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Flurochloridone-based herbicides induced genotoxicity effects on *Rhinella arenarum* tadpoles (Anura: Bufonidae)

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ABSTRACT

Acute toxicity and genotoxicity of the flurochloridone (FLC)-containing commercial formulation herbicides Twin Pack Gold® (25 percent a.i.) and Rainbow® (25 percent a.i.) were evaluated on *Rhinella arenarum* (Anura: Bufonidae) tadpoles exposed under laboratory conditions. Lethal effect was evaluated as end point for lethality, whereas frequency of micronuclei (MN) and single cell gel electrophoresis (SCGE) were employed as end points for genotoxicity. Lethality studies revealed equivalent $LC\text{-}50_{96\ h}$ values of 2.96 and 2.85 mg/L for Twin Pack Gold® and Rainbow®, respectively. Twin Pack Gold® did not induce DNA damage at the chromosomal level, whereas Rainbow® increased the frequency of MN only when the lowest concentration (0.71 mg/L) was used. However, all concentrations of Twin Pack Gold® and Rainbow® increased the frequencies of primary DNA lesions estimated by alkaline SCGE. This study represents the first evidence of the acute toxic and genotoxic effects exerted by two FLC-based commercial formulations, Twin Pack Gold® and Rainbow®, on tadpoles of an amphibian species native to Argentina under laboratory conditions. Finally, our findings highlight the importance of minimizing the impacts on nontarget living species exposed to agrochemicals.

1. Introduction

The exhaustive use of agrochemicals has led to the widespread presence of pesticides in every area of the environment, and such contamination may produce toxic effects in freshwater communities, which is one of the most important anthropogenic pressures on aquatic systems. Flurochloridone (FLC) is a selective preemergence and persistent herbicide applied widely worldwide. FLC is used to control a range of broadleaf weeds and annual grasses in umbelliferous, cotton, sunflower, sugar cane, and pea crops, among others. The herbicide is absorbed by roots and stems, interfering with the biosynthesis of carotenoid, chlorophyll, and abscisis acid metabolites, thus causing bleaching of the leaves (Klíčová et al., 2002; Lay and Niland, 1983). Very little is known about the toxicological information of FLC. It has been reported that the herbicide does not reveal genotoxic, carcinogenic, or neurotoxic potential in rodents. However, FLC could be considered a potential endocrine disruptor because it induces adverse effects in the reproductive functions and hormonal systems of male rats (EC, 2009; EFSA, 2010, 2013). Furthermore, when FLC is administered by oral, dermal, or inhalational routes, low or moderate acute toxicity has been reported in rats (EFSA, 2010). The levels of acute toxicity exerted by FLC when other models were used, *e.g.*, birds, fish, and aquatic invertebrates, were found to be moderate, in general (CASAFE, 2011; EFSA, 2010). However, using aquatic plants and algae, moderate to high levels of toxicity have been reported, respectively (EFSA, 2010).

Available information on the genotoxic and cytotoxic activities of FLC is scarce. So far, Yüzbasioglu et al. (2003) have reported that FLC induced abnormal cell-cycle progression and cellular mitodepressive activity in *Allium cepa* root meristematic cells. *c*-Metaphase, multipolarity, polyploidy, chromosome lagging, chromosomal stickiness, chromosome breaks, bridges, fragments, sister union, and micronuclei (MN) were the most frequently observed alterations after herbicide exposure (Yüzbasioglu et al., 2003). We demonstrated previously, employing mammalian cells *in vitro*, that FLC and its formulations Twin Pack Gold® and Rainbow® increased sister chromatid exchange frequencies, delayed cell-cycle progression, altered mitotic activity, and inhibited cell growth in CHO-K1 cells after 24 h of continuous exposure (Nikoloff et al., 2012). Furthermore, using the same *in vitro* cell system, we recently demonstrated the ability of FLC to induce DNA single-strand breaks measured by the comet assay (Nikoloff et al., 2012).

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There is an increasing interest in biomonitoring markers to provide a measurement as well as an estimation of biological exposure to genotoxic pollutants. To achieve this goal, several end points for testing both genotoxicity and cytotoxicity have been employed on aquatic organisms, including fishes and amphibians. Analysis of MN frequency and the induction of DNA single-strand breaks by the single cell gel electrophoresis (SCGE) assay (Brodeur et al., 2012; Cavaş and Könen, 2007; Mouchet et al., 2006; Vera Candioti et al., 2010b) are the most frequently recommended and employed end points for detecting DNA damage in circulating blood erythrocytes.

Amphibians have certain characteristics rendering them useful indicator species for measuring the effects of changes of the environment (Brodeur et al., 2012). However, in recent decades, amphibian populations have been reported to suffer significant decline worldwide (Brodeur et al., 2012), a phenomenon in most cases attributed to pollution of agricultural areas with the use of pesticides (Mann et al., 2009). 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), *Pseudacris regilla, Hyla chrysoscelis*, *Lithobates sphenocephalus*, *Acris crepitans*, *Rana boylii*, and *Gastrophryne olivacea* exposed to chlorpyrifos (Sparling and Fellers, 2009; Widder and Bidwell, 2006, 2008), *Rana boylii* exposed to carbaryl (Davidson et al., 2007), *Rana pipiens* exposed to diazinon (Relyea, 2009), and *Rana temporaria* exposed to azoxystrobin, cyanazine, esfenvalerate, MCPA ([4-chloro-2-methylphenoxy] acetic acid), permethrin, and pirimicarb (Johansson et al., 2006) have been 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. Furthermore, a correlation between the use of agrochemicals and the decline of amphibian populations has been reported (Beebee, 2005; Jones et al., 2009). The effects of pesticides, including insecticides as well as herbicides, are particularly detrimental to amphibian species because of their aquatic habitat, sensitive skin, and unprotected eggs, among other factors (Blaustein et al., 1994; Bradford et al., 2011; Brühl et al., 2011; Sparling and Fellers, 2009).

Previous studies have stressed that *Rhinella arenarum* can be considered as a suitable *in vivo* model for detecting lethal and sublethal effects (including genotoxic and cytotoxic properties) induced by several agrochemicals. Among them, the insecticides pirimicarb (Vera Candioti et al., 2010b), malathion, and azinphos-methyl (Lascano et al., 2011), and the herbicides atrazine (Brodeur et al., 2009), and 2,4-D (Aronzon et al., 2011), can be included.

The aim of the present study is characterize the acute lethality and biomarkers of genotoxicity, namely, MNs and SCGE of the FLC-containing technical herbicide formulations Twin Pack Gold® (25 percent a.i.) and Rainbow® (25 percent a.i.) on *R. arenarum* tadpoles exposed under laboratory conditions. The results should provide useful information for a more comprehensive understanding of the toxicity and genotoxicity exerted by commercial formulations of the FLC and their potential risks to amphibian populations.

2. Materials and methods

2.1. Chemicals

FLC [3-chloro-4-(chloromethyl)-1-[3-(trifluoromethyl) phenyl]-2-pyrrolidone; CAS 61213-25-0; recommended application field ratios of 7.5 to 137.5 g a.i/ha (CASAFE, 2011)] commercial grade trade formulations Twin Pack Gold® (25 percent a.i., excipients q.s.) and Rainbow® (25 percent a.i., excipients q.s.) were kindly provided by Syngenta Agro S.A. (Buenos Aires, Argentina) and Magan Argentina S.A. (Buenos Aires, Argentina), respectively. Cyclophosphamide (CP; CAS 6055-19-2) was obtained from Sigma Chemical Co. (St. Louis, MO, USA), whereas $K_2Cr_2O_7$ [Cr (VI); CAS 7778-50-9] was obtained from Merck KGaA (Darmstadt, Germany). All other chemicals and solvents were of analytical grade and were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Quality control

Concentration levels of FLC in test solutions were analyzed by high-performance liquid chromatography with an Agilent 1100 LC system (Agilent Technologies) equipped with a binary pump and an MSD VL quadrupole mass spectrometer (Agilent Technologies) with an electrospray ionization interface. A Rheodyne 7725i injector with a 20 μl loop was used. Data acquisition and analysis were performed using an LC/MSD Agilent ChemStation. The chromatographic separations were performed on a C-18 XSelect CSH column (75 × 4.6 mm, 2.5 μm pore size). The column was maintained at 25 ± 1 °C using isocratic elution of MeOH:formic acid 0.1 percent (70:30) at 0.5 mL/min. The electrospray ionization was performed using nitrogen to assist nebulization, at a flow rate of 7 L/min, and a nebulizer pressure of 30 psi. The capillary temperature and voltage were set at 330 °C and 4000 V, respectively. The fragmentor energy was set at 100 eV. Subsequently, positive single-ion monitoring (SIM^+) was used for analyte quantification by measuring of the ion $[M+H]^+$. Active ingredient samples from test solutions correspond to values obtained immediately after preparation (0 h) and 24 h thereafter. The detection limit was 0.01 mg/L.

2.3. Anuran tadpoles

R. arenarum tadpoles were selected as test organisms. This species has an extensive distribution in the Neotropical America including Argentina, Bolivia, Brazil, Uruguay, and Paraguay. This toad species from the Bufonidae family has an extensive distribution, 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. This species is easy to handle and acclimate to laboratory conditions (www.iucnredlist.org). Egg masses used for this study were collected from a temporary pond free from pluvial runoff from agricultural areas, in the vicinity of La Plata City (Buenos Aires Province, Argentina) at the late cleavage stage, stage 9 according to Gosner (1960) classification. Hatches were transported to the laboratory and then acclimatized to 16/8 h light/dark cycles in aquaria at 25 °C with dechlorinated tap water with artificial aeration and boiled lettuce as a food source until the beginning of the experimental procedures. Fertilized eggs were divided into two equal-size groups. One of the groups was used for the mortality test when they reached Gosner stage 33 (range 32–34). The second group was kept in the laboratory until individuals reached a body size that allowed extraction of blood for MN and SCGE (Gosner stage 37) and then used for the second set of experiments. Physical and chemical parameters of the water were (mean ± SE) as follows: temperature, 25.0 ± 1 °C; pH 7.5 ± 0.1; dissolved oxygen, 6.3 ± 0.3 mg/L; conductivity, 994 ± 8.5 μS/cm; hardness, 143 ± 23.5 mg $CaCO_3$/L.

2.4. Determination of LC-50

Concentrations assessed throughout the study represent the nominal concentrations of active ingredient present within Twin Pack Gold® and Rainbow® FLC-based formulations. Experiments for toxicity assessment were performed following recommendations proposed by the USEPA (1975, 2002) and ASTM (2007) standardized methods with minor modifications reported previously for native species (Brodeur et al., 2009; Natale et al., 2006; Vera Candioti et al., 2010a). Experiments were performed using 10 tadpoles at Gosner development stage 33 for each experimental point, maintained in a 1 L glass container, and exposed to six different concentrations of Twin Pack Gold® and Rainbow® ranging from 1 to 3.33 mg/L during 96 h. Negative (dechlorinated tap water; pH 7.5 ± 0.1; hardness, 143 ± 23.5 mg $CaCO_3$/L) and positive controls [23 mg/L Cr(VI)-treated tadpoles] were conducted and run simultaneously with Twin Pack Gold®- and Rainbow®-exposed tadpoles. All test solutions were prepared immediately before use and replaced every 24 h. Tadpoles were not fed throughout the experiment. Mortality was evaluated by visual observation every 24 h. Individuals were considered dead when no movement was detected after gently prodding the tadpoles with a glass rod. Experiments were performed in quadruplicate and run simultaneously for each experimental point.

2.5. Micronuclei and other nuclear abnormalities

Experiments were performed using 5 tadpoles at Gosner development stage 37 for each experimental point, maintained in a 1 L glass container, and exposed to three different concentrations of the test compound equivalent to 25 percent, 50 percent, and 75 percent of the corresponding $LC\text{-}50_{96\ h}$ value. To achieve these

concentrations, tadpoles were exposed to 0.74, 1.48, and 2.22 mg/L and 0.71, 1.42, and 2.13 mg/L Twin Pack Gold® and Rainbow®, respectively (see Section 2.4). Negative (dechlorinated tap water; see Section 2.4) and positive controls (40 mg/L CP) were conducted and run simultaneously with Twin Pack Gold®- and Rainbow®-exposed tadpoles. All test solutions were prepared immediately before each experiment. The frequency of MN was determined in peripheral mature erythrocytes at 48 and 96 h after initial treatment. Experiments were performed in triplicate and run simultaneously for each experimental point. Tadpoles were killed according to American Society of Ichthyologists and Herpetologists (ASIH, 2004) criteria. At the end of each experiment, tadpole anesthetized was induced by immersion in ice water, and blood samples were obtained by sectioning behind the operculum. Two peripheral blood smears were performed for each animal onto clean slides, air dried, fixed with 100 percent (v/v) cold methanol (4 °C) for 20 min, and then stained with 5 percent Giemsa solution for 12 min. Slides were coded and blind-scored by one researcher at 1000 × magnification. Data are expressed as a total number of MNs per 1000 cells, as suggested previously (Vera Candioti et al., 2010a). MN frequency was determined following the examination criteria reported previously (Fenech, 2007; Vera Candioti et al., 2010a). Briefly, the criteria employed in identifying MN were as follows: a diameter smaller than 1/3 of that of the main nuclei, nonrefractibility, the same staining intensity as or staining intensity lighter than that of the main nuclei, no connection or link with the main nuclei, no overlapping with the main nuclei, MN boundary distinguishable from the main nuclei boundary, and no more than four MNs associated with the nuclei.

Other erythrocytic nuclear abnormalities were blind-scored from 1000 erythrocytes per experimental point from each experiment at 1000 × magnification. Examination criteria followed those established previously (Cavaş and Ergene-Gözükara, 2003; Strunjak-Perovic et al., 2009). Briefly, cells with two nuclei were considered as binucleates (BN) whereas cells with one nucleus presenting a relatively small evagination of the nuclear membrane which contains euchromatin were classified as blebbed nuclei (BL). Nuclei with evaginations of the nuclear membrane larger than the BL which could have several lobes were considered as lobed nuclei (LB). Finally, nuclei with vacuoles and appreciable depth into a nucleus without containing nuclear material were recorded as notched nuclei (NT).

2.6. Single cell gel electrophoresis assay

Tadpoles were exposed under the same concentrations and conditions described for MN assay (see Section 2.5). Negative (dechlorinated tap water; see Section 2.4) and positive controls (40 mg/L CP) were conducted and run simultaneously with Twin Pack Gold®- and Rainbow®-exposed tadpoles. The SCGE assay was performed following the alkaline procedure described by Singh (1996) with minor modifications. After severing the spinal column behind the opercula, two drops of peripheral blood from each specimen were collected. Blood samples were diluted with 1 mL phosphate-buffered saline, centrifuged (2000 rpm, 8 min), and resuspended in a final volume of 50 μL of phosphate-buffered saline. An aliquot of 30 μL the diluted samples was mixed with 70 μL 0.5 percent low-melting-point agarose and was then layered on a slide precoated with 100 μL 0.5 percent normal-melting-point agarose. The slide was covered with a coverslip and placed at 4 °C for 10 min. After solidification, the coverslip was removed, and the slide was covered with a third layer of 50 μL 0.5 percent low-melting-point agarose. After solidification, the coverslip was removed, and slides were immersed in ice-cold freshly prepared lysis solution (1 percent sodium sarcocinate, 2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, pH 10.0, 1 percent Triton X-100, 10 percent DMSO) and then lysed in darkness for a 1 h period at 4 °C. Then, slides were placed in an electrophoresis buffer (1 mM Na_2EDTA, 300 mM NaOH) for 25 min at 4 °C to allow the cellular DNA to unwind, followed by electrophoresis in the same buffer and temperature for 30 min at 25 V and 250 mA. Finally, the slides were neutralized with a solution comprising 0.4 m Tris–HCl, pH 7.5, stained with 4′,6-diamino-2-phenylindole (DAPI; Vectashield mounting medium H1200; Vector Laboratories, Burlingame, CA, USA). Slides were examined under an Olympus BX50 fluorescence photomicroscope equipped with an appropriate filter combination. The extent of DNA damage was quantified by the length of DNA migration, which was visually determined in 100 randomly selected and nonoverlapping cells. DNA damage was classified in four classes (0-I: undamaged; II: minimum damage; III: medium damage; IV: maximum damage), as suggested previously (Cavaş and Könen, 2007). Data are expressed as the mean number of damaged cells (sum of Classes II, III, and IV) and the mean comet score for each treatment group. The genetic damage index (GDI) was calculated for each test compound following Pitarque et al. (1999).

2.7. Statistical analysis

Mortality data were analyzed using the U.S. EPA Probit Analysis, version 1.5 statistical software (http://www.epa.gov/nerleerd/stat2.htm.) and based on Finney's method (Finney, 1971). Data of MN and SCGE were analyzed by a one-way ANOVA with Dunnett's test (Zar, 1999) to determine significant differences from the control group. ANOVA assumptions were corroborated with Barlett's test for homogeneity of variances and χ^2 test for normality. Differences in BN, BL, LB, NT, and GDI in treated and control cells were evaluated by χ^2 test. The relationships between concentration and GDI and MN data were evaluated by simple linear regression and correlation analyses. Concentration–response (C–R) curves at 96 h were estimated with their 95 percent confidence limits. Regression (a and b) and correlation (r) coefficients were calculated for each C–R curve. Tests of significance of the regressions and correlation coefficients were performed following Zar (1999). The level of significance was 0.05 for all tests, unless indicated otherwise.

3. Results

Results of chemical analyses showed no significant changes ($P > 0.05$) in the concentration of the pure analyte in treatments during the 24 h interval renewals of the testing solutions (concentration range 97 ± 5 percent recovery). Concentrations assessed throughout the study represent the nominal concentrations of active ingredient present within technical formulations.

Probit analysis of mortality data yielded values of 2.96 mg/L (LC95%, 2.89–3.03) and 2.85 mg/L (LC95%, 2.74–2.97) for the $LC50_{96\ h}$ values of Twin Pack Gold® and Rainbow®, respectively.

Table 1 shows the results of the analysis of Twin Gold Pack®- and Rainbow®-induced MN in peripheral blood erythrocytes of *R. arenarum* tadpoles. An increased the frequency of MN was observed in those CP-exposed tadpoles (positive control) at 48 and 96 h ($P < 0.001$). Results revealed that the frequency of MN in tadpoles exposed to Twin Pack Gold® did not differ from that in the negative controls, regardless of both concentration and

Table 1
MN frequencies (‰) in peripheral blood erythrocytes of *Rhinella arenarum* tadpoles exposed to Twin Pack Gold® and Rainbow®.

Chemicals	Exposure concentration (mg/L)	Exposure time (h)	Number of animals observed	Number of cells observed	MN/1000 erythrocytes ± SE
Negative control		48	15	15,000	7.64 ± 1.24
		96	15	15,000	7.00 ± 1.24
Twin Pack Gold®	0.74	48	15	15,000	8.84 ± 1.39
		96	15	15,000	9.89 ± 1.38
	1.48	48	15	15,000	11.10 ± 0.99
		96	15	15,000	11.20 ± 2.98
	2.22	48	15	15,000	11.22 ± 1.77
		96	15	15,000	11.88 ± 1.88
Rainbow®	0.71	48	15	15,000	19.05 ± 1.84 ***
		96	15	15,000	11.27 ± 1.10
	1.42	48	15	15,000	9.13 ± 1.28
		96	15	15,000	10.69 ± 1.81
	2.13	48	15	15,000	11.03 ± 0.86
		96	15	15,000	8.07 ± 0.80
CP[a]	40.00	48	15	15,000	17.50 ± 2.13 ***
		96	15	15,000	17.42 ± 1.79 ***

[a] Cyclophosphamide (CP) was used as positive control.
*** $P < 0.001$; significant differences with respect to negative control values.

exposure time ($P>0.05$). On the other hand, tadpoles only exposed to 0.71 mg/L of Rainbow® for 48 h showed a significant increase in MN frequency compared with negative controls ($P<0.001$). Overall, a regression analysis demonstrated that frequency of MN values decreased as a negative linear function of the Rainbow® concentration within the 0–48 h treatment period ($r=-0.62$, $P<0.001$). When the frequencies of other nucleoplasmic abnormalities were analyzed, Twin Pack Gold® and Rainbow® treatments, regardless of the concentration, did not modified the frequencies compared to control values ($P>0.05$) (data not shown).

Results of the SCGE assay obtained in peripheral blood erythrocytes of *R. arenarum* tadpoles exposed for 48 and 96 h to Twin Pack Gold® and Rainbow® are presented in Table 2. While different nucleoid damage categories are visualized in Fig. 1, mean frequencies of cells from each damage grade are depicted in Fig. 2. CP treatment (positive control) induced an enhancement of the GDI as well as in the frequency of damaged cells compared to negative controls in specimens exposed for either 48 or 96 h ($P<0.001$) (Table 2, Fig. 2). In those tadpoles exposed to Twin Pack Gold®, a significant increase of the GDI was observed at 48 h of treatment when concentrations of 0.74 mg/L ($P<0.001$) and 2.22 mg/L ($P<0.01$) were employed (Table 2). Statistical analysis revealed that the GDI increase induced by 0.74 mg/L was due to an increase in the frequency of type IV comets ($P<0.001$) and a concomitant decrease of type I comets ($P<0.001$) (Fig. 2A). However, the GDI increase induced by 2.22 mg/L was due only to an increase in the frequency of type IV comets ($P<0.01$) (Fig. 2A). When the analysis was performed in tadpoles exposed during 96 h, increased GDI values were found regardless of the concentration ($P<0.001$) (Table 2). For all tested concentrations, this effect was due to an increase in the frequency of type IV comets ($P<0.001$) and a decrease in the frequency of type I nucleoids ($P<0.001$) (Fig. 2B). Nevertheless, in those 0.74 mg Twin Pack Gold® L^{-1}-treated tadpoles, an increment in the frequency of type III comets was also observed ($P<0.05$) (Fig. 2B). Overall, a regression analysis demonstrated that the GDI varied as a negative function of the Twin Pack Gold® concentrations in those tadpoles treated for 48 h ($r=-0.43$, $P<0.01$) but not in those exposed for 96 h ($r=0.05$, $P>0.05$). Rainbow® treatments induced DNA damage revealed by an increase in the GDI values of those tadpoles exposed for both 48 and 96 h ($P<0.05$ to $P<0.001$) (Table 2). In those tadpoles

Table 2
Analysis of DNA damage measured by comet assay in *Rhinella arenarum* tadpoles cells exposed to Twin Pack Gold® and Rainbow®.

Chemicals	Exposure dosage (mg/L)	Exposure time (h)	Number of animals observed	(%) of damaged cells (II+III+IV)	GDI[a] ± SE
Negative control		48	15	43.64	1.70 ± 3.93
		96	15	38.13	1.66 ± 5.04
Twin Pack Gold®	0.74	48	15	76.00 ***	2.51 ± 5.42 ***
		96	15	91.92 ***	3.30 ± 4.00 ***
	1.48	48	15	41.87	1.83 ± 5.19
		96	15	75.38 ***	2.52 ± 3.82 ***
	2.22	48	15	52.86	2.13 ± 5.24 **
		96	15	77.08 ***	2.59 ± 5.16 ***
Rainbow®	0.71	48	15	66.92 ***	1.98 ± 3.51 *
		96	15	85.77 ***	2.69 ± 4.73 ***
	1.42	48	15	72.85 ***	2.25 ± 3.58 ***
		96	15	60.46 **	2.08 ± 4.30 **
	2.13	48	15	57.08 *	2.10 ± 5.13 **
		96	15	73.14 ***	2.63 ± 2.70 ***
CP[b]	40.00	48	15	92.00 ***	2.93 ± 1.58 ***
		96	15	85.88 ***	2.94 ± 3.22 ***

[a] GDI Genetic damage index.
[b] Cyclophosphamide (CP) was used as positive control.
* $P<0.05$ significant differences with respect to negative control values.
** $P<0.01$ significant differences with respect to negative control values.
*** $P<0.001$ significant differences with respect to negative control values.

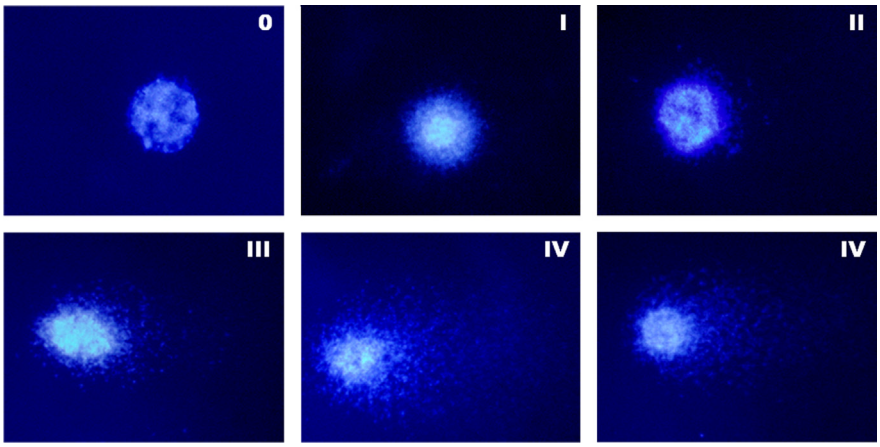

Fig. 1. Digitized comet images showing undamaged and damaged DNA in *Rhinella arenarum* red blood cells. They represent classes 0-IV as used for visual scoring (0-I: undamaged, II: minimum damage, III: medium damage, IV: maximum damage). Cells were stained with DAPI and capture at fluorescent microscope. Magnification (1000 ×).

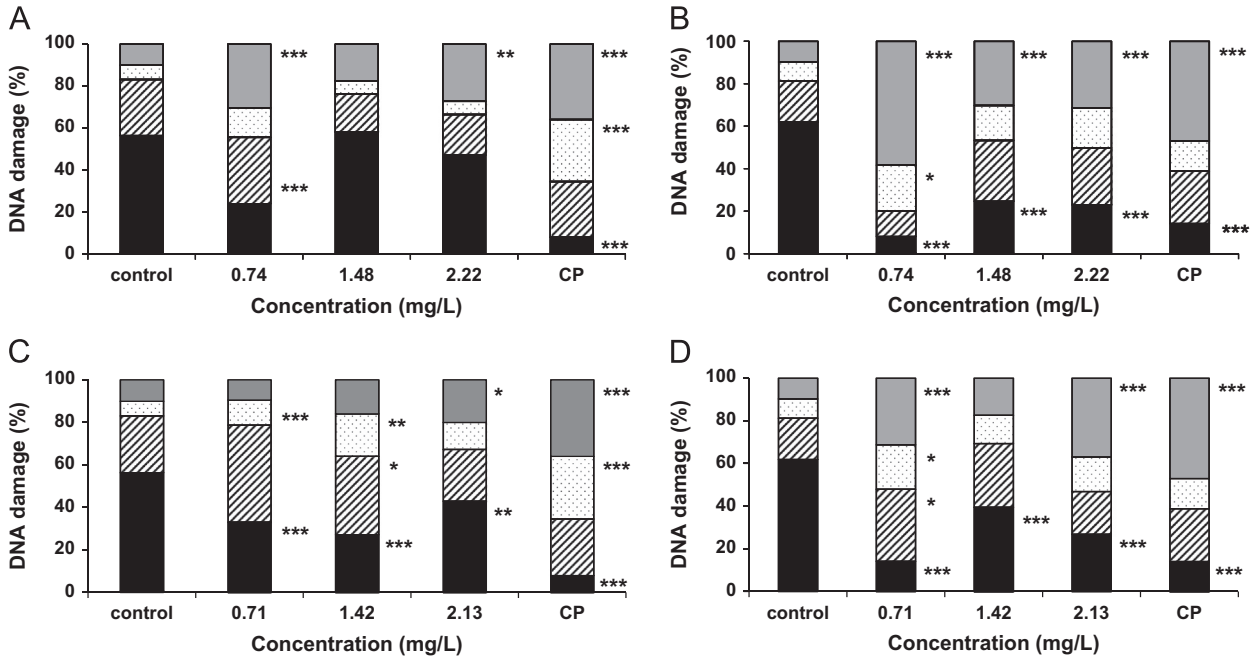

Fig. 2. Twin Pack Gold® (A) and (B) and Rainbow® (C) and (D)-induced DNA damage measured by comet assay in circulating blood cells from *Rhinella arenarum* (Anura, Bufonidae) tadpoles exposed for 48 h (A) and (C) and 96 h (B) and (D). The frequencies of undamaged (type 0-I nucleoids; black bar sections), type II (stripped bar sections), type III (dotted bar sections), and type IV (grey bar sections) were determined by analyzing 100 nucleoids from each tadpole. Results are presented as percentages of pooled data from three independent experiments. Negative (untreated tadpoles) and positive controls (CP, 40 mg cyclophosphamide/L-treated tadpoles) were conducted and run simultaneously with Twin Pack Gold®- and Rainbow®-exposed larvae. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$; significant differences with respect to control values.

exposed for 48 h, a decreased frequency of type I comets within the 0.71–2.13 mg/L concentration range was observed ($P<0.01$ to $P<0.001$). On the other hand, an increase in the frequency of type II-III comets within the 0.71–1.42 mg/L concentration range was found ($P<0.05$ to $P<0.001$) as well as in the frequency of type IV comets when tadpoles were exposed to 2.13 mg/L concentration ($P<0.05$) (Fig. 2C). When treatments lasted 96 h, the frequency of type I comets decreased for all concentrations tested ($P<0.001$) (Fig. 2D). In addition, in those tadpoles exposed to 0.71 mg/L, an increase in the frequency of type II ($P<0.05$), type III ($P<0.05$), and type IV comets ($P<0.001$) was also observed (Fig. 2D). However, in those tadpoles exposed to 2.13 mg/L Rainbow®, only the frequency of type IV comets was enhanced ($P<0.001$) (Fig. 2D). A regression analysis revealed that, overall, the GDI varied as a negative function of Rainbow® concentrations within both the 0–48 h and 0–96 h treatment periods ($r=-0.44$, $P<0.01$ and $r=-0.40$, $P<0.05$, respectively).

4. Discussion

In the current study, we evaluated acute lethal and sublethal toxicity induced by two FLC-based commercial herbicide formulations, Twin Pack Gold® (25 percent FLC) and Rainbow® (25 percent FLC), on *R. arenarum* tadpoles exposed under laboratory conditions. Lethality studies revealed equivalent $LC50_{96\ h}$ values of 2.96 mg/L (range, 2.89–3.03 mg/L) and 2.85 mg/L (range, 2.74–2.97 mg/L) for Twin Pack Gold® and Rainbow®, respectively. The results demonstrate that Twin Pack Gold® did not induce DNA damage at the chromosomal level. On the other hand, Rainbow® increased the frequency of MN in peripheral erythrocytes of *R. arenarum* only when the lowest concentration (0.71 mg/L) was employed. Furthermore, none of the FLC-based formulations induced other nuclear abnormalities, *i.e.*, binucleated cells, blebbed, lobed and notched nuclei. We observed that acute exposure to all concentrations of both formulated products increased the frequencies of primary DNA lesions estimated by alkaline SCGE. Additionally, our data revealed that the SCGE assay was more sensitive than the MN test in detecting early DNA damage when the same FLC concentrations were employed for tadpole exposure.

The MN analysis is routinely employed in cytogenetic studies to detect small chromosomal fragments like acentric fragments and chromatid fragments induced by clastogens or vagrant chromosomes produced by aneugens (Fenech, 2007; OECD, 2007). Several previous reports demonstrated the induction of MN in amphibian tadpoles as a consequence of pesticide treatments (Bouhafs et al., 2009; Lajmanovich et al., 2005; Li et al., 2010; Vera Candioti et al., 2010a). However, we did not observe a significant increase in MN frequency with any of the concentrations of Twin Pack Gold® tested, but an enhanced MN frequency was noticed at the lowest concentration of Rainbow®. So far, we do not have any explanation for this particular observation. However, a plausible possibility could be related to the presence of toxic coformulants in the commercial preparation of Rainbow®, but not in Twin Pack Gold®, that may pose an increased risk for DNA damage at the chromosomal level and have cytotoxic properties as well. Thus, they are able to increase MN frequency at the lower concentration tested and exert a more evident cytotoxicity when higher doses are employed, preventing the most severe cells from completing one cell-cycle division by the harvesting time, and thus not inducing an increase of MN. Furthermore, the possibility of induction of a selective cell loss by herbicide-induced cell death of the most damaged cells after treatment, leaving only a reduced proportion of cells capable of reaching the M_1 status, could not be ruled out. Whether the latter is true or not, the present results could confirm the importance of studying complete agrochemical formulations in toxicity screenings because the excipient(s) may have toxicological properties completely different from those of the active ingredients alone, and their impacts may be quite different, as demonstrated previously in different biotic matrices (Cox and Surgan, 2006; González et al., 2007; Nikoloff et al., 2012; Soloneski and Larramendy, 2010).

The SCGE test has become extensively valuable as a biomarker in amphibians to monitor contaminated areas (*in situ* assay) (Burlibasa and Gavrila, 2011; Maselli et al., 2010; Ralph and Petras, 1998) as well as for screening xenobiotics after direct or indirect exposure (*in vivo* assay) (Binelli et al., 2009; Knakievicz et al., 2008; Mouchet et al., 2007; Zavala-Aguirre et al., 2007). We observed that, regardless of the concentration, acute exposure to Twin Pack Gold® or Rainbow® increased the frequency of primary DNA lesions estimated by alkaline SCGE, a result opposite that of the MN test. One possible explanation for this observation could be related to the different cellular status of the target cells that are included for analysis in each end point. Whereas DNA damage is estimated by SCGE in resting cells, MN are determined on proliferating cells with lesions that have lasted for at least one mitotic cell cycle and that probably retain their repair property (He et al., 2000).

Although reports in which the frequency of agrochemical-induced DNA single-strand breaks has been used as a bioassay for evaluating genetic damage induced in amphibians are well documented by the extensive bibliography worldwide (Mouchet et al., 2006), no data are available for native Argentinean amphibians. To the best of our knowledge, our current results represent the first evidence of the acute toxic and genotoxic effects exerted by two FLC-based commercial formulations, Twin Pack Gold® and Rainbow®, on tadpoles under laboratory conditions. Furthermore, no other SCGE study has been previously reported employing *R. arenarum* as the test organism.

Our observations revealed 2.96 mg/L (range, 2.89–3.03) and 2.85 mg/L (range, 2.74–2.97) for the LC-$50_{96\ h}$ values of Twin Pack Gold® and Rainbow®, respectively. Although the *in vivo* FLC treatments in this study covered a wide range of concentrations, the concentration range represents a relatively high end of the threshold value of 0.1 μg FLC/L found in ground, surface and drinking water reported by EFSA (2010), even considering the recommended application field ratios of 7.5 to 137.5 g a.i/ha reported for Argentina (CASAFE, 2011). Thus, the concentrations of FLC employed in this investigation would be expected to be almost improbable in the environment, perhaps only observed when specific events occurred (*e.g.*, direct application, drainage ditches or by accidental discharge). Although, we cannot rule out that amphibian populations and also occupationally exposed human workers could be exposed accidentally to these agrochemicals to this range of concentrations.

In accordance with the WHO (2009), the herbicide pyrrolidone FLC is classified as a slightly toxic compound (class III) and a compound harmful if absorbed through the skin. FLC is strongly mutagenic in plants than in mammalian cells, producing in the former a 3–4 times abnormalities in cell-cycle progression and cellular mitodepressive activity than in the later (Nikoloff et al., 2012; Yüzbasioglu et al., 2003).

Concerning the acute lethal toxicity of the studied formulations on the species, both formulated herbicides can be classified as toxic compounds (category II) following either the European Union directives (Mazzatorta et al., 2002) or the classification criteria proposed by the United Nations (UN) (2011). Despite this fact, the herbicides hold identifiable sublethal effects at nearly four-fold lower concentrations than those corresponding to the lethal end-point, which are associated with the induction of DNA single-strand breaks as well as MN in circulating erythrocytes. To the best of our knowledge, acute lethality data of LC-$50_{96\ h}$ values reported here for FLC represent novel information for this type of effect not only for the herbicide but also for amphibians.

Although amphibians have been extensively employed as target organisms for analysing acute lethal effects induced by different pesticides worldwide (Burlibasa and Gavrila, 2011; Li et al., 2010; Liu et al., 2011; Ossana et al., 2010; Vera Candioti et al., 2010a), so far none of the aforementioned studies have been aimed to evaluate the effects exerted by FLC. To the best of our knowledge, our current results represent, then, the first evidence of the acute lethal and sublethal toxic effects exerted by two FLC-based commercial formulations Twin Pack Gold® and Rainbow® on tadpoles of an amphibian species native to Argentina, *R. arenarum*. Finally, our findings support the view that the SCGE assay is a highly sensitive method for the detection of DNA damage induced by environmental pollutants.

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