mg of protein) in brain, liver and muscle of *Leporinus obtusidens* exposed to commercial herbicides containing clomazone and propanil at rice paddy field concentrations, 376 μg a.i./L and 1644 μg a.i./L, respectively, for a 90-days period. Data represent the means ± SD ($n = 24$). *Significant difference between groups and control values ($P \leqslant 0.05$).

Table 1
Liver, muscle and plasma metabolites of *Leporinus obtusidens* exposed to herbicides clomazone and propanil at rice paddy field concentrations, 0.376 mg a.i./L and 1.644 mg a.i./L, respectively, for a 90-days period.

Metabolites	Control	Clomazone	Propanil
Liver			
Glycogen (μmol/g tissue)	35.0 ± 0.5	57.0 ± 0.7*	62.0 ± 0.6*
Lactate (μmol/g tissue)	4.9 ± 0.3	4.4 ± 0.2	4.5 ± 0.3
Glucose (μmol/g tissue)	7.7 ± 6.2	7.6 ± 5.9	7.3 ± 3.1
Protein (mg/g tissue)	84.0 ± 7.5	105.0 ± 10.8*	95.0 ± 8.6*
Muscle			
Glycogen (μmol/g tissue)	5.1 ± 0.46	1.6 ± 0.2*	4.0 ± 0.8*
Lactate (μmol/g tissue)	10.4 ± 1	7.9 ± 0.8*	8.8 ± 0.6*
Glucose (μmol/g tissue)	4.8 ± 0.5	3.6 ± 0.6*	4.2 ± 0.2
Protein (mg/g tissue)	97.0 ± 5.3	101.4 ± 4.5	97.4 ± 4.0
Plasma			
Lactate (μmol/mL plasma)	2.8 ± 0.1	3.7 ± 0.2*	3.5 ± 0.2*
Glucose (mg/dL plasma)	113.0 ± 6.2	115.0 ± 6.1	105.3 ± 7.9*
Protein (mg/mL plasma)	17.6 ± 1.8	16.0 ± 2.6	19.8 ± 3.0

* Indicates difference between treatment and the control ($P \leqslant 0.05$).

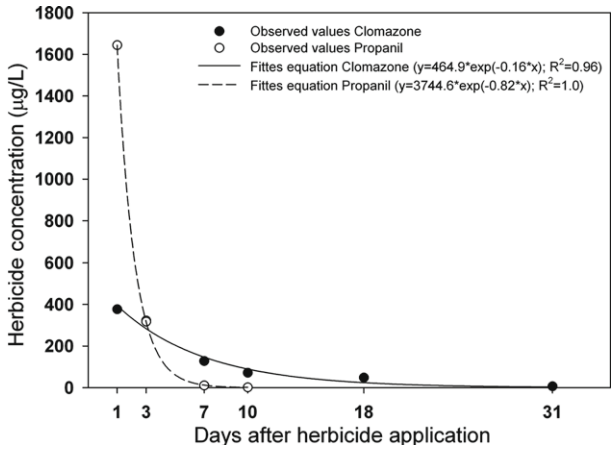

Fig. 4. Clomazone and propanil herbicides concentration (µg a.i./L) in water of rice paddy field.

Persistence (days) and First-order rate constant (µg/L/day) were showed in Table 2. Clomazone herbicide persists in the water until 31 days and propanil herbicide until 10 days after application in the rice field.

5. Discussion

AChE activity decreased significantly in the brain and muscle after exposure to commercial formulations containing clomazone and propanil, when compared to the control values. AChE activity in the brain and muscle of fish exposed to clomazone and propanil was reduced by 53% and 56%, respectively, in the brain and about 30% and 60%, respectively, in the muscle. In another study using the same commercial formulation containing clomazone, AChE was inhibited in the brain of *L. obtusidens* exposed for 30 days in rice field condition [31]. The results of the present study are in agreement with those obtained by Miron et al. [15], where clomazone was identified as a potent inhibitor in the brain and muscle of silver catfish. On the other hand, AChE activity in the brain of *L. obtusidens* and *Rhamdia quelen* exposed to sublethal concentrations of a commercial herbicide containing glyphosate was lower than that of the control group, but the same herbicide did not cause significant alterations in muscle AChE activity [32,33]. AChE inhibition was also observed in the brain and muscle of *Oreochromis mossambicus* exposed to a phosphorothionate (RPR-V) insecticide (0.017 mg/L) for a period of 30 days [34]. AChE inhibition was found in European eels (*Anguilla anguilla*) exposed to diazinon insecticide (0.042 mg/L) [35]. AChE activity in the brain of *A. Anguilla* exposed to molinate herbicide decreased by between 16% and 31% as a function of exposure time [3]. The inhibition of AChE can influence the process of cholinergic neurotransmission and promote undesirable effects. The reduction in AChE in the brain and muscle found in our investigation may be caused by the herbicides themselves during short term period and persists after long time. The effects could be cumulative due to herbicide retention in tissues or caused also by surfactants used in the commercial herbicides formulations. In fact considering results presented in Fig. 4 and Table 2 clomazone residues were found in water until 31 days and propanil until 10 days. Thus the sum of results observed in prolonged time exposure could be due to commercial formulations and not only for clomazone and propanil per se.

The present study showed a decrease of liver catalase activity in *L. obtusidens* after exposure to both herbicides tested. This reduction ranged from 37% for clomazone to 57% for propanil. In other experiments from our laboratory, a reduction in catalase activity was also observed in the liver of silver catfish exposed to clomazone (0.5 or 1.0 mg/L) after 12, 24 and 96 h [36]. In addition, there was a decrease in TBARS levels in all tissues studied for both herbicides. Different classes of pesticides or their metabolites can induce lipid peroxidation. In this study the reduction of tissue TBARS formation seems to be related to the reduction of liver catalase after exposure to the herbicides. The sum of results concerning TBARS and catalase levels may indicate a lack of antioxidant response represented by the increase in catalase. This response could be a survival strategy in response to herbicide toxicity and other enzyme or non-enzyme antioxidants defense protect tissues against TBARS formation. Catalase has shown an increase of activity after exposure to several contaminants [37]. Besides the importance of catalase, the antioxidant defense system includes other enzymes and non enzymatic antioxidants [16].

Liver glycogen levels were increased significantly after exposure to both herbicides. This result differs from that of other authors that showed a glycogen reduction after pesticide exposure [21,38]. Considering the long exposure time in the present study (90 days), the liver glycogen level can be indicative of tissue adaptation to herbicide exposure. Hepatic lactate levels were not significantly altered by either herbicide. Similarly, the liver glycogen results observed in this study were in accordance with those obtained by [39] in silver catfish exposed to clomazone at rice field concentrations. In that study, *R. quelen* exhibited an increase in protein levels in liver tissue when exposed to clomazone, similarly to observed in this study when herbicides exposure increase liver protein levels. Results concerning liver protein suggest that fish exposed to clomazone and propanil may have a compensatory mechanism to deal with possible tissue protein loss by means an increasing protein synthesis. Oruç and Üner [40] observed liver protein increase in carps after exposure to 2,4-D for 30 days. For other side, herbicides exposure did not cause any alterations on liver glucose.

In the present study, muscle tissue showed a significant reduction of glycogen and lactate levels after exposure to both herbicides. Protein levels in the muscle tissue were not affected by exposure to either herbicide and only exposure to clomazone

Table 2
Herbicide concentration, persistence and first-order rate constant (K^c) in water ponds. Data were expressed as means ± S.D. and represent the mean of three measurements of each pond.

Herbicides	Rate (g/ha)	Concentration (µg/L)[a]/(days)						(days)[b]	K^c
		1st	3rt	7th	10th	18th	31th		
Clomazone	500	376 ± 1.2	322 ± 5	127 ± 9	72 ± 9	47 ± 8	5.6 ± 1	31	0.135
Propanil	3600	1644 ± 9	317 ± 8	10 ± 0.5	0.5 ± 0	nd[d]	nd	10	0.889

[a] The quantification limit of the analytical methods for herbicides was 0.1 µg/L.
[b] Date of the last collection when the herbicide was quantified.
[c] *K*, First-order rate constant (µg/L)/day.
[d] n.d, non detectable.

caused a decrease in glucose levels. A reduction in the muscular glycogen level after exposure to both clomazone and propanil may indicate a classic response against energy depletion, by using the muscle glycogen store. The decrease of muscle lactate also indicated tissue adaptation and clearly a gluconeogensis response against herbicide toxicity. Reductions in glycogen in the liver and muscle after pesticide exposure have been reported in several studies [41]. Fish demonstrate different responses concerning metabolic parameters such as glycogen and glucose [42].

Changes in metabolic blood parameters are indicated in Table 1. Fish exposed to propanil showed a significant decrease in the plasma glucose levels. Plasma lactate levels increased significantly after exposure to both herbicides, and this may be related to lactate export to the liver for metabolism, considering that the reduced muscle lactate tends to increase blood lactate levels. No significant changes were observed in plasma protein levels after exposure to clomazone and propanil. Considering that clomazone was measured until 31 days in rice field condition and propanil until 10 days, the results showed in this study could represent the sum of toxic effects caused by herbicides or their adjuvants present in commercial formulation. The fish was affected and change metabolic and oxidative stress parameters.

Results concerning metabolic parameters are early indicators of rice field herbicide exposure. More specific studies concerning tissue herbicide bioaccumulation in the fish *L. obtusidens* are needed. The present study showed that commercial formulations containing clomazone and propanil herbicides at rice field concentrations may cause changes in toxicology and metabolic parameters of *L. obtusidens*. The parameters changes found in this study after 90 days of exposure to herbicides indicate that the toxicity is due to commercial formulation, but not only of active ingredient. These parameters can be used to evaluate herbicide toxicity in fish.

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ACETYLCHOLINESTERASE ACTIVITY IN THE BRAIN AND MUSCLE OF *Cyprinus carpio* AND *Aristichthys nobilis* EXPOSED TO AZIMSULFURON AND METSULFURON-METHYL

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Common carp (*Cyprinus carpio*) and bighead carp *(Aristichthys nobilis)* were exposed to azimsulfuron and metsulfuron-methyl (50, 100 and 200 mg L^{-1}). These herbicides are used in rice crop in Southern Brazil. Fishes survived to all tested concentrations of both herbicides and showed normal feeding and swimming behavior. Azimsulfuron inhibits significantly acetylcholinesterase (AChE) in brain and muscle of both species, and metsulfuron-methyl increase AChE activity in brain and inhibits in muscle. The present study showed that azimsulfuron and metsulfuron-methyl did not affect *C. carpio* and *A. nobilis* behaviors (feeding and swimming), but inhibited AChE activity in brain and muscle tissues of these species.

KEY-WORDS: HERBICIDES, AChE, CARP; SULFONYLUREA.

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1 INTRODUCTION

The rice production in Brazil demands the intense use of agrochemicals, mainly herbicides, due to the adopted flooded cultivation, that frequently benefits the emergence of aquatic weed species, which reduces rice growth. Among the rice herbicides registered in Brazil, two of the most used are sulfonylurea, as azimsulfuron [1-(4,6-dimetoxipirimidine-2)-3-(1-methyl-4-(2-methyl-2H-tetrazol-5)pyrazol-5-sulfunylurea, and metsulfuron-methyl (methyl 2-[[[(4-metoxi-6-methyl-1,3,5-triazine-2-il)amine]carbonyl]amine] sulfonylbenzoate-sulfonylurea) that are selective pre-emergence and post-emergence herbicides, used in pre germinated system, to control broadleaf weeds and some grasses in Southern Brazil (SOSBAI, 2007). The chemical characteristics for azimsulfuron are: water solubility = 1050 mg L^{-1} at pH 7 and coefficient of partition octane-water (log K_{ow}) = -1.37 at pH 7 for metsulfuron-methyl, water solubility = 2790 mg l^{-1} at pH 7 and log K_{ow} 0.018 at pH 7 (SENSEMAN, 2007). When used in the vicinity of aquatic ecosystems, these pesticides may enter water bodies as a result of spray drift, leaching from the soil and surface runoff during precipitation in concentrations which may exert adverse effects on non-target organisms inhabiting the area (CHANDRASEKARA and PATHIRATN, 2007). Adverse effects of agrochemicals used in rice culture on biochemical parameters of non-target organisms like fish were previously observed (MIRON et al., 2005; CRESTANI et al., 2007; MORAES et al., 2007; CATTANEO et al., 2008; FONSECA et al., 2008; GOLOMBIESKI et al., 2008; MORAES et al., 2009). Rice-fish culture has been suggested as an alternative for fish and/or rice farmers in South Brazil (SATO, 2002), and the carps *Ctenopharyngodon idella, C. carpio, A. nobilis and Hypophthalmichthys molitrix* had been the main most frequent species used in this system (SATO, 2002; MARCHEZAN et al., 2006; GOLOMBIESKI et al., 2005; 2007).

Pesticides produce many physiological and biochemical changes in freshwater organisms by influencing the activities of several enzymes (SANCHO et al., 1998). The enzyme acetylcholinesterase plays an essential role in acetylcholine-mediated neurotransmission. It is contained in cholinergic synapses in the central nervous system and in neuromuscular synapses where it rapidly hydrolyzes acetylcholine (DOWNES et al., 2004). Acetylcholinesterase (AChE; EC 3.1.1.7) activity is one of the most frequently used indicators to verify carbamate and organophosphate effects (CHUIKO, 2000; AGUIAR et al., 2004). However, brain AChE activity was inhibited by pesticides or herbicides of another classes as insecticide organochlorine (endosulfan) (DUTTA and ARENDS, 2003) and herbicides isooxazolidinone (clomazone) (MIRON et al., 2005), glycine (glyphosate) (GLUSCZAK et al., 2006) and aryloxialcanoic acid (FONSECA et al., 2008). The inhibition of AChE for herbicides can affect locomotion and equilibrium in exposed organisms and may impair feeding, escape, and reproductive behavior (SAGLIO and TRIJASSE, 1998; BRETAUD, TOUTANT & SAGLIO, 2000).

Common carp (*C. carpio*), and bighead carp (*A. nobilis*) (Cypriniformes) are omnivorous and zooplanktonic species, respectively. This species also could be used in association with fish-rice fields, where herbicides such as azimsulfuron and metsulfuron-methyl are common. Thus, the aim of this study was to investigate the effects of azimsulfuron and metsulfuron-methyl on behavior and AChE activity in brain and muscle tissues of common carp and bighead carp as a possible early indicator of toxicity.

2 MATERIAL AND METHODS

The herbicides used in this study were obtained commercially as follows: metsulfuron-methyl (Ally - 50% purity), and azimsulfuron (Gulliver - 50% purity) both from Dupont® (Brazil), and dissolved in water. Acetylthiocholine (AChE, EC 3.1.1.7), DTNB (5,5'dithio-bis 2 nitrobenzoic acid), and bovine serum albumin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents used in the experiments were of the highest analytical grade (Aldrich).

Common carp (weight 10.78 ± 3.48 g and length 8.64 ± 0.81 cm) and bighead carp (weight 9.42 ± 0.6 g and length 8.86 ± 0.38 cm) were obtained from a commercial fish farm near Santa Maria, Rio Grande do Sul State, Brazil, and transported to the Fish Physiology Laboratory of the Universidade Federal de Santa Maria, Santa Maria, Brazil. Fish were acclimated to laboratory conditions for seven days. They were kept in tanks (250 L) and the water was constantly aerated in a static system. Water parameters were measured every day, and were as follow: temperature 17.8 ± 0.16°C, pH 7.6 ± 0.02 units, dissolved oxygen 5.5 ± 0.16 mg L^{-1}, non-ionized ammonia 0.02 ± 0.002 mg L^{-1}, nitrite 0.03 ± 0.014 mg L^{-1}, alkalinity 45 ± 3.25 mg L^{-1} $CaCO_3$ and hardness 21 ± 3.56 mg L^{-1} $CaCO_3$. During acclimation, fish were fed once a day with commercial fish pellets (42% crude protein, Supra, Brazil). Sewage and pellet leavings were removed every other day by suction.

After the acclimation period, groups of 10 fish/box (36 L glass box) (triplicate) were exposed for 96h, as usually performed in acute experiments (MIRON et al., 2008), to different nominal concentrations of azimsulfuron and metsulfuron-methyl (mg L^{-1}): 50, 100 e 200. Control fish were maintained in the same condition, but in water without the herbicide. Herbicides were added to the water only at the beginning of the experiment. During the experimental period fish were fed every day with commercial feed (44% crude protein) (Purina Brazil). Feces and pellet residues were removed daily by suction removing the minimal water quantity.

Water pH was measured electrometrically (pHmeter Schott Handylab 1), water temperature and dissolved oxygen with an oxygen meter (Oximeter Oakton) and water hardness and alkalinity according to APHA (1992). Total ammonia and nitrite were determined by kits (Alfatecnoquímica, Florianópolis, Brazil). Throughout experiment swimming activity (normal, erratic swimming, lethargy, immobility) and feeding behavior (feeding or not) were observed for 30 min at the time of feeding, registered, and compared to control. At the end of exposure period (96h), six fish were randomly chosen from each box and killed by punching the spinal cord behind the opercula, placed in ice and, tissues (brain and muscle) were removed on ice, frozen in liquid nitrogen and then stored at -20°C until AChE assay.

Enzyme activity was determined in fishes exposed to all tested concentrations of both herbicides: 50,100 and 200 mg L^{-1}. Brain and muscle tissues were weighed and homogenized in 150 mM NaCl (15 mg mL^{-1}) using a Potter-Elvejhem glass/Teflon homogenizer. The homogenates were centrifuged for 15 min at 3000 g at 5°C and the supernatant was used as the enzyme source. AChE activity was measured as described by ELLMAN, COURTNEY & ANDRES (1961) and modified by MIRON et al. (2005). Aliquots of supernatant (50-100 μL) (brain and muscle, respectively) were incubated at 25°C for 2 min with 0.1 M phosphate buffer, pH 7.5; 1 mM DTNB as chromogen. After 2 min, the reaction was initiated by the addition of acethylthiocholine (0.08 M) as substrate for the reaction mixture. The final volume was 2.0 mL. Absorbances were determined at 412 nm during 2 min. Enzyme activity was expressed as μmol of acethylthiocholine (AcSCh) hydrolyzed per min per gram of protein.

The values for common carp were for azimsulfuron (minimum and maximum): dissolved oxygen 6.1-6.8 mg L^{-1}, temperature 17.2-18.4°C, pH 7.6-7.9, alkalinity

32-46 mg L^{-1} $CaCO_3$, hardness 24-32 mg L^{-1} $CaCO_3$, total ammonia 0.5-3 mg L^{-1}, non-ionized ammonia 0.01-0.02 mg L^{-1}, and nitrite 0-0.05 mg L^{-1}; metsulfuron-methyl: dissolved oxygen 6.8-7.4 mg L^{-1}, temperature 15-16.7°C, pH 7.4-7.8, alkalinity 34-49 mg L^{-1} $CaCO_3$, hardness 20-32 mg L^{-1} $CaCO_3$, total ammonia 0.5-2 mg L^{-1}, non-ionized ammonia 0.007-0.018 mg L^{-1}, and nitrite 0.02-0.05 mg L^{-1}.

For bighead carp the parameters were: azimsulfuron: dissolved oxygen 6.1-7.1 mg L^{-1}, temperature 16.6-20.1°C, pH 7.5-7.9, alkalinity 24-40 mg L^{-1} $CaCO_3$, hardness 24-36 mg L^{-1} $CaCO_3$, total ammonia 0.5-2 mg L^{-1}, non-ionized ammonia 0.011- 0.038 mg L^{-1} and nitrite 0.03-0.05 mg L^{-1}; metsulfuron-methyl: dissolved oxygen 6.0-6.5 mg L^{-1}, temperature 19.4-20.7°C, pH 7.7-8.0, alkalinity 34-39 mg L^{-1} $CaCO_3$, hardness 20-24 mg L^{-1} $CaCO_3$, total ammonia 0.5-1 mg L^{-1}, non-ionized ammonia 0.024-0.038 mg L^{-1}, and nitrite 0-0.02 mg l^{-1}.

Statistical analyses were performed using one-way analysis of variance (ANOVA). Means were compared by Tukey test and expressed as mean ± standard error ($n = 6$) (BANZATO and KRONCA, 1995). Differences were considered to be significant at a probability level of $P < 0.05$ between treatments and controls.

3 RESULTS

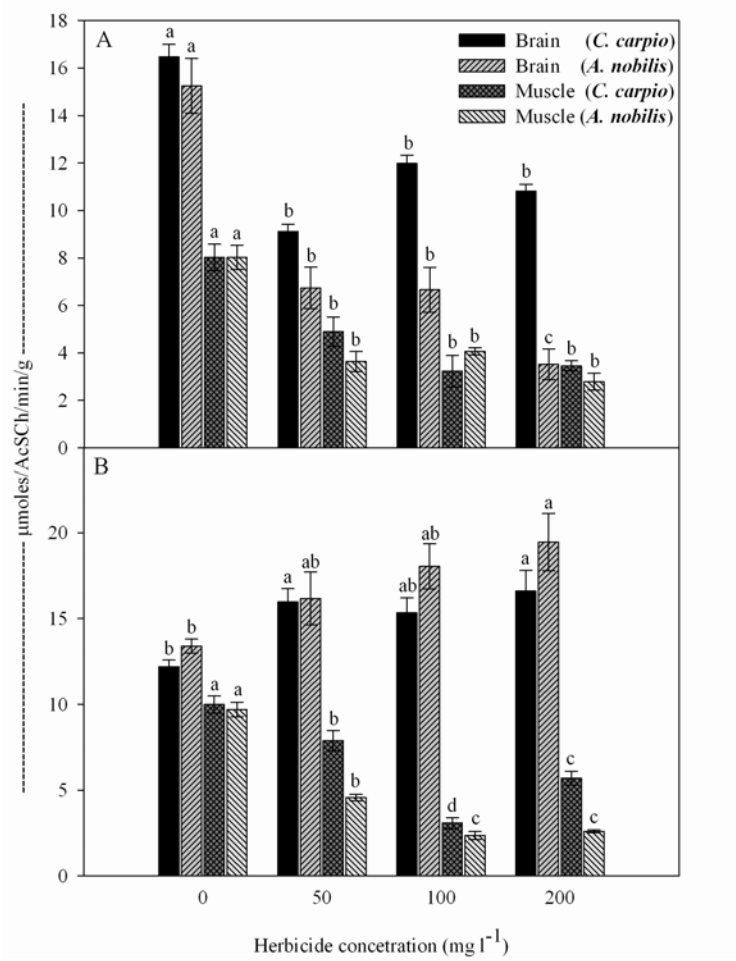
All common and bighead carps fingerlings exposed to azimsulfuron and metsulfuron-methyl survived even at the highest concentration used (200 mg L^{-1}), and showed normal feeding and swimming behavior at all concentrations tested (Table 1).

TABLE 1 - EFFECT OF DIFFERENT CONCENTRATIONS OF AZIMSULFURON AND METSULFURON-METHYL ON FEEDING AND SWIMMING BEHAVIORS, AND BRAIN AND MUSCLE AChE ACTIVITY IN CARPS

Herbicides/ species	Feeding behavior	Swimming behavior	Brain AChE activity (% of control)	Muscle AChE activity (% of control)
			Common Carp	
Azimsulfuron				
Control	Feeding	Normal	100	100
50 mg L^{-1}	Feeding	Normal	55	61
100 mg L^{-1}	Feeding	Normal	73	40
200 mg L^{-1}	Feeding	Normal	66	43
Metsulfuron				
Control	Feeding	Normal	100	100
50 mg L^{-1}	Feeding	Normal	131	79
100 mg L^{-1}	Feeding	Normal	126	31
200 mg L^{-1}	Feeding	Normal	136	57
			Bighead carp	
Azimsulfuron				
Control	Feeding	Normal	100	100
50 mg L^{-1}	Feeding	Normal	44	45
100 mg L^{-1}	Feeding	Normal	44	51
200 mg L^{-1}	Feeding	Normal	23	35
Metsulfuron				
Control	Feeding	Normal	100	100
50 mg L^{-1}	Feeding	Normal	121	47
100 mg L^{-1}	Feeding	Normal	135	24
200 mg L^{-1}	Feeding	Normal	145	27

Brain of unexposed control fish showed higher specific AChE activity than muscle for azimsulfuron (Figure 1 - A); control: common carp 16.47 against 8.02 and bighead carp 15.6 against 8.02 μmol/min/g protein, respectively. After azimsulfuron exposure, AChE activity was significantly lower (P<0.05) at all concentrations tested (Figure 1- A and B) in both tissues of the two species, after 96h exposure to azimsulfuron. Azimsulfuron inhibition reached maximum of 45% in the brain and 60% in the muscle of common carp and 77% in the brain and 65% in the muscle of bighead carp (Table 1).

FIGURE 1 - EFFECTS ON THE AChE ACTIVITY IN BRAIN AND MUSCLE OF CARPS (*Cyprinus carpio* AND *Aristichthys nobilis*) AFTER 96h EXPOSURE FOR AZIMSULFURON (A) AND METSULFURON-METHYL (B)



Values are mean ± sem (N = 6). Data are expressed as μMol/acsch/min/g protein. Different letters indicate significant difference of AChE activity considering the tissue analyzed in each fish species (P<0.05).

For metsulfuron-methyl (Figure 1- B) brain AChE activity was higher than muscle for two carps species in control treatment; common carp 12.2 against 9.99 and bighead carp 13.4 and 9.7 μmol/min/g protein. Maximum metsulfuron-methyl inhibition in the muscle common carp and bighead carp was 69 and 76%, respectively.

Conversely, exposure to all metsulfuron-methyl concentrations tested for 96h significantly increased AChE activity in the brain of both species (Figure 1 - B) (common carp 31-36% and

bighead carp 21-45% as compared to control values). However, for muscle tissue significantly decreased AChE activity to all metsulfuron-methyl concentrations for two carps.

4 DISCUSSION

In Southern Brazil azimsulfuron and metsulfuron-methyl are applied to rice crop at 0.005 and 0.002 mg L^{-1}, respectively (SOSBAI, 2007). In the current study, no fish mortality or changes in feeding and swimming behavior was observed even in the highest concentration (200 mg L^{-1}) for both herbicides, what represents 40.000 and 100.000 times higher than the concentrations of rice fields water. Therefore, in carps these parameters can not be used as indicators of water contamination by these herbicides. RESGALLA et al. (2002) found for common carp fingerlings (2-8 cm) 96h LC_{50} 26 mg L^{-1} for metsulfuron-methyl (concentration tested 1-64 mg L^{-1}). SENSEMAN (2007) reported carps 96h LC_{50} for azimsulfuron > 1000 mg L^{-1} and for rainbow trout 492 mg L^{-1}, and the 96-h LC_{50} was 150 mg L^{-1} for metsulfuron-methyl.

The herbicides can affect various types of behavior in fish, directly as well as indirectly by altering the chemical perception of natural substances of ecoethological importance (SAGLIO and TRIJASSE, 1998). The results obtained for carps exposed to azimsulfuron and metsulfuron-methyl showed feeding and normal swimming behavior at all concentrations. Silver catfish (*Rhamdia quelen*) fingerlings exposure to metsulfuron-methyl (200 to 1200 mg L^{-1}) showed normal feeding behavior but burst swimming reactions at all tested concentration (400, 800 and 1200 mg L^{-1}) (MIRON et al. 2004). This change of swimming activity was also observed in goldfish exposed of the 0.001 to 10 mg L^{-1} nicosulfuron (sulfunylurea) (SAGLIO et al., 2001).

The activity of AChE enzyme is extremely important for many physiological functions, such as fast locomotion, predator evasion, and orientation toward food. When AChE activity decreases, ACh is not broken and accumulates within synapses which therefore cannot function in a normal way (DUTTA and AREND, 2003).

The results of the present study showed that, in unexposed fish brain to azimsulfuron, AChE-specific activity was two-fold higher than that in muscle tissue. Higher brain AChE activity was also observed in channel catfish (*Ictalurus punctatus*) after exposure to organophoshorus compounds (STRAUS and CHAMBERS, 1995). The AChE activity in juvenile goldfish (*Carassius auratus*) exposed to nicosulfuron herbicide (sulfunylurea) was higher in brain and did not induce effect in the muscle (BRETAUD, TOUTANT & SAGLIO, 2000). *Labeo rohita* exposure to cypermetrin (organophosphorus) (DAS and MUKHERJEE, 2003) had significantly decreased in brain AChE activity in 45 days.

The AChE inhibition observed in fish exposed to azimsulfuron, leads to an accumulation of acetylcholine, causing over-stimulation of the receptors. According to FERNÁNDEZ-VEGA et al. (1999; 2002) and MIRON et al. (2005) AChE inhibition in brain and muscle produces adverse effects in movement because AChE participates in neural and neuromuscular transmissions. However, AChE activity in the brain of carps exposed to metsulfuron-methyl was higher than in control fish, but no change on feeding and swimming behaviors, with active carps until 96h exposition. This study is in agreement with MIRON et al. (2005) that obtained the same results for silver catfish *(Rhamdia quelen)* exposed to several metsulfuron-methyl concentrations (400, 800 and 1200 mg L^{-1}).

The activation observed in AChE activity after exposure to metsulfuron-methyl could represent an increase in the hydrolysis of the neurotransmitter acetylcholine, with a

consequent decreased activation of nicotinic and muscarinic receptors. Thus, activation or inhibition of AChE can influence the process of cholinergic neurotransmission and promote undesirable effects. Therefore, azimsulfuron and metsulfuron-methyl appears safe for common and bighead carps.

5 CONCLUSION

The present study showed that azimsulfuron and metsulfuron-methyl did not affect *C. carpio* and *A. nobilis* behavior, but inhibited AChE activity in brain and muscle tissues of these species.

RESUMO

ATIVIDADE DA ACETILCOLINESTERASE EM CÉREBRO E MÚSCULO DE CARPAS *Cyprinus carpio* E *Aristichthys nobilis* EXPOSTAS A AZIMSULFURON E METSULFURON-METIL

Alevinos de carpa húngara (*Cyprinus carpio*) e carpa cabeça grande *(Aristichthys nobilis)* foram expostas a azimsulfuron e metsulfuron-metil (50, 100 and 200 mg L^{-1}), herbicidas utilizados em lavouras de arroz no Sul do Brasil. Os peixes sobreviveram a todas as concentrações testadas de ambos os herbicidas e mostraram comportamento alimentar e natatório normal. Azimsulfuron inibiu significativamente a enzima acetilcolinesterase (AChE) em cérebro e músculo de ambas as espécies e metsulfuron-metil aumentou a atividade da AChE no cérebro e a inibiu em músculo. O presente estudo mostrou que azimsulfuron e metsulfuron-metil não afetam os comportamentos (alimentar e natatório) de *C. carpio* e *A. nobilis*, mas inibiram a atividade da AChE em tecido cerebral e muscular dessas espécies.

PALAVRAS-CHAVE: HERBICIDAS; AChE; CARPAS; SULFUNILURÉIA.

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