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Evaluation of genotoxicity in *Rhamdia quelen* (Pisces, Siluriformes) after sub-chronic contamination with Fipronil

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Abstract Diverse genetic biomarkers have been used to evaluate the effects of pollution by mutagenic agents such as metals and pesticides, as well as a large variety of chemical substances derived from human activities. This work researched the effects that an exposure of 60 days to the insecticide Fipronil (concentrations of 0.05, 0.10 and 0.23 μg/L) can cause in the fish *Rhamdia quelen* using Comet assay with gills, histopathological analysis of gills and the Piscine Micronucleus test and Nuclear Morphological Alterations. The results for the Comet assay and for gills histopathological injuries showed no difference between the control group and the contaminated groups. In the Piscine Micronucleus test, the smallest concentration of Fipronil (0.05 μg/L) was similar as the control group, while concentrations of 0.10 and 0.23 μg/L caused more damage to the DNA. These results suggested that only the highest concentrations of Fipronil tested cause damage in erythrocytes, but none of these concentrations was sufficient to alter the DNA in the gill cells. *R. quelen* may be a less sensitive bioindicator than other fish that have been tested. On the other hand, the concentrations used may not have been sufficient to detect alterations in the DNA of *R. quelen* with the chosen tests. Works like this take on great importance given the enormous quantity of substances that are thrown daily into the environment in an uncontrolled way, without evaluation of the consequences. The application of these tests with other concentrations, tissues and exposure times is suggested for future works.

Keywords Pesticide · Mutagenesis · Comet assay · Piscine Micronucleus test

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Introduction

Various chemical products are used to minimize damage done by insects, fungi and weeds in order to reduce agricultural production losses

(Ecobichon 2000). Agriculture is currently extremely dependent on the use of pesticides, and the abandonment or reduction in their use would cause a fall in agricultural production, an increase in production costs a rise in prices and, in some places, hunger and malnutrition (Knutson 1999). However, use of these pesticides is excessive and has led to important environmental pollution (Townsend et al. 2006). According to the European Community, the established limit for each pesticide is 0.10 μg/L for surface waters (CEE 1980). Among these pesticides is the Fipronil, a broad spectrum insecticide and acaricide. Its degradation produces the byproducts Fipronil sulfide, Fipronil sulfone and desulfinylfipronil. These metabolites are more toxic to aquatic organisms than the parent compound (US EPA 1996). According to Le Faouder et al. (2007), Fipronil may affect non-target insects like honey bees. For this reason, France has prohibited the commercialisation of Fipronil since 1999. Three other countries have prohibited its application since then: Italy, Germany and Slovenia. Non-target environments like the milk of cows fed Fipronil-treated corn may also be affected. The Fipronil of the food was transferred to the milk under its sulfone form (Le Faouder et al. 2007).

The impact of toxic materials on the integrity and functioning of DNA has been investigated in various ways under different conditions (McCarthy and Shugart 1990). The use of biomarkers as a measure of biological responses in affected organisms is a very important factor in the simplification and cost reduction of biological monitoring, especially in aquatic environments. These biomarkers consist of DNA adducts, chromosome aberrations, DNA breaks, micronuclei frequency and other nuclear abnormalities (Bombail et al. 2001). Fishes are one of the most appropriate organisms for aquatic environment monitoring (Van der Oost et al. 2003); currently, however, there are few toxicity studies being carried out in South America with endemic freshwater fishes (Akaishi et al. 2004; Rabitto et al. 2005).

One particularly well-established method that is useful in the evaluation of genotoxic effects of a wide scale of compounds, both in fishes (Al-Sabti and Metcalfe 1995) and other species (Grisolia et al. 2004), is the micronucleus induction assay (Schmid 1975). According to Hose et al. (1987), this test revealed great potential because it can be executed rapidly, is inexpensive and an excellent indicator of chemical contamination in fish.

Micronuclei are small masses of cytoplasmic chromatin outside the main nucleus of cells, which can originate from a chromosome break or a malfunction of the mitotic spindle apparatus (Heddle et al. 1991), i.e. there are entire or partial chromosomes that were not incorporated inside the nucleus of the daughter cell during cell division and that appear as a small roundish dark structure, identical in appearance to the cell nucleus (Bombail et al. 2001).

Although there was a basal level of measurable spontaneous micronuclei formation in most of the fish species (Al-Sabti and Metcalfe 1995), exposure to clastogens on a large scale, both in the laboratory and in the field (Bombail et al. 2001; Grisolia and Starling 2001; Rodriguez-Cea et al. 2003) has shown to elevate the frequency of micronuclei.

The Comet assay, in turn, was primarily applied to ecotoxicology about 15 years ago and became one of the most popular tests for the detection of strand breaks in aquatic animals, whether in in vitro, in vivo or in situ exposures (Ohe et al. 2004).

The advantages of the Comet assay include: (a) genotoxic damage is detected at an individual cell level; (b) most of the eukaryotic cell types are appropriate for the Comet assay; (c) only a small number of cells is required; (d) it is generally easier to carry out and more sensitive than other methods that evaluate strand breaks; (e) DNA strand breaks form quickly after genotoxic exposure, allowing an early evaluation of the response in the biota (Frenzilli et al. 2008).

Histopathological damage together with genetic alterations like nuclear aberrations is also an effect that has been related in studies of aquatic organisms in impacted areas (Akaishi et al. 2004; Mouchet et al. 2006; Katsumiti et al. 2008). Effects in the structures of the cells and tissues constitute an important parameter to be considered in the evaluation of the toxic potential of contaminants on living organisms. This biomarker has been used in toxicology works as they allow the evaluation of possible xenobiotic effects in target organs and tissues (Fent 1996).

The objective of the present work was to evaluate the effects of the pesticide Fipronil on the neotropical fish *Rhamdia quelen* (sub-chronic exposure of 60 days in concentrations of 0.05, 0.10 and 0.23 μg/L) through the Piscine Micronuclei test and nuclear morphological alterations, the Comet assay with gills and histopathological analysis with gills. The reason for choosing the tested doses was based on: (1) the upper concentration permitted by European Community legislation which is 0.10 μg/L, for all pesticides individually in water for human consumption (CEE 1980), (2) we used a concentration half that set by the European community (0.05 μg/L); and (3) 0,23 μg/L was the median concentration of Fipronil found at sampling sites in streams draining basins with intensive rice cultivation, so an realistic environmental concentration (Mize et al. 2008).

Material and methods

Experimental design

R. quelen, a neotropical fish popularly known as *Jundiá*, was chosen for the bioassays. Each group had 15 animals (Fipronil exposures of 0.05, 0.10 and 0.23 μg/L). A control group was kept unexposed. Each group was housed in a separate aquarium and acclimatized in aired tanks at constant water temperature (22°C) under a 12-h light/dark photoperiod. The animals had a mean weight of 37.4 g (standard deviation of ±7 g) and 17.3 cm in length (standard deviation of ±1 cm). The Fipronil was used in the commercial formula, specifically under the label of Termidor 25 EC - BASF S/A ®.

After 60 days of exposure the fishes were anaesthetized using 20% benzocaine to avoid suffering.

Piscine Micronuclei test

Thus, the procedures of Heddle (1973) and Schmid (1975), with modifications of Ferraro et al. (2004), were used for the Piscine Micronuclei Test (MNT). The erythrocytes of each fish were examined under ×1,000 magnification and scored for the presence of both typical micronuclei and nuclear alterations manifested as changes in the normal elliptic shape of the nuclei (Ayllon and Garcia-Vazquez 2000). Both features were considered nuclear abnormalities and scored together. In addition, 1,000, 2,000, 3,000 and 4,000 erythrocyte nuclei were analyzed from each control fish in order to verify if higher counts could cause results different from those found with 1,000 nuclei.

Comet assay

The Comet assay with gills was performed according to Speit and Hartmann (1999), with modifications of Ferraro et al. (2004) and Cestari et al. (2004). The gill cells used for the Comet assay were homogenized (Potter-type homogenizer at 1,500 rpm). A 15-μL sample was taken from each Eppendorf tube and was mixed with 120 μL of low-melting-point agarose (0.5%). The suspension was spread on slides previously coated with a normal agarose layer. The rest of the protocol followed Ferraro et al. (2004). Comets were scored using a Leica epifluorescence microscope. One hundred nucleoids from each fish were analyzed (Kobayashi et al. 1995) using the visual classification based on the migration of DNA fragments from class 0 (no visible damage), class 1 (little damage), class 2 (medium damage), class 3 (extensive damage) and class 4 (maximally damaged) nuclei. The score was calculated by multiplying the number of nuclei in a class by the class number.

Micronucleus frequency and other nuclear morphological abnormalities, as well as comet assays comparing negative and positive control groups and contaminated ones, were evaluated through Kruskal–Wallis tests. Results with $p < 0.05$ were considered statistically significant.

Histopathology

Gill samples were preserved in Alfac fixative solution for 16 h (85 mL of 80% ethanol; 10 mL of 40% formalin; 5 mL of glacial acetic acid per 100 mL of solution), dehydrated in a graded series of ethanol baths, and embedded in Paraplast-Plus resin (Sigma). Sections (5 mm thick) were

stained with haematoxylin/eosin and observed under a Leica photomicroscope. Morphological lesions were graded according to the injury index described by Bernet et al. (1999), wherein observations of the injury in the gills were classified under three severity factors (minimal, moderate and marked pathological importance). A qualitative analysis was carried out based on this severity factor.

Results

There were no micronuclei in the Piscine Micronucleus assay, only nuclear morphological alterations. Using this test, the counting of only 1,000 cells is sufficient to obtain a reliable result (Table 1). Therefore, the same result may be obtained in the counting of one, two, three or four thousand cells (Fig. 1), i.e. the group contaminated with 0.05 μg/L of Fipronil did not differ from the control, but the two higher concentrations of Fipronil caused a significant elevation in the damage to the DNA when compared to the control group and the group exposed to 0.05 μg/L of Fipronil. The group with thee highest concentration of Fipronil showed the highest rate of damage.

In regard to the comet assay with gill cells, there was no difference in the treatments in relation to rate of damage to the DNA, i.e. none of the contaminated groups differed from the negative control group (Fig. 2).

Among the histopathological alterations found in the gills, aneurisms and lamellar fusion were conspicuous in all of the groups, including the control (Fig. 3). They are stage I (w) and stage II (a few) changes, both of which are less severe classes, despite the possibility of moderately compromising gill function. No difference was verified between the control group and the different contaminated treatments (referring to the histological analysis of the gills), which corroborates the Comet assay result of this same tissue.

Table 1 Comparison of the counting of 1,000, 2,000, 3,000 and 4,000 cells in each treatment group

Treatments	H
Control group	$H_{(3,52)} = 3,97$ n.s.
0,05 μg/L Fipronil	$H_{(3,60)} = 0,99$ n.s.
0,10 μg/L Fipronil	$H_{(3,60)} = 0,97$ n.s.
0,23 μg/L Fipronil	$H_{(3,60)} = 0,06$ n.s.

H Kruskal–Wallis test result, *n.s.* not significant

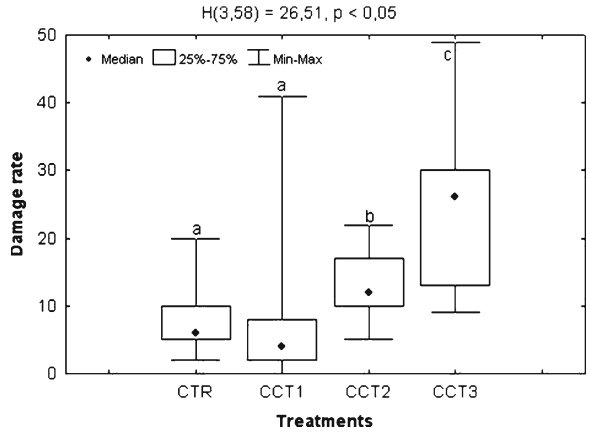

Fig. 1 Comparison between exposed treatments—Micronucleus test. *H* Kruskal–Wallis test result; *CTR* control group; *CCT1* group contaminated with 0.05 μg/L of Fipronil; *CCT2* group contaminated with 0.10 μg/L; *CCT3* group contaminated with 0.23 μg/L

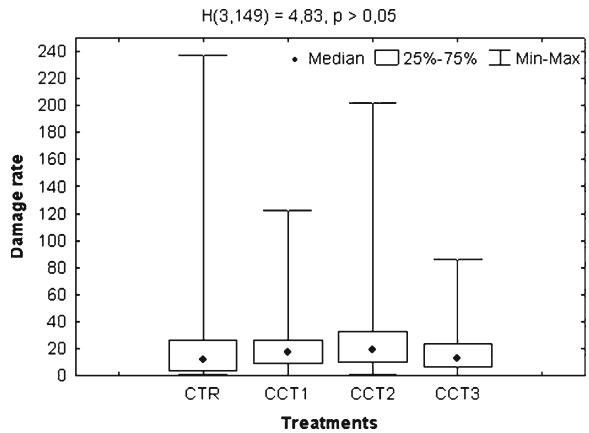

Fig. 2 Comet assay result. *H* Kruskal–Wallis test result; *CTR* control group; *CCT1* group contaminated with 0.05 μg/L of Fipronil; *CCT2* group contaminated with 0.10 μg/L; *CCT3* group contaminated with 0.23 μg/L

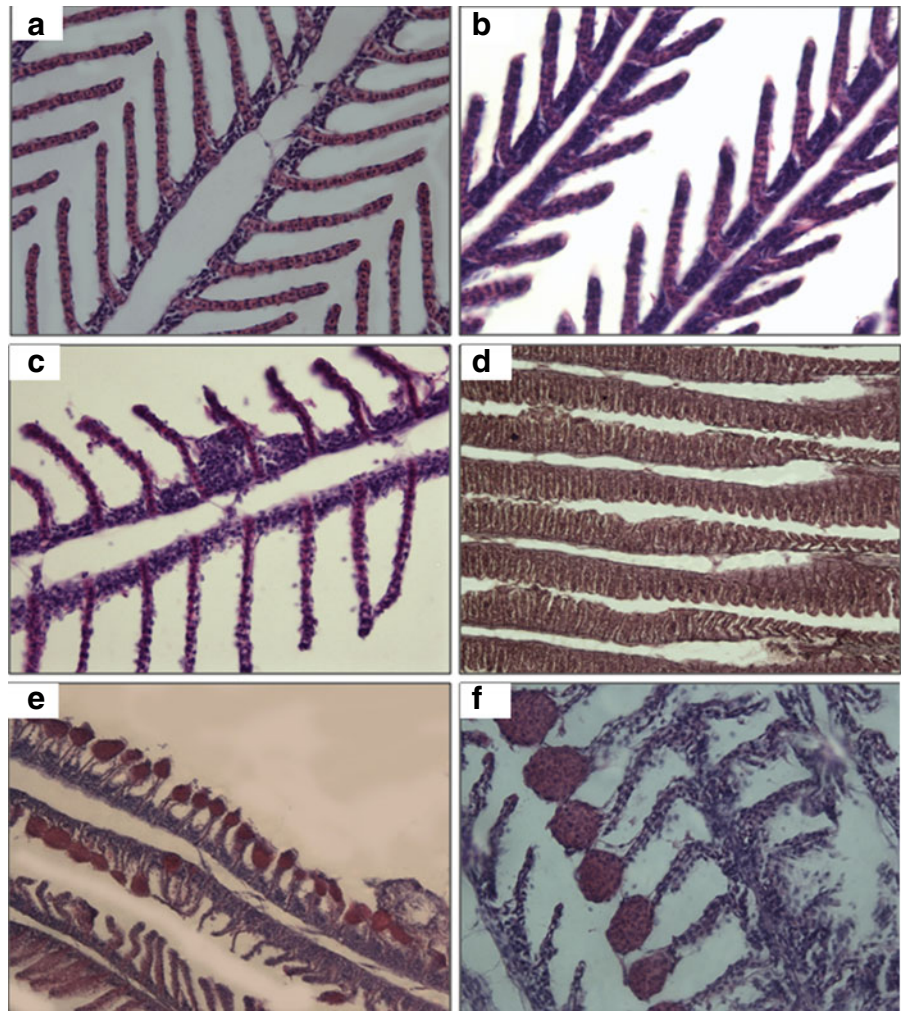

Fig. 3 Photomicrograph of gill tissue: **a**, **b** lamellae normal, **c** partial hyperplasia of lamellas, **d** total fusion of lamellae; **e**, **f** aneurysms in smaller and larger zoom

Discussion and conclusions

In the present work the Piscine Micronucleus test revealed that the contaminations at concentrations of 0.10 and 0.23 μg/L of Fipronil caused a higher rate of damage to the DNA compared to the negative control group and the lower concentration (0.05 μg/L). However, there was no difference between the control group and the lowest concentration of Fipronil. This test suggests that higher concentrations of Fipronil may induce damage to the DNA of *R. quelen*.

In respect of the Comet assay with gills, all of the contaminated groups had damage similar to the control group, which was corroborated by histopathological analyses of this same tissue.

The results suggest that only the highest concentrations of Fipronil (tested) cause damage in erythrocytes, but none of the concentrations was sufficient to alter the DNA or the morphology of the gill cells.

A few alterations in the gills of *R. quelen*, mainly aneurisms, hyperplasia and lamellar fusion, were found in the histopathological analyses. The alterations were the same in all of the treatments, including the control group. In addition, most of the injuries are considered minor

with regression possible if the source of stress is eliminated.

These results may thus be related to the low concentrations of contaminant used and to the sub-chronic time of the assay duration because the presence of gill injuries in fish may be interpreted as a result of *acute* effects of xenobionts (Zodrow et al. 2004).

Some authors have demonstrated that in prolonged exposures the frequency of micronuclei in the erythrocytes of fishes shows a decrease after 15 and 21 days of exposure (Campana et al. 1999; De Lemos et al. 2001). These differences seem to be associated with the kinetics of cell removal (Çavas and Ergene-Gozukara 2003) or with the development of adaptive mechanisms of tolerance to stress caused by toxic chemicals that cause an increase in the replacement rate of dead or damaged cells to maintain normal physiological conditions (Mersch et al. 1996).

Al-Sabti and Metcalfe (1995) demonstrate that the maximum induction of micronuclei normally occurs one to five days after exposure.

The European Community established a limit of 0.10 μg/L for each pesticide in surface waters for human consumption (CEE 1980). This concentration limit, in addition to two others (0.05 and 0.23 μg/L of Fipronil) was used in this work. This may justify the fact that, in general, the tests used here did not reveal an increased damage rate for contaminated groups relative to the control group, i.e. the limit established by the European community is a safe limit.

Therefore, the 0.10 μg/L dose of Fipronil accepted by the European Community, when tested with the biomarkers used in this work, did not reveal an altered damage rate. The 0.05 μg/L dose is below this standard dose, and perhaps 0.23 μg/L is not a high enough concentration to significantly elevate the rate of damage to the DNA in the comet assay. In order to obtain a more demonstrative result with this fish additional studies are needed, with concentrations varying between decimal places (e.g. 0.1, 1, 10 μg/L) and not only in the same decimal place.

A lethal dose of Fipronil [50% (LC_{50})] is, for example, 0.246 mg/L for the rainbow trout, 0.083 mg/L for the bluegill and 0.130 for the sheepshead minnow (*Cyprinodon variegates variegates*) (US EPA 1996), which supports this hypothesis. These doses are well above those tested here, with the lowest (0.083 mg/L) being approximately 360 times the highest concentration used in the present work.

Wirth et al. (2004) analyzed the impacts of Fipronil on a mesocosm replica and found that there was no effect associated with Fipronil in shellfish (*Mercenaria mercenaria*), oysters (*Crassostrea virginica*) or fish (*Cyprinidon variegates*). In this work, Fipronil affected only shrimp (*Palaemonetes pugio*). The highest concentration used by Wirth et al. (2004) was 5 μg/L, i.e. 21 times higher than the highest concentration used in the present work. The studies cited above corroborate the hypothesis that the concentrations studied here are insufficient for a perceptible response.

Fipronil is a "new generation" insecticide/acaricide and is highly toxic to many aquatic species (US EPA 1996) and can bioaccumulate in some of them. In one bioaccumulation work, rainbow trout (*Oncorhynchus mykiss*) were fed food contaminated with Fipronil (10 μg/g of food). The exposure did not affect the health of the fish. They did not die, and their behavior and coloration were similar to the control fish. As regards bioaccumulation in this work, Fipronil was quickly eliminated and was no longer detected after the 34th clearance day (Konwick et al. 2004).

On the other hand, a more recent work (Miranda et al. 2008) observed the presence of Fipronil in the muscle and liver of the fish *Hoplias malabaricus* from Lago de Ponta Grossa (Paraná State, Brazil). This pesticide was found in 30% of the individuals (both tissues), with maximum concentrations of 42.3 and 58.6 ng g^{-1} of dry weight, respectively. In addition, there was a strong Pearson correlation between the concentration of lipids and Fipronil.

Fipronil is a class of insecticides known as phenylpyrazole that are recognized as a disruptor of chloride channels connected to γ-aminobutyric acid (GABA) in the nerve cells, leading to the hyper-excitement and eventual mortality of the insects (Gant et al. 1998). It is much more toxic in invertebrates than vertebrates due to its different GABA receptor connection affinities (Hainzl et al. 1998).

In this sense laboratory tests of Fipronil and its products of degradation have revealed acute lethal toxicity in "very low" concentrations [(LC_{50}) from <0.5 μg/L for selected aquatic macroinvertebrates] (Mize et al. 2008). This means that the concentration used in the present work is less than LC_{50} for aquatic macroinvertebrates, which are more sensitive than fish. Fipronil is more toxic in invertebrates because of its specificity regarding their GABA channels (Hainzl et al. 1998).

Chandler et al. (2004) reported that the fertility, reproduction and development of an estuary copepod were affected by a 0.22 μg/L concentration of Fipronil.

If the contaminant is also a pollutant depends on its level in the environment, the organism considered, and if the organism is damaged. Thus, a compound may be a pollutant for one organism, but not for another (Walker et al. 2006).

For example, ppt levels (ng/L) of Fipronil affect "Mysid" shrimp and ppb levels (μg/L) affect freshwater Daphnia and Bluegill sunfish (Gunasekara and Troung 2007). In the present work ppt (ng/L) levels were used, i.e. the tested concentrations correspond to 50, 100 and 230 ng/L, respectively, which supports the idea that low concentrations of this pollutant reveal responses only from invertebrates (more sensitive than fish due to the specificity of Fipronil).

R. quelen is a rustic species that is adapted to various environments and is resistant to variations in temperature and salinity (Barcellos et al. 2001). In addition, previous works have documented physiological and biochemical responses in studies of exposure to pesticides (e.g. herbicides)(Miron et al. 2005; Crestani et al. 2006; Glusczak et al. 2007). These studies show that various parameters are altered (e.g. glycogen, lactate, levels of glucose in the tissues and acetylcholinesterase activity in the brain.

As regards the histopathological analyses, Thophon et al. (2003) also state that the *acute exposure* to some environmental pollutant affects gill function and can lead to death. The effects of environmental contaminants on fish gills can be particularly serious because the gills are the main organ for respiration and osmotic and ionic regulation.

Hyperplasia, found in this study in the gills of *R. quelen*, is characterized by the increase in the proliferation of cells, which can lead to the fusion of the lamellas and, more rarely, the filaments (Heath 1987). Lamellar fusion is a natural defense mechanism to protect the epithelium of the lamella from direct contact with toxic agents (Heath 1987; Ojha 1999).

Fipronil, sold in Brazil as Regent®, is used to control (in broad spectrum) plagues in the field and can also affect non-target insects. For example, it is considered one of the most relentless enemies of honeybees (Le Faouder et al. 2007), which are important in pollination. For this reason, France has prohibited the commercialization of Fipronil since 1999. Three other countries have prohibited the application of Fipronil: Italy, Germany and Slovenia.

Le Faouder et al. (2007) suggest that non-target environments, like milk produced by cows fed daily with corn silage made from kernels treated with Fipronil, can be affected. This work shows the transfer of Fipronil from food to milk under its sulfonic form. What is more, traces of Fipronil residues in the corn, soybeans, wheat and straw show diffuse contamination of this pesticide in the environment.

In a pesticide monitoring work in two springs in the state of Rio Grande do Sul (Brazil), Fipronil represents 16% of the analyses of pesticide residues, which were tested by gas chromatography with an electron capture detector (GC-ECD). In this same work, the insecticide Fipronil was detected only on the first date off the seven collections; however, at this time, residues were found at all of the collection points. Thee maximum concentration found for this insecticide was about 380 times above the detection limit (1.14 μg/Kg), which corresponds to 0.003 μg/Kg (Grützmacher et al. 2008). The concentration cited above (1.14 μg/Kg) corresponds to almost five times the highest concentration tested in this dissertation (0.23 μg/L) and more than 11 times the limit permitted by the European Community (0.10 μg/L).

Fipronil (in concentrations of 70, 140 and 280 mg/kg) can affect thyroid function in rats due to the decrease in the concentrations of the plasma of total thyroxine (T4), probably through

the increase in the unblocking of T4 and the increase in the plasmatic concentrations of thyrotropin (TSH), in addition to decreasing the concentrations of total T3 and free TH in the plasma (Leghait et al. 2009). Furthermore, Fipronil altered the normal function of the endocrine system and caused adverse reproductive effects in female "Winstar" rats (Ohi et al. 2004).

The metabolites of the degradation of Fipronil (Fipronil sulfide, Fipronil sulfone and desulfinylfipronil) are more toxic to aquatic organisms than the parent compound. For example, desulfinylfipronil, a photodegraded form of Fipronil, is extremely stable and is more toxic than the compound from which it is derived (US EPA 1998).

In a study about the degradation of Fipronil in sediment, the half-life of Fipronil was shorter in higher concentrations, which was contrary to what occurs to most pesticides. While initially it seems advantageous that Fipronil has a shorter half-life in sediments, this degradation is insignificant given that Fipronil degrades into fipronil-sulfide and fipronil-sulfone, which are similar to Fipronil in toxic potential and exhibit higher environmental stability under tested conditions (Brennan et al. 2009).

Tan et al. (2008) examined the microbiological degradation of a racemic mixture of chiral forms of Fipronil, enantiopures Fipronil-R and Fipronil-S, in an aerobic environment and flooded meadow soils. Their results suggest that different types of degradation of enantiomers of Fipronil, as well as the formation of different toxic metabolites under aerobic and flooded conditions, should undergo precise evaluations as regards environmental and ecological risks for chiral pesticides.

Recent studies demonstrate the potential of pesticides to inhibit or induce enzymes that metabolize xenobionts in humans. Exposure of human hepatocytes to doses of Fipronil of 0.1 to 25 μM resulted in an increase dependent on the dose in the expression RNAm CYP1A1 as measured by the branched DNA method, in addition to inducing the formation of CYP isoforms and cytotoxicity in human hepatocytes (Das et al. 2006).

The Environmental Protection Agency (USA) says that Fipronil is highly toxic to fishes, aquatic invertebrates and flying birds but relatively less toxic to mammals, common moorhens and other bird species (US EPA 1996).

Works that have the purpose of verifying the effects of pollutants, mainly pesticides, on environmental health assume great importance, considering that the indiscriminate release of toxic substances into the environment is one of the biggest problems of the modern world. The highest concentrations of Fipronil used in the present work can affect the DNA of the erythrocytes of *R. quelen*, but none of these concentrations affected either the morphology or the DNA of its gill cells. Further studies are, therefore, suggested using higher concentrations, other exposure times and other tissues.

Although this work does point positive results in certain experimental conditions, the genotoxicity tests conducted in bioassays with fish *R. quelen* show DNA damage in erythrocytes of this species even at low concentrations of Fipronil. This, itself shows the importance of studies like this in addition to monographs institutional and governmental laws governing the use of pesticides and their residues in the environment.

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