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RESEARCH ARTICLE

Genotoxic effect of a binary mixture of dicamba- and glyphosate-based commercial herbicide formulations on *Rhinella arenarum* (Hensel, 1867) (Anura, Bufonidae) late-stage larvae

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Abstract The acute toxicity of two herbicide formulations, namely, the 57.71 % dicamba (DIC)-based Banvel® and the 48 % glyphosate (GLY)-based Credit®, alone as well as the binary mixture of these herbicides was evaluated on late-stage *Rhinella arenarum* larvae (stage 36) exposed under laboratory conditions. Mortality was used as an endpoint for determining acute lethal effects, whereas the single-cell gel electrophoresis (SCGE) assay was employed as genotoxic endpoint to study sublethal effects. Lethality studies revealed $LC50_{96\ h}$ values of 358.44 and 78.18 mg L^{-1} DIC and GLY for Banvel® and Credit®, respectively. SCGE assay revealed, after exposure for 96 h to either 5 and 10 % of the Banvel® $LC50_{96\ h}$ concentration or 5 and 10 % of the Credit® $LC50_{96\ h}$ concentration, an equal significant increase of the genetic damage index (GDI) regardless of the concentration of the herbicide assayed. The binary mixtures of 5 % Banvel® plus 5 % Credit® $LC50_{96\ h}$ concentrations and 10 % Banvel® plus 10 % Credit® $LC50_{96\ h}$ concentrations induced equivalent significant increases in the GDI in regard to GDI values from late-stage larvae exposed only to Banvel® or Credit®. This study represents the first experimental evidence of acute lethal and sublethal effects exerted by DIC on the species, as well as the induction of primary DNA breaks by this herbicide in amphibians. Finally, a synergistic effect of the mixture of GLY and DIC on the induction of primary DNA breaks on circulating blood cells of *R. arenarum* late-stage larvae could be demonstrated.

Keywords Amphibians · Dicamba-based formulations · Glyphosate-based formulations · Lethal effects · SCGE assay

Introduction

Worldwide, living species are inevitably exposed to several classes of pesticides, and they represent both a significant ecological and public health concern. Furthermore, pesticides are ubiquitous on the planet, since anthropogenic activities are continuously releasing extensive amounts of them into the environment, especially on croplands and pastures, with the aim of increasing agricultural production. In this sense, according to FAO-WHO, global agricultural production is growing, and it must increase more than 70 % by 2050 (FAO-WHO 2006). Large amounts of pesticides may be hazardous to the environment because of their persistence, bioaccumulation, and toxicity (www.epa.gov/pesticides). Unfortunately, it is difficult to decrease the use of these agrochemicals without reducing crop yields (McLaughlin and Kinzelbach 2015). It has been estimated that less than 0.1 % of pesticides applied to crops worldwide reach their specific targets, leaving large amounts of toxic residues free to move into different environmental compartments (Pimentel et al. 1993). Pesticides are able to contaminate soil and air, as well as surface and ground water, affecting then not only target but also nontarget organisms (Liang et al. 2013; Meffe and de Bustamante 2014). Thus, the agrochemical contamination of food, water, and air has become a severe concern for human and ecosystem health (WHO 2009).

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In agriculture, pesticides are, in most cases, not used as a single active ingredient but rather as part of complex commercial formulations. In addition to the active ingredient(s), the formulated products contain several types of adjuvants differing in their modes of action, including carrier substances and solvents that improve the pesticide absorption (WHO 1990). Although the additive compounds frequently comprise a large part of a commercial pesticide formulation, they are not usually included in any discussion of the effect on living cells, and their jeopardizing effects on living organisms may exceed those of the active ingredients (WHO 1990). Several studies highlight that the toxicity of nonactive ingredients present in the full commercial formulations of pesticides could be even more toxic than the active ingredient itself, and could affect the overall toxicity of the product (Nikoloff et al. 2014a, b; Soloneski et al. 2008, 2015; Soloneski and Larramendy 2010; Zeljezic et al. 2006).

Another aspect that deserves attention is related to a common modern agricultural practice, e.g., the application of more than one pesticide at the same time as mixtures of pesticides, which represent, then, an additional important group of environmental pollutants (Lydy et al. 2004). It is well known that chemicals in mixtures interact with each other and with the biological matrices at different levels, and they can significantly alter the toxic and genotoxic responses exerted by individual chemicals (Brodeur et al. 2014; Lajmanovich et al. 2013; Lazarová and Slameňová 2004; Roustan et al. 2014). Following the prevailing concept, three basic classes of joint action are widely accepted for evaluating the effects of mixtures of toxicants in the environment, i.e., additivity, synergism, and antagonism (Warne 2003). It is possible then that mixtures of pesticides can produce additive or synergistic effects, or can even produce antagonism (Lydy et al. 2004). As a result, the analysis of the potential toxic and genotoxic effects of the chemical binary mixtures rather than single components is strongly recommended for better and proper risk assessment evaluation.

Amphibians are an integral part of aquatic biota, at least in one developmental stage, and they are sensitive to any change in the environment not only at the species level but also according to the developmental stage. Several studies have demonstrated that these vertebrates can be used as valid indicator species for environmental monitoring (Brodeur et al. 2012; Kehoe et al. 2015). Nevertheless, in recent decades, amphibian populations have been reported to suffer a significant decline worldwide, a phenomenon in several cases committed to pollution of both natural and agricultural areas due to the use of pesticides (Mann et al. 2009; Wagner et al. 2014). Although environmental pollution might interfere with normal amphibian growth, development, and susceptibility to disease, the induction of genetic damage into DNA after acute and chronic exposure to agrochemicals is ultimately the most relevant jeopardizing effect. Several studies support the concept that this biomarker has been of great interest in assessing the risk posed by a toxic chemical. It is considered an appropriate tool to evaluate the effects of exposure to xenobiotic on organisms, including amphibians, with the final aim to relate cause and effect (de Lapuente et al. 2015; Pérez-Iglesias et al. 2014, 2015; Vera Candioti et al. 2010). Furthermore, a positive correlation between amphibian population decline and the use of agrochemicals has been well documented (Beebee 2005; Jones et al. 2009). The deleterious effects of pesticides, including herbicides and insecticides, are particularly detrimental to amphibian species because of their aquatic habitat, sensitive skin, and unprotected eggs, among other factors, e.g., overexploitation, diseases, habitat loss and/or modification, introduced species, and climate change (Bradford et al. 2011; Brühl et al. 2011; Mann et al. 2009).

Argentina has approximately 60 species of amphibians, and the common South American toad *Rhinella arenarum* occurs widely in the humid pampas where agrochemicals are largely employed for economical crops. As an adult, *R. arenarum* is terrestrial and reproduces in shallow temporary, and semitemporary ponds formed in low-lying areas within this agroecosystems are important habitats for amphibians, including *R. arenarum*, and other wildlife that may be affected by the agronomical activities carried out in the adjacent fields (Agostini et al. 2009; Natale et al. 2006). Previous studies have demonstrated that the common South American toad *R. arenarum* can be considered as a suitable in vivo model for detecting both lethal and sublethal effects (including genotoxic and cytotoxic properties) exerted by several agrochemicals. Among them, the insecticides *p*,*p*-DDT (Juárez and Gusmán 1984), carbaryl (Ferrari et al. 2011), pirimicarb (Vera Candioti et al. 2010), malathion (Lascano et al. 2011), parathion (Guzmán and Guardia 1978), chlorpyrifos (Sotomayor et al. 2015), and azinphos-methyl (Ferrari et al. 2011; Lascano et al. 2011) and the herbicides glyphosate (GLY) (Lajmanovich et al. 2013), atrazine (Brodeur et al. 2009), flurochloridone (Nikoloff et al. 2014c), and 2,4-D (Aronzon et al. 2011) can be included.

The single-cell gel electrophoresis (SCGE) test, also called the comet assay, is a well-established, simple, inexpensive, and sensitive method to assess single and double DNA strand breaks as well as alkali-labile lesions in the DNA of a single cell in both in vitro and in vivo systems (Collins et al. 2014). Due to the large number of advantages and applications of this methodology, the number of publications concerning the SCGE assay has consistently grown in the last years, making the SCGE assay a field of great interest and application in genotoxicology studies (Collins et al. 2014).

Hence, the purpose of this study was to evaluate the acute toxicity of two herbicide commercial formulations, namely, the 57.71 % dicamba (DIC)-based Banvel® and the 48 % GLY-based Credit®, separately as well as the binary mixture

of these herbicides on *R. arenarum* late-stage larvae exposed under laboratory conditions employing a static acute experimental method. Whereas mortality was used as an endpoint for determining acute lethal effects, the SCGE assay was employed as genotoxic endpoint to study sublethal effects on circulating blood cells. Furthermore, whether the test substances as a mixture had any additive, synergistic, or antagonistic effects was also investigated. These herbicides were selected because they are commonly used as agricultural chemicals with intensive and overlapping applications in agricultural fields, not only in Argentinean soybean crops (www.senasa.gov.ar), but also around the world in many applications for the treatment of transgenic and nontransgenic agronomic crops (Behrens et al. 2007; Green et al. 2008).

Material and methods

Chemicals

Agrochemicals used included the 57.71 % dicamba-based (3, 6-dichloro-2-methoxybenzoic acid; CAS 1918-00-9) commercial grade trade formulation Banvel® (kindly provided by Syngenta Agro S.A., Buenos Aires, Argentina) and the 48 % isopropylamine salt of glyphosate-based [N-(phosphonomethyl] glycine; CAS1071-83-6) commercial grade trade formulation Credit® (Dow AgroSciences Argentina S.A.). Cyclophosphamide (CP; CAS 6055-19-2) was purchased from Sigma-Aldrich Co. (St. Louis, MO), whereas $K_2Cr_2O_7$ [Cr(VI); CAS 7778-50-9] was obtained from Merck KGaA (Darmstadt, Germany). All other chemicals and solvents of analytical grade were purchased from Sigma-Aldrich Co.

Quality control

Concentration levels of DIC and GLY in test solutions were analyzed by QV Chem Laboratory (La Plata, Buenos Aires, Argentina) according to U.S. Geological Survey Report 01-4134 (Furlong et al. 2011) and OSHA Analytical Method PV2067, respectively. Analyte levels were estimated by high-performance liquid chromatography using an ultraviolet detector and derivatization with fluorenylmethyloxycarbonyl. Active ingredient samples from test solutions (100.0 and 720.0 mg L^{-1} for Credit® and Banvel®, respectively) correspond to values obtained immediately after preparation (0 h) and 24 h thereafter. The detection limits were 0.5 and 0.2 mg L^{-1} for DIC and GLY, respectively.

Anuran late-stage larvae

R. arenarum late-stage larvae were selected as test organisms. *R. arenarum*, the Argentine toad, also called the Argentine common toad or the common South American toad, is a terrestrial and freshwater anuran species in the family Bufonidae found in Argentina, southern Brazil, Uruguay, Bolivia, and possibly Paraguay, from sea level up to 2600 m asl. This species is easy to handle and acclimate to laboratory conditions (Kwet et al. 2004). 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's classification (Gosner 1960). Hatchlings were collected with the permission of the Flora and Fauna Direction from the Buenos Aires Province (Buenos Aires, Argentina; code 22500-22339/13), and experimental procedures were approved by the Ethical Committee of the National University of La Plata (codes 11/N699 and 11/N746). Hatchlings were transported to the laboratory and then acclimatized to 16 h/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. Specimens were kept in the laboratory until individuals reached Gosner development stage 36 (range, 35–37) and were then used for the 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, 6.4 ± 0.3 mg L^{-1}; conductivity, 994 ± 8.5 μS/cm; hardness, 142 ± 21.5 mg $CaCO_3$ L^{-1}.

Determination of LC50

Experiments for toxicity assessment were performed following the recommendations proposed by the U.S. EPA (1975, 1982, 2002) and ASTM (2007) standardized methods with minor modifications reported previously for native species (Nikoloff et al. 2014c; Pérez-Iglesias et al. 2014; Ruiz de Arcaute et al. 2014; Vera Candioti et al. 2010). Experiments were performed using ten late-stage larvae at Gosner development stage 36 (range, 35–37) for each experimental point, maintained in a 1-L glass container, and exposed to ten different concentrations of Banvel® (concentration range, 96.0–720.0 mg L^{-1}) and Credit® (concentration range, 50.0–100.0 mg L^{-1}) for 96 h. Negative (dechlorinated tap water; pH, 7.5 ± 0.1; hardness, 142 ± 21.5 mg $CaCO_3$ L^{-1}) and positive controls [23 mg L^{-1} Cr(VI)-treated late-stage larvae] were conducted and run simultaneously with Credit®- and Banvel®-exposed late-stage larvae. All test solutions were prepared immediately before use and replaced every 24 h. Late-stage larvae 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 late-stage larvae with a glass rod. Experiments were performed in quadruplicate and run simultaneously for each experimental point.

Single-cell gel electrophoresis assay

Experiments were performed using five late-stage larvae at Gosner development stage 36 (range, 35–37) for each experimental point, maintained in 1-L glass containers and exposed to two different concentrations of the test compounds equivalent to 5 and 10 % of the corresponding $LC50_{96\ h}$ values, either alone or in their mixtures. To achieve these concentrations, late-stage larvae were exposed to 17.92 and 35.84 mg L^{-1} Banvel® and 3.91 and 7.82 mg L^{-1} Credit® (see "Determination of LC50" section). Negative (dechlorinated tap water; see "Determination of LC50" section) and positive controls (40 mg L^{-1} CP) were conducted and run simultaneously with Credit®- and Banvel®-exposed late-stage larvae. All test solutions were prepared immediately before each experiment. Experiments were performed in triplicate and run simultaneously for each experimental point. Late-stage larvae were euthanized 96 h after initial treatment according to the American Society of Ichthyologists and Herpetologists (ASIH 2004) criteria. At the end of each experiment, late-stage larvae were anesthetized by immersion in ice water, and blood samples were obtained by sectioning behind the operculum. 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. Aliquots of 30 μL of the diluted samples were mixed with 70 μL of 0.5 % low-melting-point agarose and was then layered on slides precoated with 100 μL of 0.5 % normal-melting-point agarose. The slides were covered with coverslips and placed at 4 °C for 10 min. After solidification, the coverslips were removed, and the slides were covered with a third layer of 50 μL of 0.5 % low-melting-point agarose. After solidification, the coverslips were removed, and the slides were immersed in ice-cold freshly prepared lysis solution (1 % sodium sarcosinate, 2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, pH 10.0, 1 % Triton X-100, 10 % 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 five classes (0–I, undamaged; II, minimum damage; III, medium damage; IV, maximum damage), as suggested previously (Cavas and Konen 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) using the formula GDI = [I (I) + 2 (II) + 3 (III) + 4 (IV) / N (0–IV)], where 0–IV represent the nucleoid type, and N_0–N_{IV} represent the total number of nucleoids scored.

Statistical analysis

Mortality data were analyzed using the U.S. EPA Probit Analysis statistical software, version 1.5 (http://www.epa.gov/nerleerd/stat2.htm), and based on the method of Finney (1971), or by the linear interpolation method when the Probit was not applicable (U.S. EPA 1989). Statistical analyses were performed using the Statgraphics Plus software, version 5.1. SCGE data were analyzed by factorial ANOVA with Tukey's test to determine significant differences between factor levels. ANOVA assumptions were corroborated with Barlett's test for homogeneity of variances and a χ^2 test for normality. Concentration-response (*C-R*) curves at 96 h were estimated with their 95 % confidence limits. Regression (a and b) and correlation (r) coefficients were calculated for each *C-R* curve. Tests determined the significance of the regressions and correlation coefficients. The level of significance was 0.05 for all tests unless indicated otherwise.

Results

Chemical analysis

Results of chemical analyses showed no significant changes ($P > 0.05$) in the concentration of the pure analyte in treatments with 24 h interval renewals of the testing solutions (concentration range, 97 ± 5 % recovery). Concentrations assessed throughout the study represent the nominal concentrations of active ingredient present within the DIC-based and GLY-based formulations Banvel® and Credit®, respectively.

Lethal endpoints

Probit analysis of the mortality data allowed determination of the LC50 values of DIC and GLY present within the formulations Banvel® and Credit® after 24, 48, 72, and 96 h of exposure, respectively.

For the DIC-based herbicide formulation Banvel®, results revealed the following mean LC50 values: 24 h, 742.08 mg L^{-1} (range, 514.97–1069.38 mg L^{-1}); 48 h, 525.05 mg L^{-1} (range, 506.69–548.62 mg L^{-1}); 72 h, 484.10 mg L^{-1} (range, 469.73–501.93 mg L^{-1}); and 96 h, 358.44 mg L^{-1} (range, 340.11–375.74 mg L^{-1}). Overall, LC50 values were directly affected by the exposure time ($r = -0.96$, $P < 0.05$).

Similarly, mortality data yielded concentrations of GLY equivalent to 89.44 mg L^{-1} (range, 82.68–96.36 mg L^{-1}), 85.96 mg L^{-1} (range, 65.02–113.66 mg L^{-1}), 82.08 mg L^{-1} (range, 80.16–92.20 mg L^{-1}), and 78.18 mg L^{-1} (range, 75.77–81.22 mg L^{-1}) for the 24-, 48-, 72-, and 96-h LC50 values, respectively. As revealed by regression analysis, LC50 values were directly affected by the exposure time ($r = -0.99$, $P < 0.001$).

Sublethal endpoints: DNA damage

The SCGE assay results obtained in peripheral blood cells of *R. arenarum* late-stage larvae exposed for 96 h to Banvel®, Credit®, and their binary mixtures are presented in Table 1 and Fig. 1. While the mean frequencies of different nucleoid damage categories and total damage induction are summarized in Table 1, the genetic damage indexes are depicted in Fig. 1. CP treatment (positive control) induced an enhancement in the frequency of damaged nucleoids as well as in the GDI compared to negative controls ($P < 0.001$) (Table 1 and Fig. 1). Such alteration was due to an enhanced frequency of type II ($P < 0.01$), III ($P < 0.001$), and IV nucleoids ($P < 0.001$) and a concomitant decrease of type 0–I nucleoids ($P < 0.001$; Table 1).

DNA damage in dicamba-based herbicide-treated late-stage larvae

In late-stage larvae treated with the DIC-based formulation Banvel®, an equal significant increase of the GDI was observed compared to negative control values regardless of the concentration of the herbicide assayed ($P < 0.001$; Fig. 1). The jeopardizing effect was due to an enhanced frequency of type II ($P < 0.001$) and IV nucleoids ($P < 0.001$) and a concomitant decreased frequency of type 0–I nucleoids ($P < 0.001$; Table 1). In addition, a significant increase in the frequency of type III nucleoids in late-stage larvae treated with 10 % of the Banvel® $LC50_{96\ h}$ concentration was observed ($P < 0.01$; Table 1).

Table 1 Analysis of DNA damage measured by comet assay in peripheral blood cells of *Rhinella arenarum* late-stage larvae exposed to Banvel® and Credit®

Chemicals	Number of organisms observed	Number of cells analyzed	Nucleoid categories, % ± SE				% of damaged cells (II + III + IV)
			Type 0 + I	Type II	Type III	Type IV	
Negative control	15	1835	89.81 ± 14.23	8.12 ± 1.49	2.02 ± 0.59	0.05 ± 0.06	10.19
Banvel®							
5 % $LC50_{96\ h}$	15	1839	44.97 ± 7.97***	45.02 ± 5.63***	6.31 ± 2.09	3.70 ± 0.91***	55.03***
10 % $LC50_{96\ h}$	15	1799	44.47 ± 8.12***	41.75 ± 4.62***	7.17 ± 1.49**	6.61 ± 1.47***	55.53***
Credit®							
5 % $LC50_{96\ h}$	15	1838	41.02 ± 7.36***	53.26 ± 4.88***	3.26 ± 0.72	2.45 ± 1.08	58.98***
10 % $LC50_{96\ h}$	15	1745	30.49 ± 5.79***	57.94 ± 3.87***	7.34 ± 1.24**	4.24 ± 0.83***	69.51***
Mixture							
5 % $LC50_{96\ h}$ Banvel® + 5 % $LC50_{96\ h}$ Credit®	15	1881	21.21 ± 4.03***	61.94 ± 3.18***	11.22 ± 2.73***	5.64 ± 1.21***	78.79***#
10 % $LC50_{96\ h}$ Banvel® + 10 % $LC50_{96\ h}$ Credit®	15	1749	8.75 ± 1.57***	75.36 ± 2.37***	7.55 ± 1.91**	8.35 ± 1.57***	91.25***#
Positive control[a]	15	2010	10.20 ± 3.12***	31.09 ± 7.33**	24.13 ± 4.89***	34.58 ± 6.02***	89.8***

**$P < 0.01$; ***$P < 0.001$, significant differences with respect to negative control values; #$P < 0.001$, significant differences with respect to herbicide alone exposed tadpole values

[a] Cyclophosphamide (CP, 40 mg L^{-1}) was used as positive control

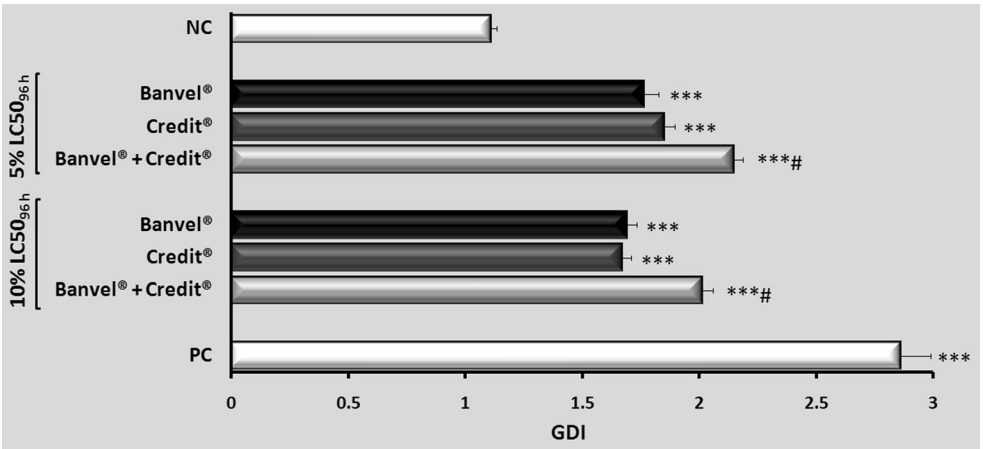

Fig. 1 DNA damage induced by dicamba-based Banvel® (*black bars*) and glyphosate-based Credit® (*dark gray bars*), evaluated by the single-cell gel electrophoresis assay, on *Rhinella arenarum* late-stage larvae under laboratory conditions. Late-stage larvae were exposed for 96 h to either 5 and 10 % Banvel® $LC50_{96\ h}$ concentrations or 5 and 10 % Credit® $LC50_{96\ h}$ concentrations and to the binary mixtures (*light gray bars*) of 5 % Banvel® plus 5 % Credit® $LC50_{96\ h}$ concentrations and 10 % Banvel® plus 10 % Credit® $LC50_{96\ h}$ concentrations. Results are expressed as pooled data of genetic damage indexes from three independent experiments. Negative (*NC*, untreated late-stage larvae) and positive controls (*PC*, 40 mg L^{-1} cyclophosphamide-treated late-stage larvae) (*white bars*) were conducted and run simultaneously with herbicide-exposed late-stage larvae. ***$P < 0.001$ (significant differences with respect to negative control values). $^{\#}P < 0.001$ (significant differences with respect to herbicide alone-exposed late-stage larvae values)

DNA damage in glyphosate-based herbicide-treated late-stage larvae

In late-stage larvae treated with the GLY-based formulation Credit®, an equal significant increase of the GDI was observed compared to negative control values regardless of the concentration of the herbicide assayed ($P < 0.001$; Fig. 1). For both concentrations, such alteration was due to an enhanced frequency of type II ($P < 0.001$) and a concomitant decreased frequency of type 0–I nucleoids ($P < 0.001$; Table 1). In addition, a significant increase in the frequency of type III ($P < 0.01$) and type IV nucleoids ($P < 0.001$) in late-stage larvae treated with 10 % of the Credit® $LC50_{96\ h}$ concentration was observed (Table 1).

DNA damage in dicamba- plus glyphosate-based herbicide-treated late-stage larvae

The mixtures of Banvel® and Credit® induced a significant increase of the GDI compared to negative control values as well as to those for late-stage larvae exposed only to Banvel® or Credit® ($P < 0.001$; Fig. 1). For both mixture concentrations, such observation was due to an enhanced frequency of type II, III, and IV nucleoids and a concomitant decrease of type 0–I nucleoids ($P < 0.001$; Table 1). Furthermore, no statistically significant differences in the GDI were observed between late-stage larvae exposed to Banvel® plus Credit® at 5 % $LC50_{96\ h}$ concentrations and those exposed to Banvel® plus Credit® at 10 % $LC50_{96\ h}$ concentrations ($P > 0.05$; Fig. 1). Overall, treatment with Banvel® plus Credit® at 5 % $LC50_{96\ h}$ concentrations induced equivalent 1.19- and 1.20-fold increases of the GDI over those induced by 5 % Banvel® $LC50_{96\ h}$ and 5 % Credit® $LC50_{96\ h}$ treatments, respectively. Similarly, 1.22- and 1.16-fold increases in the GDI were observed in late-stage larvae exposed to the mixture comprising Banvel® plus Credit® at 10 % $LC50_{96\ h}$ concentrations compared to those induced by 10 % Banvel® $LC50_{96\ h}$ and 10 % Credit® $LC50_{96\ h}$ concentrations, respectively (Fig. 1).

Discussion

R. arenarum is listed as one of the least concerns in view of its wide distribution, tolerance of a broad range of habitats, and presumed large population, and because it is unlikely to be declining fast enough to qualify for listing in a more threatened category (Kwet et al. 2004). Previous studies highlighted that these anuran frog late-stage larvae can be considered appropriate reference organisms for the evaluation and quantification of genomic instability in aquatic environments (such as polluted aquatic areas and agroecosystems) induced by pesticides and other emerging pollutants (for review, see http://cfpub.epa.gov/ecotox/ and references therein). Among these, cyclophosphamide; heavy metals; nonylphenol; bisphenol; epichlorohydrin; polyaniline nanoparticles and nanofibers; the fungicide trifloxystrobin; the insecticides pirimicarb, chlorpyrifos, cypermethrin, diazinon, carbaryl, azinphos-methyl, and endosulfan; and the herbicides 2,4-D, glufosinate-

ammonium, picloram, bispyribac-sodium, glyphosate, metsulfuron-methyl, flurochloridone, as well as atrazine, can be included (for review, see http://cfpub.epa.gov/ecotox/ and references therein).

Regarding the acute lethal effects of the herbicides obtained in the current study, DIC and GLY tested herbicides could be ranked, according to the scoring used by the Office of Pollution Prevention and Toxics of the U.S. EPA (2001), as compounds with moderate/medium and low ecotoxicity concerns, respectively, for larvae of *R. arenarum* at late-stage larvae. Additionally, DIC and GLY can be classified as harmful compounds for aquatic organisms (category III) following the classification criteria proposed by the European Union directives (UN 2011). Furthermore, whereas GLY can be ranked also as a harmful chemical, DIC should be considered a compound that may cause long-term adverse effects in the aquatic environment, according to the hazard risk assessment categories of the European Union directives (Mazzatorta et al. 2002).

It is very well known that pesticide-induced toxicity is a phenomenon that varies among different amphibian species, not only for the same active ingredient but also for different commercially available formulations based on the same active ingredient (Nikoloff et al. 2014c; Pérez-Iglesias et al. 2014, 2015; Relyea 2009; Ruiz de Arcaute et al. 2014; Vera Candioti et al. 2010). Our current results reveal a concentration of 358.44 mg L^{-1} (confidence limit, 340.11–375.74 mg L^{-1}) as the LC50 value for *R. arenarum* late-stage larvae when exposed to DIC for 96 h. To the best of our knowledge, acute lethality data of DIC have not been reported so far for *R. arenarum* late-stage larvae. Accordingly, this study represents the first experimental evidence of the acute lethal effects exerted by DIC on late-stage larvae of a Neotropical anuran species native to Argentina.

However, a different scenario can be envisioned for the case of GLY formulations. It is known that the acute toxicity of GLY is considered to be very low by the World Health Organization (WHO-FAO 1997) and depends greatly on the coadjuvants, such as surfactants, humectants, and dispersants, always included in technical formulations, to enhance and improve its adsorption and effectiveness (Mann et al. 2009; Wagner et al. 2013). Very recently, in March 2015, the World Health Organization's International Agency for Research on Cancer published a summary of its forthcoming Monograph 112, including GLY, which was classified as "probably carcinogenic in humans" (category 2A) based on epidemiological, animal, and in vitro studies (Guyton et al. 2015).

Since the expiration in 2000 of GLY patent protection from Monsanto Agricultural Products Co (St. Louis, MI), several new GLY-based herbicides have been released into the market worldwide (http://www.pesticideinfo.org). Numerous reports agree in demonstrating that commercial GLY-based formulations are more acutely toxic than the pure herbicide to aquatic organisms (Cedergreen and Streibig 2005; Peixoto 2005; Pereira et al. 2009; Sobrero et al. 2007). It has been observed that formulations containing trimethylsulfonium salt of glyphosate, e.g., Avans®, are more toxic to aquatic organisms than those in which GLY is present as an isopropylamine salt, e.g., Roundup Original® (Pettersson and Ekelund 2006). Furthermore, laboratory studies demonstrated for the latter, the first GLY-based herbicide to be commercialized all over the world, that the toxicity is largely due the presence of the surfactant polyethoxylated tallow amine (POEA). The surfactant POEA is the principal toxic component in the GLY-based formulation Roundup Original®, and several reports demonstrate that this product may have a toxicity several times higher than GLY itself, making the formulated mixture of greater toxicity than both the active ingredient and GLY-based formulations not containing POEA (Bolognesi et al. 1997; Moore et al. 2012; Tsui and Chu 2003, 2008). In agreement with these observations, reported previously for *R. arenarum* late-stage larvae exposed to several POEA-free GLY-based formulations were LC50 values 20–26 times greater than the values normally reported in late-stage larvae exposed to Roundup Original® (Brodeur et al. 2014; Lajmanovich et al. 2011). Furthermore, the 96-h LC50 value of 78.18 mg L^{-1} (range, 75.77–81.22 mg L^{-1}) calculated for the 48 % GLY-based formulation Credit® is consistent with LC50 values reported previously for the same anuran species and POEA-free GLY-based formulations, e.g., Infosato®, Glifoglex®, and C-K Yuyos FAV® (Brodeur et al. 2014; Lajmanovich et al. 2011).

Previous studies agree in demonstrating that the SCGE assay represents an extensively valuable biomarker in amphibians to monitor contaminated areas (in situ assay) (Burlibasa and Gavrila 2011; Maselli et al. 2010; Meza-Joya et al. 2013) as well as for xenobiotic screening after direct or indirect exposure (in vivo assay) (Meza-Joya et al. 2013; Mouchet et al. 2007; Pérez-Iglesias et al. 2014, 2015; Ruiz de Arcaute et al. 2014; Vera Candioti et al. 2010). Our current findings are in accord with the previous concept and highlight that the SCGE assay is a highly sensitive method for the detection of DNA damage induced by environmental pollutants in aquatic organisms. Although reports of the frequency of agrochemical-induced DNA single-strand breaks are well documented by an extensive bibliography worldwide (Feng et al. 2004; Mouchet et al. 2007; Ralph and Petras 1997; Yin et al. 2008), data for Argentinean amphibian species are scarce. Recently, we used *R. arenarum* late-stage larvae to evaluate the lethal and sublethal toxicity of flurochloridone-based commercial herbicide formulations (Nikoloff et al. 2014c). Similarly, the jeopardizing effects of the insecticide imidacloprid and an imidacloprid-based

formulation (Pérez-Iglesias et al. 2014; Ruiz de Arcaute et al. 2014), as well as the imazethapyr-based herbicide formulation on Montevideo tree frog *Hypsiboas pulchellus* late-stage larvae were reported (Pérez-Iglesias et al. 2015). Based on the results obtained in this study with the commercial DIC-based herbicide formulation Banvel®, it can be demonstrated that, regardless of the length of the treatment within the 17.92- to 35.8-mg-L^{-1} concentration range, the two tested concentrations increased the frequency of DNA strand breaks. To the best of our knowledge, no previous studies have addressed the induction of DNA strand breaks exerted by DIC on amphibians. Accordingly, our current results represent the first in vivo evidence using the SCGE assay of the induction of primary DNA damage exerted by DIC on erythrocytes of an anuran species, *R. arenarum* late-stage larvae.

It is known that most pesticides can chemically interact when combined in mixtures (Baas et al. 2010; Rodney et al. 2013). Sometimes, the metabolism of one of compound may interfere with the metabolism of the other chemical(s) present in the mixture, causing several side effects in living organisms, both target and nontarget species (Belden and Lydy 2000). This interference can be exerted during the absorption, distribution, or metabolism or at the site of toxic action and excretion of the compound (Baas et al. 2010; Rodney et al. 2013). It is known that pesticides, when applied in mixtures, can interact to have additive, synergistic, antagonistic, or enhancement effects (Blouin et al. 2004). According to McCarty and Borgert (2006), there are several models to determine the interaction between toxic compounds. However, the simplest, and also considered the best, model proposed commonly accepts that synergistic effects, where chemicals interact to produce a higher response than that obtained from each of the individual agrochemicals, may increase weed or other pest management. On the other hand, antagonistic effects, where interactions reduce the effect exerted by an individual agrochemical, can lead, then, to the reduction of the effectiveness of each of the compounds. Our results clearly demonstrate that the binary mixtures of a 5 % Banvel® $LC50_{96\ h}$ concentration plus 5 % Credit® $LC50_{96\ h}$ concentration and 10 % Banvel® $LC50_{96\ h}$ concentration plus 10 % Credit® $LC50_{96\ h}$ concentration induced equivalent significant increases of the GDI over values obtained when late-stage larvae were exposed only to Banvel® or Credit®, regardless of the concentration of the active ingredient present in the combination. Accordingly, a synergistic effect of the mixture of DIC and GLY on the induction of primary DNA strand breaks on circulating blood cells of *R. arenarum* late-stage larvae could be demonstrated. Similar observations were recently reported by Olszyk et al. (2015) on the effect of a GLY and DIC herbicide tank mixture on native and nongenetically engineered soybean seedlings when analyzing plant growth. In general, plant growth responses to combinations of GLY and DIC were less than the sum of growth responses to each individual herbicide (Olszyk et al. 2015).

Although studies on the toxic effects induced by the binary mixture of the herbicides DIC and GLY have not yet been performed, some authors have investigated the combination of GLY with other pesticides and reported synergistic effects for most of them (Brodeur et al. 2014). For example, Tatum et al. (2012) evaluated the toxicity of the GLY in combination with the herbicides imazapir and triclopyr and a modified vegetable oil surfactant in the water flea *Ceriodaphnia dubia* (Cladocera, Daphniidae) and in fathead minnow fish *Pimephales promelas* (Cypriniformes, Cyprinidae) at environmentally relevant concentrations. The results revealed that the mixture of these chemicals had a slight synergism on LC50 values for both invertebrate models employed (Tatum et al. 2012). Similarly, Santos et al. (2011) evaluated the combination of the binary mixture of GLY and the insecticide dimethoate in two invertebrates, the earthworm *Eisenia andrei* (Annelida, Lumbricidae) and the woodlouse *Porcellionides pruinosus* (Isopoda, Porcellionidae) and in seeds of the field mustard *Brassica rapa* (Brassicales, Brassicaceae). Regarding germination success, a synergistic effect was observed at a realistic binary mix concentration, but an antagonistic effect was observed at 5 and 10 times the field concentration (Santos et al. 2011). When enzymatic biomarkers were analyzed, a similar synergistic pattern in earthworm and isopods was observed only after exposure to 5 and 10 times the field concentration (Santos et al. 2011). Equitoxic and nonequitoxic mixtures of GLY-based and the insecticide cypermethrin-based commercial formulations were significantly synergic in both combinations, affecting the mortality of *R. arenarum* late-stage larvae (Brodeur et al. 2014). However, in a previous study, the common wheat *Triticum aestivum* was exposed to GLY in binary mixtures with two organophosphorous compounds at concentrations around the respective EC50 values, and mostly antagonistic interactions were reported (Bielecki et al. 2004).

Based on the results obtained in our current study, it could be verified and demonstrated that DIC and GLY alone did interact with DNA and lead to primary strand break lesions in peripheral blood cells on the nontarget species *R. arenarum* when late-stage larvae were exposed for a short treatment lasting no more than 96 h. In mixture, both herbicides continued to induce DNA damage in the cells assayed at higher frequencies than those observed for each pesticide alone, thereby indicating that these herbicides act synergistically when applied together. Furthermore, although speculative, the fact that early-stage anuran larvae are generally sensitive to pesticide exposure compared to later (Jones et al. 2010), that a short-time exposure of late-stage larvae can enhance mortality at metamorphosis (Wagner et al. 2015), and finally that bufonids are often more resistant to pesticides compared

to other anuran families (Jones et al. 2009) can speak on behalf of the results of the present study gained with late-stage larvae of a bufonid species as "best-case-scenario" for anurans exposed to the tested formulations and their mixtures.

The environment modifications are generally accepted to be a major causal factor in the decline of amphibian populations (Beebee 2005; Jones et al. 2009). Under realistic conditions, amphibians as well as other nontarget organisms are exposed to different mixtures of agrochemicals by regular discharge into the environment (Lydy et al. 2004). The knowledge of the relationship between two or more agrochemicals effects at individual level is a key for knowing the dynamics in amphibian populations. Thus, genotoxic studies evaluating their joint effects are helpful to predict genotoxicity patterns that may occur, e.g., when DIC and GLY are present in the environment. Our current results highlight that the comet assay methodology reveals DNA damage exerted by DIC and GLY higher than expected if one regards the genotoxicity of both herbicides acting individually. The high level of DNA damage inflicted by the mixture of DIC and GLY may give rise to a higher level of deleterious mutations that can negatively influence several processes in organisms exposed, including uptake, metabolization, and excretion, among others determining the tadpoles' survival (Beebee 2005; Jones et al. 2009). Because mixtures of DIC and GLY herbicides produce synergistic DNA damage, they could magnify these effects inside freshwater amphibian populations.

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Compliance with ethical standards

Conflict of interest The authors declare that there are no conflicts of interest.

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