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Markers of Pesticide Exposure in Irrigated Rice Cultures

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The objective of this research is to verify the genotoxicity caused by pesticides used in irrigated rice cultures in Araranguá city in the southern Brazilian state of Santa Catarina through the alkaline comet assay in peripheral blood of *Geophagus brasiliensis* and to analyze the toxicity of the water using *Daphnia magna* as sentinel organism. Three collections of water and fish were made in the main rice ditch, and one collection for the control group was taken in the Araranguá River. The toxicity test with *D. magna* and the comet assay followed protocols previously described. The toxicity factor for the control group and collections 1, 2, and 3 were, respectively, 0, 1, 0, and 2. The comet assay demonstrated significant differences just in collection 2, in comparison to the control group and collections 1 and 3. These results, despite significant statistical data, are not a biological problem, because the values were not so large but serve to warn of a possible disruption of the balance in this environment system.

KEYWORDS: Pesticides; *Daphnia magna*; comet assay; *Geophagus brasiliensis*

INTRODUCTION

Pollution is a modification capable of provoking an interruption in the natural sequence of biological evolution, causing a break in ecosystem balance (*1*). Contamination sites pose significant environmental hazards for terrestrial and aquatic ecosystems. They are important sources of pollution and may result in ecotoxicological effects (*2*). Aquatic environmental pollution is a serious and growing problem (*3*), with the increasing number of industrial, agricultural, and commercial chemicals in the aquatic environmental having led to various deleterious effects on organisms (*4*).

Due to the model of agriculture adopted in Brazil, pesticide usage has become intensive, and many environmental problems of contamination are occurring (*5*). The most frequent impact happens through the accumulation of compositions that are not excreted by the organisms and are transmitted to a higher level in the food chain (*6*). Although agriculture is only one of the nonpoint sources of pollution, usually it is indicative as the most important contributor among all of the pollutant categories (*7*).

In this context, DNA damage has been proposed as a useful biomarker for assessing the genotoxic properties of environmental contaminants in biomonitoring studies (*8*−*12*), the comet assay being one of the most used test to this propose (*8*). The use of fish biomarkers as indices of pollution effects are of increasing importance and can permit early detection of environmental problems (*13*−*15*). Fish can bioaccumulate toxic substances (*16*) in a direct way through the skin or breathing, absorbing through the gills, or indirectly when they ingest these pollutants (*17*). Species such as *Daphnia* constitute an important food source for fish and are widely used as bioindicators in ecotoxicological tests (*18*, *19*). Their descendants are genetically identical, which ensures greater uniformity in the results of the assays (*18*).

Ecotoxicological effects occur at all levels of biological organization, from the molecular to the ecosystem level (*2*). One of the ecosystems that stands out with the presence of these effects is irrigated rice (*20*, *21*), which is predominant in Araranguá city. The culture of irrigated rice is the largest consumer of water on a world scale (*22*). The water that is removed for use from the Araranguá River stays stagnant quiet a long time in the rice paddy, especially during the period of application of the pesticides. This water is used by species of fish that enter and are then found in the ditches used in the cultivation of the rice paddy, being exposed to the pesticides.

Despite the growing pollution originating from agriculture, a study of the genotoxicity of these pollutants in the fish that inhabit the used area, as well as ecotoxicological analysis of these waters, was not still done. In this study, we verified the genotoxicity caused by the pesticides used in the agriculture of irrigated rice, in the city of Araranguá, through the comet assay in peripheral blood of *Geophagus brasiliensis*, and we analyzed the toxicity of the water using *Daphnia magna* as a bioindicator.

MATERIALS AND METHODS

Site Description. We studied the toxicity of pesticides used in the agriculture of irrigated rice in Araranguá city, located in the southern Brazilian state of Santa Catarina. The study area is a total of 40 ha of planted and irrigated rice, located in the Itopaba neighborhood of Araranguá city, close of the Araranguá River, divided by the SC 449 (Km 26) highway.

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The cultivation of irrigated rice has been practiced for more than 40 years in this area. During this period different pesticides have been used. The water that irrigates the rice paddies of this area is removed directly from the Araranguá River and does not cross any neighboring plantation.

Species Analyzed. *D. magna* Straus, 1820 (Cladocera, Crustacea), is known popularly as "water flea" and constitutes a food source for fish and other animals (*19*). Several countries use this species as a bioindicator. In France, for example, *D. magna* was chosen for the monitoring of industrial effluents and other toxicant agents. In the United States, its use is recommended by the Environmental Protection Agency (EPA) for evaluation of the toxicity for pesticides (*23*).

G. brasiliensis (acará) is of the Cichlidae family, of fresh water, and are appreciated as a food and are used as an ornamental. The feeding constituted of sediments deposited in the mud, where the residues of the pesticides cannot act with the DNA (*24*). This species is easy to capture and the most abundant in the study place.

Collection of Samples. Three collections of water and fish were made in the main rice ditch (28° 55′ 2″ S and 49° 31′ 20″ W) that receives pumped water from the Araranguá River, and one collection for the control group was accomplished in the same river, at a distance of 500 m from the sampling area.

The first collection was during the planting of the rice (November 2006). The rice seeds were not treated or immunized before the planting. The fish were collected in the ditch by use of fish nets. To perform the comet assay 10 *G. brasiliensis* fishes were collected independent of size, weight, and sex.

Peripheral blood samples were drawn from the cardiac vein using heparinized syringes to avoid clotting. After collection, the blood was stored in a fresh and protected place from light until the completion of the analysis in the laboratory. Simple water samples were collected in duplicate and stored in polyethylene flasks that were maintained refrigerated until the analysis. Peripheral blood cells are among the most used cells for genotoxicity studies, mainly with the comet assay (*25*−*27*). They circulate through the entire body and are easily obtained.

Twenty days after the planting of the rice there was an application of the pesticides Ricer 240 SC (Dow Agro Sciences) and Basagran 600 (BASF) to the rice. The pesticides Glyfosate (Nortox) and DMA 806 BR (Dow Agro Sciences) were used for the control of harmful herbs in the small land levees of the rice fields and in the ditches and were twice applied, the first one in the period of preparation of the soil for planting and other just days before the planting of the rice crop. In December 2006, 10 days after the application of the pesticides Ricer 240 SC and Basagran 600, the second collection was made, following the same procedures as the first. In February 2007, the beginning of the rice crop and the collection of water and fish took place, following the same procedures as for the previous collection of samples.

The control group was collected in April 2007 from the Araranguá River, again following the same procedures of the previous collections, using 27 *G. brasiliensis*. The water samples were collected according to the previous procedures.

Toxicity Test with *Daphnia magna*. The toxicity tests with *D. magna* were accomplished in agreement with the norms established by the Associação Brasileira de Normas Técnicas (ABNT) present in NBR 12713 (*28*), in the Laboratório de Ecotoxicologia do Instituto de Pesquisas Ambientais e Tecnológicas (IPAT), of the Universidade do Extremo Sul Catarinense (UNESC), Criciúma (SC), Brazil.

Juvenile *D. magna* were obtained by cultivation in the laboratory, with age varying from 2 to 24 h, were fed daily with green algae of the species *Senedesmus subspicatus*, and were maintained in a stove incubator with a controlled temperature of 20 °C on a 16 h light/dark cycle for day. In each test, controls were used for validation of the experiment that consisted in the maintenance of the organisms in culture medium. The tests were in duplicate, and they contained 10 organisms per flask (50 mL). In this test we determined water pH, dissolved oxygen (DO) content, and the hardness of the solution test in each experiment.

The period of the test was for 48 h. In the end it was registered for the number of immobile organisms in each flask. The organisms were considered to be immobile when they did not present movement after 10 s of observation, accompanied by light agitation of the flasks.

Tests of sensibility of the organisms with a standard of potassium dichromate solution ($K_2Cr_2O_7$) were made periodically for greater safety in the answers to the tests. DO, pH, and hardness were also verified in agreement with the American Public Health Association (APHA) (*29*).

Table 1. Toxicity Factor of the Water Samples Collected in the Study Area [Adapted from Knie and Lopes (*18*)]

dilution	ml/L of sample (50 mL)	toxicity factor	%
1:1	50.00	1	100.00
1:2	25.00	2	50.00
1:3	16.67	3	33.34
1:4	12.50	4	25.00
1:6	8.33	6	16.67
1:8	6.25	8	12.60
1:12	4.17	12	8.34
1:16	3.125	16	6.25
1:24	2.084	24	4.167
1:32	1.562	32	3.125
1:48	1.042	48	2.083

Comet Assay. The alkaline comet assay was performed as described by Tice et al. (*30*), with adaptations for fish cells described by Andrade et al. (*14*). The blood samples were diluted 1:120 (v/v) with RPMI-1640 medium and used immediately. Briefly, 5 μL of each diluted blood sample was added to 95 μL of 0.75% (w/v) molten low-melting-point agarose, and a portion of the mixture was spread on a microscope slide precoated with 1.5% (w/v) normal-melting-point agarose and covered with a coverslip. After a brief period on ice to solidify the agarose, the coverslip was carefully removed, and the slide was placed in lysis buffer (2.5 M NaCl, 100 mM EDTA, and 10 mM Tris, pH 10.0−10.5, with freshly added 1% Triton X-100 and 10% dimethyl sulfoxide) for a minimum of 1 h at 4 °C. The slide then was incubated in freshly made alkaline buffer (10 N NaOH and 200 mM EDTA, pH > 13) for 10 min. The slides were then submitted to electrophoresis in the same buffer: 20 min at 25 V and 300 mA. All of these steps were carried out under dim indirect yellow light. Following electrophoresis, the slides were neutralized in 0.4 M Tris (pH 7.5) and the DNA stained with a solution containing 2 mg/mL ethidium bromide.

To demonstrate the sensitivity of the assay, negative and positive controls from human blood were included in each electrophoresis.

Images of 100 randomly selected cells (50 cells from each of two replicate slides) were analyzed from each animal using a fluorescence microscope equipped with an excitation filter of BP546/12 nm and a 590 nm barrier filter. The damage index (DI) was evaluated by using the sum of classes of the 100 cells analyzed and may vary from 0 (all cells undamaged − 0 × 100) to 400 (all cells damaged − 4 × 100). The DI is based on the length of migration and on the amount of DNA in the tail [see figures in Heuser et al. (*31*)], and it is considered to be a sensitive measure of detectable DNA damage. Damage frequency was calculated as the percentage of cells with a tail. International guidelines and recommendations for the comet assay consider that visual scoring of comets is a well-validated evaluation method. Although the DI parameter is subjective, it is highly correlated with computer-based image analysis (*30*, *32*).

Statistical Analysis. For the toxicity test with *D. magna* the tab of dilutions was used (**Table 1**), where the toxicity factor was determined through the direct visualization of the mobility of the organisms in the dilutions test, corresponding to the smallest dilution that did not cause the immobility or death of > 10% of the organisms. The result was expressed in whole number, and it corresponds to the dilution of the solution tests.

All data of the comet assay are presented as means ± SD. Differences among the collections were determined by one-way ANOVA, followed by the Tukey post hoc test. In all experiments, P values of < 0.05 were considered to indicate statistical significance.

RESULTS

The water samples of collection 1 presented larger amounts of DO (6.7 mg/L), compared to the control group (4.8 mg/L). The water samples of collection 3 had the smallest amount of DO (4.6 mg/L) in relation to the control group and collection 1.

Analysis of the pH (**Figure 1**) showed that the water samples of collection 1 presented a smaller pH value (6.8) in relation to the control group (7.19). There was an increase in water pH in collection 2 (7.91) in relation to the control group and collection 1.

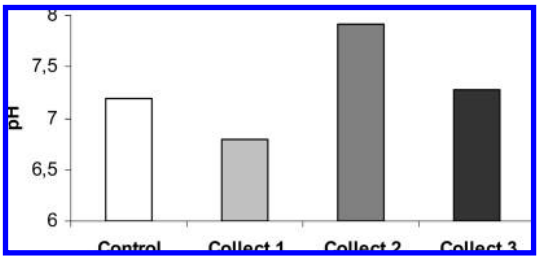

Figure 1. pH variation found in the water samples collected in the study.

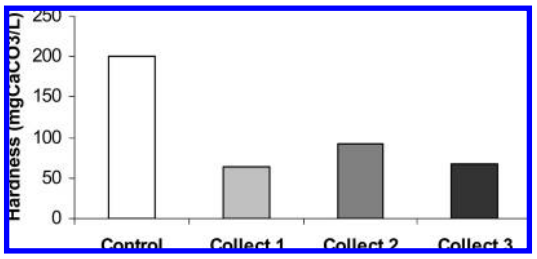

Figure 2. Hardness (mg of $CaCO_3$/L) of the water samples collected in this study.

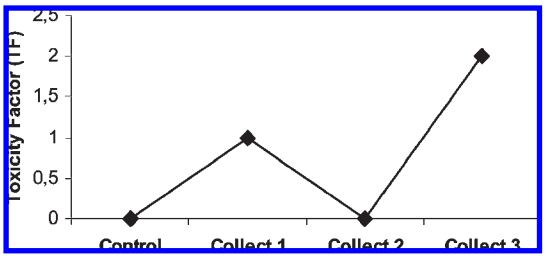

Figure 3. Toxicity factor of the water samples collected in this study.

Water pH in collection 3 (7.27) was lower when compared to collection 2 and higher in relation to collection 1.

The hardness of the samples of collection 1 was 64.68 mg of $CaCO_3$/L, contrasting with 92.12 mg of $CaCO_3$/L in collection 2 and 68 mg of $CaCO_3$/L in collection 3. The control group presented a hardness of 200 mg of $CaCO_3$/L (**Figure 2**).

The toxicity factor (**Figure 3**) for the control group and collections 1, 2, and 3 were, respectively, 0, 1, 0, and 2.

In **Figures 4** and **5**, the damage index and the damage frequency, respectively, are presented. Collection 2 presented larger damage index and damage frequency (11.1 ± 5.43 and 10.5 ± 4.8, respectively), followed by collection 3 (8.9 ± 2.54 and 8.3 ± 3.25) and collection 1 (8.1 ± 2.47 and 7.3 ± 2.0), when compared to the control group (2.41 ± 1.5 and 2.34 ± 1.38).

DISCUSSION

The physicochemical characteristics of the water samples collected in the study area are important and can influence the result of the toxicity tests. The DO is of great importance for the aquatic airborne organisms, being the main variable for the characterization of the effects of the waters for organic pollutants (*18*). The amount of DO present in the samples is within the limit (O_2 > 2 mg/L) established by Decreto Estadual 12.486 of 20/10/78 (Brazilian law) (*33*), which defines and classifies waters considered to be drinkable.

The pH is an important environmental variable, and it is a factor that limits the survival of aquatic organisms, because most require pH values between 6 and 9. Values below 5.5 can be lethal for several microorganisms, and the limits of tolerance for many

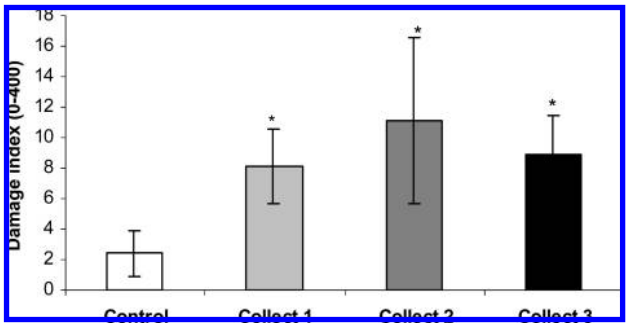

Figure 4. Damage index for the comet assay in the collections of this study. Values are expressed as means ± SD [n = 10 for collections 1, 2, and 3; n = 27 for control group; one-way analysis of variance (ANOVA), Tukey's post hoc, $P < 0.05$; ∗, different from control].

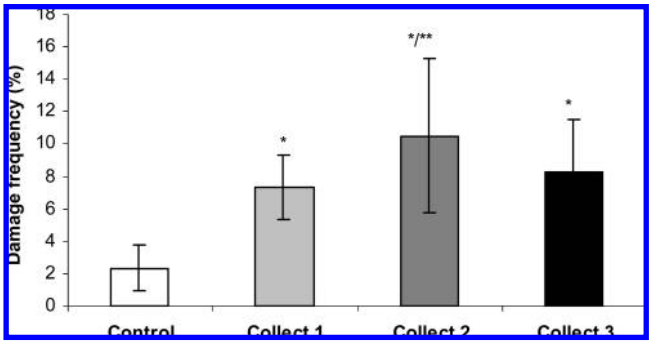

Figure 5. Damage frequency for the comet assay in the collections of this study. Values are expressed as means ± SD [n = 10 for collections 1, 2, and 3; n = 27 for control group; one-way analysis of variance (ANOVA), Tukey's post hoc, $P < 0.05$; ∗, different from control; ∗∗, different from collections 1 and 3].

fishes are between 4 and 10.8 (*18*). The pH values found in the water samples of this study were not divergent enough to interfere in the answers of the tests.

The total hardness parameter verifies the amount of calcium and magnesium present in the sample, expressed in terms of $CaCO_3$ (*18*). All collected samples are within the values recommended by Decreto Estadual 12.486 (*33*), that is, 100 mg/L, with tolerance up to 200 mg/L.

The toxicity factor is determined through the direct observation of the mobility of the organisms in the series of dilutions applied in the toxicity tests, statistical calculation not being necessary. The results show that collections 1 and 3 presented larger toxicity in relation to the control group.

Collection 1 seems to have presented toxicity due to application of the pesticides Glyfosate and DMA 806 BR for the control of harmful herbs in the small land levees and in the ditches of the plantations, in the period of soil preparation for the planting of the rice.

The low toxicity presented in collection 2 can be a function of the intense rain that occurred in the week that preceded the collection and the flow and quantity of water that dispersed the residue of the applied pesticides Ricer 240 SC and Basagran 600 over a wider area of the collection point, with lower levels of these residues.

Collect 3 presented larger toxicity levels in relation to the other collections, which seems to be related with the second application of the pesticides Glyfosate and DMA 806 BR on the small land levees and the ditches of the paddies, added to the new pesticides applied.

The comet assay has the advantage, among others, of speed in obtaining the results (*13*), and this methodology can be adapted for many organisms (*3*, *8*−*13*).

The DNA damage presented in collection 1 seems to be related to the application of the pesticides Glyfosate and DMA 806 BR used at the beginning of the rice planting. These pesticides, added to the newly applied (Ricer 240 SC and Basagran 600), could induce the DNA damage observed in the second collection. This result is the opposite of was observed in the water toxicity in collection 2. The intense rain that occurred during this period did overflow the rice ditches, diluting the residues of the pesticides. The water was led to a final destination, justifying the low toxicity of the water of the collection point.

Before the second application of the pesticides Glyfosate and DMA 806 BR, the fact that the fish can excrete these pollutants, and accumulate them in the body, facilitating a reduction the DNA damage, can explain the data observed in the organisms of collection 3.

In sum, these results, despite the significant statistical evidence, are not biologically a problem, because the index of DNA damage was below 20 (on a scale from 0 to 400) and the damage frequency did not surpass 20%, compared to the control group that was below 10%. Even so, these results serve as an alert for possible breaks in the balance of this set.

The use of the comet assay in the detection of genotoxicity in the aquatic environment is a reliable measure. Pandrangi et al. (*9*), using blood of *Ameiurus nebulosus* and *Cyprinus carpio*, demonstrated that DNA damage is associated at the levels of pollutants in the sediment of North American lakes, and these results indicate that the comet assay is extremely sensitive and should be useful in detecting DNA damage caused by environmental contaminants. Andrade et al. (*14*) investigated the potential of the comet assay for monitoring genotoxicity in mullet and sea catfish and observed the potential application of the comet assay to erythrocytes of mullets and sea catfish.

There are still a few studies using the comet assay as a field bioindicator for fish in aquatic environment. Akcha et al. (*11*) accomplished a study using the fish *Limanda limanda* collected in different places of the English Channel (in southeastern France) known for being contaminated, and the results of the comet assay are in agreement with the levels of pollution of the channel.

Aquatic systems related to the rural environment gather several pollutants, including different pesticides, constituting complex mixtures (*2*). This work did not try to separate which pesticide present in the study place was the one responsible for the effects on the fish and the aquatic environmental or to indicate the interactions of this pesticide mixture with the appraised organisms and its reflection in the environment. Even so, the results are limited because the concentrations of application of the pesticides are not considered.

In conclusion, this study showed chemical and biological data of an area with fields of irrigated rice exposed to different pesticides, indicating an association of the pesticides with damage levels in the blood of fish and toxicity in the water. Despite all efforts, no relationship causes could be established. However, we suggest more detailed studies on the genotoxicity of these pollutants originating from the culture of irrigated rice on fish and analyses of the waters present in the ditches and inside the rice paddy, pointing out the importance of this information for the population.

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