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Sublethal and lethal effects on *Rhinella Arenarum* (Anura, Bufonidae) tadpoles exerted by the pirimicarb-containing technical formulation insecticide Aficida®

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ABSTRACT

Acute toxicity, genotoxicity, and cytotoxicity of the pirimicarb-containing commercial-formulation carbamate insecticide Aficida® (50% pirimicarb) were evaluated on *Rhinella arenarum* (Anura, Bufonidae) tadpoles exposed under laboratory conditions. Lethal and sublethal effects were employed as bioassays for acute toxicity, whereas micronuclei (MNi) induction and alterations in the ratio erythrocytes:erythroblasts were employed as end-points for genotoxicity and cytotoxicity, respectively. Cr(VI) (23 mg L^{-1}) and cyclophosphamide (40 mg L^{-1}) were employed as positive controls for toxicity and geno-cytotoxicity assays, respectively. In Gosner stage 25 (STD25), the results revealed mean values of 402.0 and 223.6 mg Aficida® L^{-1} for LC-$50_{24\ h}$ and LC-$50_{96\ h}$, respectively. When STD37-39 tadpoles were exposed, the LC-$50_{24\ h}$ and LC-$50_{96\ h}$ reached values of 239.4 and 181.7 mg Aficida® L^{-1}, respectively. Sublethal effects revealed a mean EC-$50_{96\ h}$ of 133.85 and 104.2 mg Aficida® in those STD25 and STD37-39 treated tadpoles, respectively. The results demonstrated that in 48-h-exposed tadpoles, a MNi increase was found only in those 80.0 mg L^{-1} Aficida®-treated individuals. When tadpoles were exposed to Aficida® for 96 h, only the 160 mg L^{-1}-treated individuals showed a significant increase in MNi frequency. Concentrations ranging from 80.0 to 250.0 mg Aficida® L^{-1} resulted in cellular cytotoxicity, revealed by a decreased proportion of circulating erythrocytes and an enhancement of erythroblasts. Accordingly, this species could provide a suitable and useful experimental model for biomonitoring aquatic ecosystems.

1. Introduction

Organisms living in agroecosystems are highly exposed to agrochemicals, which represent a significant concern at both ecological and public health levels. Furthermore, agrochemicals are hazardous on the planet, because anthropic activities are continuously introducing extensive amounts of these compounds into the environment regardless of their persistence, bioaccumulation and toxicity (www.epa.gov/pesticides/).

In epidemiological as well as in experimental genotoxic and cytotoxic studies, there is an increasing interest in biomonitoring markers to provide both a measurement and an estimation of biological exposure to genotoxic pollutants. Their effects can be monitored using a broad range of both *in vivo* and *in vitro* assays. Several end-points for testing both genotoxicity and cytotoxicity have been employed on aquatic organisms, including fishes, and amphibians. Among them, analysis of micronuclei (MNi) frequency (Lajmanovich et al., 2005; Mouchet et al., 2006, 2007; Cavaş and Könen, 2007; Peltzer et al., 2008; Ali et al., 2009) and the induction of DNA single-strand breaks by the single cell gel electrophoresis assay (Lajmanovich et al., 2005; Mouchet et al., 2006, 2007; Cavaş and Könen, 2007; Peltzer et al., 2008; Ali et al., 2009) are the most frequently employed and recommended end-points for detecting cytogenetic and DNA damage in nucleated erythrocytes, respectively. Furthermore, MNi analysis has been considered a reliable bioassay for clastogenic and/or aneugenic screening in amphibians under both laboratory and field conditions, as well as for the *in situ* estimation of water pollution (Van Hummelen et al., 1989; Lajmanovich et al., 2005; Huang et al., 2007; Peltzer et al., 2008; Shenoy et al., 2009).

In agriculture, pesticides are generally not used as a single active ingredient but as part of a complex commercial formulation. In addition to the active component, the formulated products contain different solvents and adjuvants, some of which have been reported to induce damage in mammalian cells, among others (González et al., 2007, 2009; Soloneski et al., 2008). Hence, risk assessment must also consider additional toxic effects caused by the excipient(s). Thus, both the workers and nontarget organisms

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are exposed to the simultaneous action of the active ingredient and a variety of other chemical/s contained in the formulated product. Despite the beneficial effects associated with the agricultural and household use of agrochemicals, many of these products may contain potentially hazardous compounds, and due to their extensive employment, the contamination of food, water, and air has become a severe health problem for humans and ecosystems (WHO, 1988). So far, the available information indicates that 23 formulated products containing pirimicarb as an active ingredient have been registered worldwide (www.environmentalchemistry.com). Some of these pirimicarb-containing formulations have been reported to induce toxic effects in the microbial community of freshwater sediments (Widenfalk et al., 2004, 2008), in the crustaceans *Ceriodaphnia quadrangula* (Mansour and Hassan, 1993) and *Daphnia magna* (Andersen et al., 2006; Syberg et al., 2008), in insects such as *Culex quinquefasciatus* (Magnin et al., 1988), in fishes such as *Poecilia reticulata* and *Cyprinus carpio* (OPP-EEDB, 2000), and in anuran tadpoles of *Pelophylax perezi* (Honrubia et al., 1993; Alvarez et al., 1995).

Among carbamate pesticides, pirimicarb is a selective insecticide used mostly for aphid control of crops. Its mode of action is inhibiting acetylcholinterase activity (Sultatos, 2008). Data are available in the literature about genotoxic studies are scarce (IARC, 1976; WHO-FAO, 2004). Pirimicarb has been generally recognized as nongenotoxic in bacteria, yeast, fungi, and in mammalian cells (EPA, 1974). Whereas it has been reported to be nonmutagenic in *Salmonella typhimurium* after S9 metabolic activation (EPA, 1974), a positive response was observed in mouse lymphoma L5178Y cells (WHO-FAO, 2004). Positive results have been reported in the w/w$^+$ eye mosaic system with the *Drosophila melanogaster* Oregon-K strain (Aguirrezabalaga et al., 1994). Pirimicarb did not induce chromosomal alterations in rat bone marrow cells after oral administration (Anderson et al., 1980) and in *in vitro* human lymphocytes with or without S9 metabolic activation (EPA, 1974). However, Pilinskaia (1982) observed an increased frequency of chromosomal aberrations in peripheral lymphocytes from occupational exposed workers. Finally, DNA single-strand breaks were detected by the comet assay in *in vitro* human lymphocytes (Ündeger and Basaran, 2005).

The aim of the present study is to characterize the acute toxicity of the Argentinean pirimicarb-containing commercial-formulation carbamate insecticide Aficida® (50% pirimicarb) on *Rhinella arenarum* (Anura, Bufonidae) tadpoles exposed under laboratory conditions by determining mortality, narcosis, and biomarkers of geno and cytotoxicity as MNi in mature erythrocytes and circulating erythrocyte:erythroblast ratio. This commercial formulation was chosen because it represents one of the most widely carbamate-containing formulations used for cereal production and garden insect control in Argentina and on a worldwide scale.

2. Materials and methods

2.1. Chemicals

Pirimicarb (2-dimethylamino-5,6-dimethylpyrimidin-4-yl dimethylcarbamate; commercial grade, trade name Aficida®, CAS 23103-98-2; 50% pirimicarb, excipients q.s.) was kindly provided by Syngenta Agro S.A. (Buenos Aires, Argentina). Cyclophosphamide (CAS 6055-19-2) was obtained from Sigma Chemical Co. (St. Louis, MO, USA), and $K_2Cr_2O_7$ [Cr(VI)] (CAS 7778-50-9) was obtained from Merck KGaA (Darmstadt, Germany).

2.2. Quality control

Concentration levels of pirimicarb in test solutions were analyzed by high-performance liquid chromatography (Agilent 1100) with diode array at a wavelength of 230 nm, with a 15 cm C_{18} column, 4.6 mm inner diameter, and acetonitrile in 10 mM KH_2PO_4 pH 4 buffer with a 60:40 ratio, at 0.8 ml/min, to test stability of the compound along the time of exposure by comparison of chromatographic peaks corresponding to solutions from the initial time and 24 h after treatment.

2.3. Anuran tadpoles

R. arenarum tadpoles were selected as target organisms. This toad species from the Bufonidae family has an extensive neotropical distribution, including Argentina, Bolivia, Brazil, Uruguay, and Paraguay, from sea level up to 2600 m asl. It inhabits small ponds or bogs with stagnant water in dry temperate habitats, mostly in open areas. In Brazil and Argentina, it occurs mainly in coastal regions. Oviposition (up to 40 000 eggs) occurs in these temporary bodies of water wherein tadpoles feed and develop, reaching the prometamorphic stage 25 in about 7–8 d after oviposition (Gosner, 1960). This species is easy to handle and acclimate to laboratory conditions (www.iucnredlist.org). All tadpoles used for this study were obtained from gelatinous strings containing eggs at the late cleavage stages, stage 9 according to Gosner's classification (Gosner, 1960), collected from a temporary pond free from pluvial runoff from agricultural areas, in the vicinity of La Plata city (Buenos Aires Province, Argentina). Eggs were transported to the laboratory and then acclimatized to 16:8 h light/dark cycles in aquaria at 25 °C with dechlorinated tap water (pH 7.6–8.3, hardness 250 mg/L $CaCO_3$) with artificial aeration and boiled lettuce as a food source until the beginning of the experimental procedures.

2.4. Determination of LC-50 and sublethal effects

In order to compare the existence of a differential sensitivity between different stages of development, two sets of experiments were carried out for toxicity assessment following recommendations proposed by the USEPA standardized methods (USEPA, 1989) with minor modifications reported for native species elsewhere (Natale et al., 2006). Fertilized eggs were divided into two equal-size groups. One of the groups (n = 600) was used for the first acute toxicity test when they reached Gosner stage 25 (STD25) (216 h of age). The second group was kept in the laboratory until individuals reached a body size that allowed extraction of blood for MNi (Gosner stage 37–39, 1344 h of age) and then used for the second set of experiments. The tadpoles were fed twice a week with blended lettuce *ad libitum*. They were kept in dechlorinated tap water (pH 7.6–8.3, hardness 250 mg $CaCO_3$ L^{-1}) with continuous aeration, at a temperature of 25 °C ± 1 °C, and a 16:8 h light/dark photoperiod cycle. In the first set of experiments, 10 tadpoles at Gosner development stage 25 (Gosner, 1960) maintained in a 1 L glass container were used for each experimental point, and exposed to 12 different concentrations of Aficida® (0.02, 0.05, 0.1, 0.5, 1, 10, 20, 40, 60, 80, 100 y 500 mg L^{-1}) during 96 h. Concentrations of Aficida® assessed throughout the study represent the nominal concentration of the formulated product. Two negative (dechlorinated tap water, pH 7.6–8.3, hardness 250 mg CaCO3 L^{-1}) and positive controls (23 mg Cr(VI) L^{-1}-treated tadpoles) were conducted and run simultaneously with Aficida®-exposed tadpoles. All test solutions were prepared immediately before use, and replaced every 24 h. Tadpoles were not fed throughout the experiment. Lethal (mortality) and sublethal effects (narcosis) were determined in four independent experiments by analyzing lethal and sublethal effects every 24 h, and employed as toxicity end-points. Tadpoles were visually examined and narcosis was defined as lack of sudden swimming response to gentle touching in regard to control organisms. The same experimental design was employed for the second set of experiments with tadpoles between

Gosner development stages 37 and 39 (Gosner, 1960) in treatments with 80, 160, 250, and 300 mg Aficida® L^{-1}.

2.5. Genotoxicity assay

2.5.1. Micronuclei assay

Experiments were conducted using six tadpoles following the same experimental design employed for the second set of experiments described in Section 2.4. Positive controls (40 mg cyclophosphamide L^{-1}) were conducted and run simultaneously with Aficida®-exposed tadpoles. The frequency of MNi was determined at 48 h and 96 h after initial treatment. Three independent experiments were performed and run simultaneously for each experimental point.

2.5.2. Micronuclei analysis

At the end of each experiment, tadpoles were anesthetized (30% ethanol) and two drops of peripheral blood samples were obtained by heart puncture. Two peripheral blood smears were performed for each tadpole onto clean slides, air dried, fixed with 100% (v/v) cold methanol (4 °C), and then stained with 5% Giemsa solution. Slides were coded and blind-scored by one researcher at 1000× magnification. MNi frequency was determined by analyzing 1000 erythrocytes from each tadpole as suggested elsewhere (Lajmanovich et al., 2005; Cabagna et al., 2006) and is expressed as the total number of MNi/1000 cells. MNi frequency was determined following previously reported examination criteria (Fenech, 2000; Meintières et al., 2001; Ferreira et al., 2004; Cabagna et al., 2006). Briefly, the criteria employed for MNi identification were as follows: diameter smaller than 1/3 of the main nuclei diameter; non-refractability; staining intensity similar to or lighter than that of the main nuclei; no connection or link with the main nuclei; no overlapping with the main nuclei; an MNi boundary distinguishable from the main nuclei boundary.

2.6. Cytotoxicity assay

Erythrocyte and erythroblast frequencies were blind determined by one researcher at 1000× magnification by analyzing a total of 1000 erythrocyte/erythroblast cells from each tadpole in those slides employed for MNi analysis, and are expressed as the total number of erythrocytes and erythroblasts in 1000 cells.

2.7. Statistical analyses

Data of laboratory toxicity tests were analyzed by probit analysis (Finney, 1971), and LC-50 values and the 95% confidence interval were calculated from estimated dose–response curves. The same toxicological parameters were corroborated using the Probit Analysis Program, version 1.5 (www.epa.gov). Student's *t*-test for independent samples was applied to compare data from the three independent experiments as mean values of MNi/1000 erythrocytes and erythrocytes/1000 cells between treated and control groups (Zar, 1999). The relationships between mortality, narcosis, frequency of erythrocytes/erythroblasts, and MNi data were evaluated by simple linear regression and correlation analyses. All end-point data were examined for meeting the assumption of parametric statistics by using the Levene's test for determining variance homogeneity, and the Shapiro–Wilk W test. Significance of the regression and correlation coefficients (elevations and slopes) were tested following Zar (1999). Goodness of fit test was used for data fitting to Probit model. The level of significance chosen was 0.05, unless indicated otherwise.

3. Results

3.1. Acute toxicity assays

Results of chemical analyses showed no changes in the concentration of the toxicant in treatments during the 24 h interval renewals of the testing solutions.

Probit analysis of the mortality data for the first experiment in which Gosner stage 25 (216 h of age) tadpoles were used allowed determination of the LC-50 of Aficida® after 24 h and 96 h of exposure. Also the intercept value (a) and slope value (b) are given for each concentration response plot. When the 95% confidence limits could be estimated, corresponding values are given between brackets after each LC-50. The $LC\text{-}50_{24\ h}$ was 402.0 mg Aficida® L^{-1} ($a = -7.40$, $b = 4.76$). A constant value of 223.6 mg Aficida® L^{-1} ($a = -14.36$, $b = 8.24$) was calculated for the LC-50 at 48, 72, and 96 h. Probit analysis of sublethal effects (narcosis end-point) allowed calculation of a mean $EC\text{-}50_{24-48\ h}$ 124.9 mg Aficida® L^{-1} [94.3–237.1] ($a = 0.40$, $b = 2.19$); $EC\text{-}50_{72\ h}$ 119.2 mg Aficida® L^{-1} [91.3–213.3] ($a = 0.38$, $b = 2.22$); $EC\text{-}50_{96\ h}$ of 133.8 mg Aficida® L^{-1} [87.7–572.5] ($a = 2.20$, $b = 1.32$).

Probit analysis of mortality data from the second experiment in which Gosner stage 37–39 (1344 h of age) tadpoles were employed allowed determination of the following toxicity values in mg Aficida® L^{-1}: $LC\text{-}50_{24\ h} = 239.4$ ($a = -23.43$, $b = 11.95$); $LC\text{-}50_{48\ h} = 205.7$ ($a = -4.97$, $b = 4.31$), $LC\text{-}50_{72\ h} = 149.7$ [103.2–193.4] ($a = -3.07$, $b = 3.71$), and $LC\text{-}50_{96\ h} = 181.7$ [142.6–209.8] ($a = -14.56$, $b = 8.66$). The analysis of sublethal effects yielded end-point narcosis in mg Aficida® L^{-1} of $EC\text{-}50_{24\ h} = 224.5$ [196.4–274.0] ($a = -6.29$, $b = 4.80$), $EC\text{-}50_{48\ h} = 199.0$ ($a = -1.24$, $b = 2.72$), $EC\text{-}50_{72\ h} = 152.8$ ($a = -4.04$, $b = 4.37$), and $EC\text{-}50_{96\ h} = 104.2$ ($a = -7.90$, $b = 6.39$).

3.2. Genotoxicity and cytotoxicity assays

Fig. 1 summarizes the results of the analysis of Aficida®-induced MNi in circulating erythrocytes of *R. arenarum* tadpoles. The frequency of MNi in cyclophosphamide-exposed (positive control) tadpoles was significantly increased compared to negative control values when the analysis was performed at both 48 h and 96 h of treatment ($P < 0.05$). In those tadpoles exposed for 48 h, a significant increase in the frequency of MNi was found only in those 80 mg L^{-1} Aficida®-treated individuals with respect to negative control values ($P < 0.05$). On the other hand, no increased MNi frequency was observed in those tadpoles exposed to either 160 mg Aficida® L^{-1} or 250 mg Aficida® L^{-1} ($P > 0.05$) (Fig. 1). In addition, Fig. 1 demonstrates that only those tadpoles exposed to Aficida® for 96 h with 160 mg L^{-1} showed a significant increase in the frequency of MNi in regard to negative control values ($P < 0.05$). It should be noted that the frequency of MNi in those 250-mg L^{-1}-exposed tadpoles was not determined because all but one individual were able to be analyzed at this exposure treatment. Furthermore, 300 mg/L induced lethality in all tadpoles after 24 h of treatment (Fig. 1).

The results of the analysis of the proportion of circulating erythrocytes and erythroblasts in the blood of those tadpoles exposed for 48 h and 96 h are shown in Fig. 2. The ratio erythrocytes:erythroblasts in the cyclophosphamide-exposed (positive control) tadpoles did not differ from that of negative control values when the analysis was performed at 48 h of treatment ($P > 0.05$). However, after 96 h of exposure, a significant decrease and a concomitant increase in the frequency of erythrocytes and erythroblasts, respectively, were observed in those positive control tadpoles in regard to negative controls ($P < 0.001$). The results showed that at 48 h of treatment, a significant decrease in the proportion of erythrocytes and a concomitant increase in the frequency erythro-

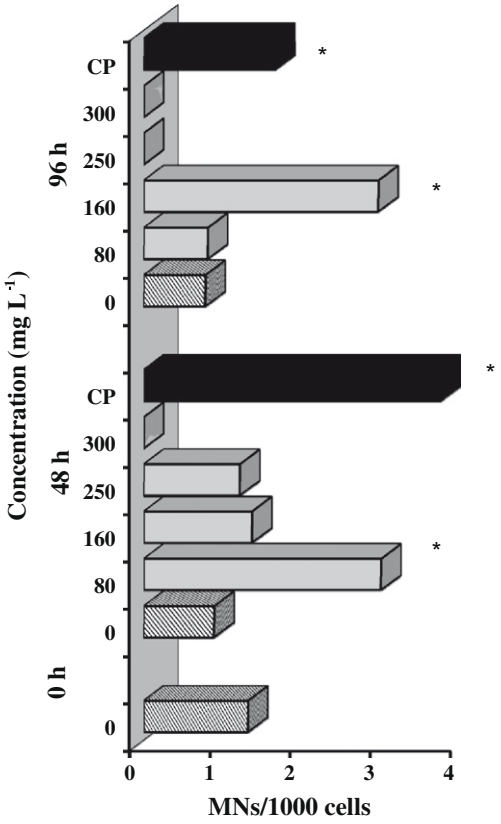

Fig. 1. Frequency of MNi in circulating erythrocytes from negative controls (stripped bars) and *Rhinella arenarum* (Anura, Bufonidae) tadpoles exposed to Aficida® (gray bars). The frequency of MNi was determined at 48 and 96 h after initial treatment. The MNi frequency was determined by analyzing 1000 erythrocytes from each tadpole and is expressed as the total number of MNi/1000 cells. Cyclophosphamide (40 mg L^{-1}) was used as positive control (black bars). $^{*}P < 0.05$.

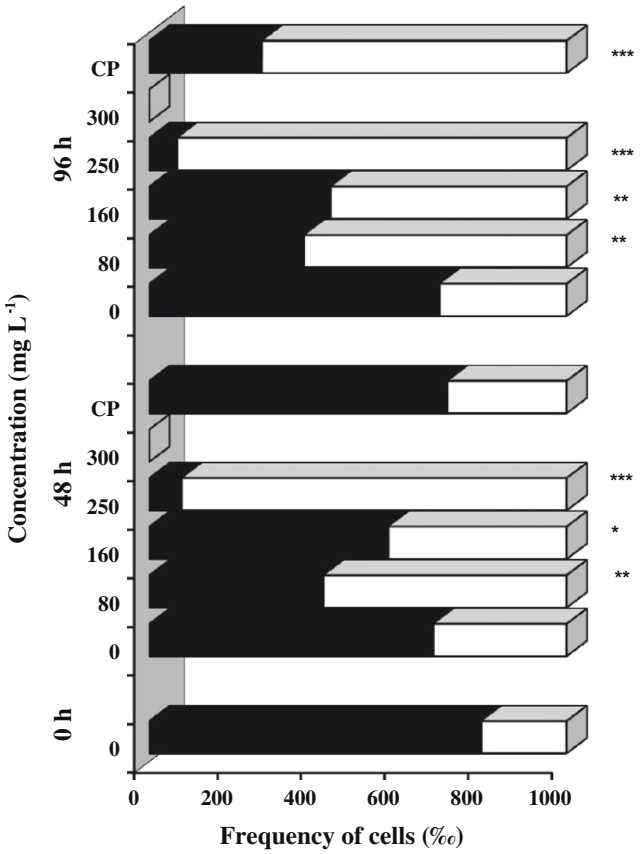

Fig. 2. Frequency of circulating erythrocytes (black bar areas) and erythroblasts (white bar areas) in circulating blood from negative controls and *Rhinella arenarum* (Anura, Bufonidae) tadpoles exposed to Aficida®. The frequency of erythrocytes and erythroblasts was determined at 48 and 96 h after initial treatment. The erythrocyte and erythroblast frequencies were determined by analyzing 1000 cells from each tadpole and are expressed as the total number of erythrocytes/erythroblasts in 1000 cells. Cyclophosphamide (40 mg L^{-1}) was used as positive control. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

blasts were observed within the 80–250 mg Aficida® L^{-1} concentration range in regard to negative controls ($P < 0.01$, $P < 0.05$, and $P < 0.001$ for 80, 160, and 250 mg Aficida® L^{-1}, respectively) (Fig. 2). Similar results were found when the analysis was performed after 96 h of treatment. A significant decrease and a concomitant increase in the frequency of erythrocytes and erythroblasts were found regardless of the concentration of Aficida® employed ($P < 0.01$ for 80 and 160 mg Aficida® L^{-1}, and $P < 0.001$ for 250 mg Aficida® L^{-1}) (Fig. 2).

Overall, a regression test revealed a positive and a negative correlation between the frequency of narcosis and mortality ($r = 0.90$, $P < 0.05$; Supplementary Fig. 1A) and the frequency of circulating erythrocytes ($r = -0.99$, $P < 0.01$; Supplementary Fig. 1B), respectively, in those 48 h Aficida®-treated tadpoles. When the analysis was performed after 96 h from the initial treatment, a negative correlation between mortality and the number of circulating erythrocytes ($r = -0.99$, $P < 0.001$; Supplementary Fig. 1C), and a positive relationship between narcosis and the frequency of induced MNi ($r = 0.99$, $P < 0.01$; Supplementary Fig. 1D), were observed.

4. Discussion

In the present report, the acute lethal toxicity, genotoxicity, and cytotoxicity of Aficida® were evaluated on *R. arenarum* (Anura, Bufonidae) tadpoles exposed under laboratory conditions. Regarding the acute lethal effects of the studied formulation on the species, the chemical could be ranked from moderate to low concern according the scoring used by the Office of Pollution Prevention and Toxics of the EPA (Smrchek et al., 1993; Wagner et al., 1995). Despite this fact, the pesticide presents identifiable sublethal effects at almost 100-fold lower concentrations than those corresponding to the lethal end-point, which are associated with changes in hematological parameters. Acute lethality data of LC-50 values reported here for pirimicarb contribute new information for this type of effect on amphibians. Our results reveal that larvae in prometamorphosis (STD37-39) are more sensitive than in premetamorphosis (STD25). However, opposite results have been previously reported for this species when exposed to the herbicide atrazine. Brodeur et al. (2009) demonstrated that larvae in prometamorphosis (STD37-39) were more resistant than STD25 ones. Previous reports by Honrubia et al. (1993) provided information about effect levels on *P. perezi* exposed under laboratory conditions with the pirimicarb-containing formulation ZZ-Aphox® at 0.02% and 0.14% over 9 weeks of exposure. Histological damage correlated well with the observed mortalities, though no LC-50 values were reported by these authors. Additionally, Alvarez et al. (1995) reported bone malformations for the same species with formulation at concentrations of 0.25 mg L^{-1} of the active ingredient after 14 weeks exposure. Johansson et al. (2006) exposed *Rana temporaria* tadpoles to pirimicarb concentrations of up to 16 mg L^{-1}, and observed over 80% survival after 72 h of exposure. If we compare the LC-50 values reported here for *R. arenarum* exposed to pirimicarb with those from the literature for several fish species from standardized toxicity tests, *R. arenarum* is within

the range between the most and least sensitive fish: *Oncorynchus mykiss*, LC-$50_{96\ h}$ = 29–129 mg pirimicarb L^{-1} (OPP-EEDB, 2000); *C. carpio*, LC-$50_{96\ h}$ = 410 mg pirimicarb L^{-1} (Svobodova, 1980). Although, much higher toxicity has been reported for the aquatic microcrustacean *D. magna* (Kusk, 1996) as well as for the mosquito *C. quinquefasciatus* (Magnin et al., 1988), with an EC-50 of 0.014 mg L^{-1} and an LC-$50_{24\ h}$ of 8.5 mg L^{-1}, respectively.

The MNi test has been extensively applied as an end-point for genotoxicity in amphibians (Wirz et al., 2005; Huang et al., 2007; Peltzer et al., 2008) to monitor contaminated areas (*in situ* assay) as well as for screening different compounds after direct or indirect exposure (*in vivo* assay) (Lajmanovich et al., 2005; Mouchet et al., 2007; Zavala-Aguirre et al., 2007; Binelli et al., 2008, 2009; Knakievicz et al., 2008). In 1986, Jaylet and coworkers (1986) described a model micronucleus test system using peripheral blood erythrocytes from the new larvae of *Pleurodeles waltl* for detecting mutagens in water pollution. Since then, several reports agree in demonstrating that quantification of MNi in the peripheral nucleated erythrocytes not only from salamanders but also from anurans provides a valid and reliable whole-animal system for studies of genomic instability induced by pesticides and other pollutants in aquatic environments worldwide. Among them, the MNi induction in tadpoles from *Lithobates catesbeianus*, *Xenopus laevis* and *Pelophylax ridibunda* committed to different anthropogenic activities, including the use of several pesticides can be included (Békaert et al., 2002; Matson et al., 2005; Wirz et al., 2005). Similarly, in tadpoles from *R. temporaria* and *X. laevis* exposed to the pyrethroid Fastac 10 EC (Rudek and Rozek, 1992), from *X. laevis* exposed to the fungicide captan (Mouchet et al., 2006), from *Fejervarya limnocharis* and *Pomoxis nigromaculatus* exposed to the pesticides imidacloprid and RH-5849 (Feng et al., 2004), as well as from *L. catesbeianus* exposed to the pyrethroid insecticide lambda-cyhalothrin (Campana et al., 2003), enhanced frequencies of micronucleated erythrocytes were observed when treated under laboratory conditions. In Argentina, in particular, reports in which the frequency of agrochemical-induced MNi has been used as bioassay for detecting genetic damage induced in native amphibian species are rare. Lajmanovich et al. (2005) demonstrated that the exposure under laboratory conditions to the synthetic chlorinated pesticide endosulfan increased the frequency of MNi in *Hypsiboas pulchellus* tadpoles. An enhanced frequency of MNi has been also observed in *Scinax nasicus* tadpoles from ponds of agricultural landscapes at large-scale production with the glyphosate-resistant soybean as the dominant crop (Van Hummelen et al., 1989; Lajmanovich et al., 2005; Huang et al., 2007; Peltzer et al., 2008; Shenoy et al., 2009). Finally, Cabagna et al. (2006) reported the induction of MNi under laboratory conditions by the pyrethroid insecticide cypermethrin in *Odontophrynus americanus* tadpoles. To the best of our knowledge, our present findings represent, then, the first evidence of the acute toxic, genotoxic, and cytotoxic effects exerted by Aficida® under laboratory conditions on tadpoles from a native amphibian anuran species, namely, *R. arenarum*.

Results from the analysis of Aficida®-induced MNi in *R. arenarum* tadpoles were obtained after continuous pesticide treatments lasting 48 h and 96 h. The analysis revealed, in spite of clear pesticide genotoxic properties, some effects related to the *in vivo* exposure protocol that could lead to the underestimation of the damage assessed by the end-point used for measuring genotoxicity, namely, the frequency of MNi in peripheral mature erythrocytes. Among them: (a) the induction of a selective cell loss by pesticide-induced cell death of the most damaged cells at an early exposure time, leaving only a reduced proportion of cells not severely damaged, and then able to persist in circulating blood, (b) the inability of the most severely damaged cells to enter into mitosis, and then not being able to be included in a further analysis, and/or (c) the possibility that tadpoles erythrocytes could be able to repair the damage induced by the pesticide cannot be ruled out. Furthermore, it should be taking into account that nuclear lesions might be altered by several other factors such as erythropoiesis, required time for maturation, and lifespan of erythrocytes, as suggested by Udroiu (2006). Thus, to achieve a further elucidation of Aficida®-induced damaging mechanism(s), the ratio of mature erythrocytes and erythroblasts circulating in those tadpoles committed to MNi analysis was analyzed. The results reveal an alteration of the erythrocyte:erythroblast ratio in Aficida®-treated tadpoles: a significant decrease in the frequency of mature erythrocytes when increasing the concentration of the pesticide titrated into experiments, regardless of the harvesting time. Our results could suggest an alteration in the erythrocyte kinetics in those pesticide-treated tadpoles if we consider that MNi-carrying erythrocytes tend to be removed faster from circulation due to any one, or a combination of several, of the putative explanations we hypothesized above. However, caution should be taken in such conclusions because the erythrocyte:erythroblast ratio can also reflect the balance between other biological factors wherein cell maturation rate, immature cell input, and cell removal by the spleen and liver should be included and considered.

Finally, many amphibian populations are declining in number worldwide, this phenomenon being, in most of the cases, associated with pollution of agricultural areas with the use of pesticides. However, other factors, e.g., over-exploitation, diseases, habitat loss and/or modification, introduced species, climate change, can also contribute with this situation (Mann et al., 2009). Among others, negative effects against wild tadpole frog populations of *Bufo bufo*, *Bufo americanus*, *Bufo boreas*, *Pseudacris crucifer*, *Pseudacris regilla*, *Hyla versicolor*, *Rana pipiens*, *Rana clamitans*, *Rana cascadae*, *Rana boylii*, *Rana catesbeiana*, and *Litoria freycineti* exposed to endosulfan (Broomhall and Shine, 2003; Brunelli et al., 2009; Jones et al., 2009; Relyea, 2009; Sparling and Fellers, 2009), *P. regilla*, *Hyla chrysoscelis*, *Lithobates sphenocephalus*, *Acris crepitans*, *R. boylii*, and *Gastrophryne olivacea* exposed to chlorpyrifos (Widder and Bidwell, 2006, 2008; Sparling and Fellers, 2009), *R. boylii* exposed to carbaryl (Davidson et al., 2007), *R. pipiens* exposed to diazinon (Relyea, 2009), and *R. temporaria* exposed to azoxystrobin, cyanazine, esfenvalerate, MCPA ([4-chloro-2-methylphenoxy] acetic acid), permethrin, and pirimicarb (Johansson et al., 2006) have been recently reported. Although environmental pollution might interfere with normal amphibian growth, development, and susceptibility to disease, the induction of genetic injury into DNA after chronic exposure to agrochemicals is, perhaps, the most relevant biological effect. The results we obtained with *R. arenarum* tadpoles exposed under laboratory conditions to the pirimicarb-containing commercial-formulation carbamate insecticide Aficida® highlight that this native species provides a suitable and useful experimental model for biomonitoring aquatic ecosystems.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chemosphere.2009.10.064.

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