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# DNA damage exerted by mixtures of commercial formulations of glyphosate and imazethapyr herbicides in *Rhinella arenarum* (Anura, Bufonidae) tadpoles

Wanessa F. Carvalho[1,2] · Celeste Ruiz de Arcaute[1,3] · Juan Manuel Pérez-Iglesias[1,3] · Milagros R. R. Laborde[1,4] · Sonia Soloneski[1,3] · Marcelo L. Larramendy[1,3]

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## Abstract

Glyphosate (GLY) and imazethapyr (IMZT) are two herbicides commonly used worldwide, either alone or in mixtures. They represent key pesticides in modern agricultural management. The toxicity that results when employed as mixtures has not been characterized so far. Acute toxicity of the 48% GLY-based herbicide (GBH) Credit® and the 10.59% IMZT-based herbicide (IBH) Pivot® H alone and their binary combinations was analyzed in *Rhinella arenarum* tadpoles exposed in a semi-static renewal test. Lethal effects were determined using mortality as the end-point, whereas sublethal effects were determined employing the single-cell gel electrophoresis (SCGE) bioassay. Based on mortality experiments, results revealed $LC50_{96\,h}$ values of 78.18 mg/L GBH and 0.99 mg/L IBH for Credit® and Pivot® H, respectively. An increase in the genetic damage index (GDI) was found after exposure to Credit® or Pivot® H at 5 and 10% of $LC50_{96\,h}$ values. The combinations of 5% Credit®-5% Pivot® H $LC50_{96\,h}$ and 10% Credit®-10% Pivot® H $LC50_{96\,h}$ concentrations significantly enhanced the GDI in comparison with tadpoles exposed only to Credit® or Pivot® H. Thus, the effect of interaction between GBH and IBH inducing DNA damage in *R. arenarum* blood cells can be considered to be synergistic.

**Keywords** Amphibians · Credit® · Comet assay · Herbicide binary mixture · Pivot® H

## Introduction

Agricultural practices introduce large amounts of xenobiotics to move freely into diverse environmental compartments (Ippolito et al. 2015; Liaud et al. 2016), since only less than 0.1% of the total amount of agrochemicals applied around the world reach their targeted pests (Pimentel 1995). Thus, large amounts of different applied agrochemicals move into the different environmental compartments affecting negatively, not only their target organisms, but also non-target biota (Larramendy 2017a, 2017b; Meffe and de Bustamante 2014; Zhelev et al. 2018).

Currently, the use of mixtures of pesticides is a frequently employed approach to control weeds and manage the herbicide resistance, regardless of the possible effect that the mixture could exert on non-target organisms (Ge et al. 2014). Indeed, it has been suggested that the mixing combinations of pesticides should be considered as a noteworthy group of new stressors for the environment (Lydy et al. 2004). Furthermore, chemicals present in mixtures can cause complex changes rather than the toxic effects exerted by individual compounds (LeBlanc and Wang 2006; Silva et al. 2015; Soloneski et al. 2016; Svartz et al. 2016; Varona-Uribe et al. 2016). It is known that a toxicant mix may exert three categories of joint action,

These authors contributed equally: Wanessa F. Carvalho, Celeste Ruiz de Arcaute, Juan Manuel Pérez-Iglesias

✉ Marcelo L. Larramendy
marcelo.larramendy@gmail.com

[1] Cátedra de Citología, Facultad de Ciencias Naturales y Museo, Universidad Nacional de La Plata, Calle 64 N° 3, La Plata 1900, Argentina

[2] Departamento de Genética, Instituto de Ciências Biológicas, Universidade Federal de Goiás, Goiânia, Brazil

[3] Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Buenos Aires, Argentina

[4] Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT), Buenos Aires, Argentina

additivity, synergism and antagonism (Blouin et al. 2010; Brodeur et al. 2014, 2016; Calabrese 1995; Lydy et al. 2004; Ruiz de Arcaute et al. 2018; Soloneski et al. 2016). So far, the complex interactions between pesticides in combination and their toxic effects have been poorly studied. Thus, the analyses of the joined effects of pesticides in mixtures, has been suggested (Güngördü et al. 2016; Warne 2003).

Amphibians represent one of the most threatened and rapidly declining organisms globally (Brooks et al. 2016; Egea-Serrano et al. 2012; Ficetola and Maiorano 2016). One of the major causes of this negative impact on anuran species is the pesticide pollution observed in both natural and agricultural areas (Beebee 2005; Egea-Serrano et al. 2012; Jones et al. 2010; Mann et al. 2009; Sparling and Fellers 2009; Wagner et al. 2013, 2015). Anurans, in particular, are threatened by aquatic contamination due to their unprotected eggs and sensitive tegument. Moreover, their populations are adversely affected by habitat modifications, diseases and the presence of exotic species (Bradford et al. 2011; Brühl et al. 2011; Mann et al. 2009; Sparling and Fellers 2009).

*Rhinella arenarum* (Hensel 1867), a toad belonging to the family Bufonidae, inhabits the Argentinean humid Pampas where the application of pesticides is a common practice in crops of economic interest. The species reproduces in shallow, temporary and semi-temporary ponds or bogs formed within this agroecosystem. Agrochemical activities performed in these or neighboring fields may affect amphibians, including *R. arenarum* (Agostini et al. 2009; Babini et al. 2016). Based on the wide distribution of the species, its broad range of habitats and large populations, *R. arenarum* has been classified as least concerns (Kwet et al. 2004). Several studies throughout the literature have demonstrated that amphibians represent useful and valid environmental indicators for environmental monitoring (Larramendy 2017a, 2017b). In addition, several previous studies have highlighted that larvae from *R. arenarum* can be considered to be an appropriate biotic matrix for the evaluation and quantification of pesticide- and other pollutant-induced toxicity, and for estimation of genomic instability in aquatic environments. Among these xenobiotics, can be included several herbicides such as 2,4-dichlorophenoxyacetic acid, atrazine, glufosinate-ammonium, bispyribac-sodium, flurochloridone, glyphosate, metsulfuron-methyl and picloram, among others (USEPA 2017 and references therein).

Different end-points for evaluating both cytotoxicity and genotoxicity are used in aquatic vertebrates, including amphibians. Detection and quantification of damage induced in DNA by the single-cell gel electrophoresis (SCGE) assay is a recommended genetic biomarker to estimate genotoxicity and oxidative damage (Mouchet et al. 2006a, 2006b, 2007; Nikoloff et al. 2014b; Pérez-Iglesias et al. 2014, 2015, 2017, 2018). In amphibians, in particular, several reports show that the SCGE assay is a valuable technique for detecting the effects of pollution in different anthropized areas (Maselli et al. 2010; Meza-Joya et al. 2013; Ralph and Petras 1998), for screening for the harmful effects of xenobiotics (Mouchet et al. 2007; Nikoloff et al. 2014b; Pérez-Iglesias et al. 2014, 2015, 2017, 2018; Ralph and Petras 1998; Ruiz de Arcaute et al. 2014), and recently, for analysing the combined effects of xenobiotics when applied as mixtures (Soloneski et al. 2016).

The purpose of the current study was to examine the acute toxicity and genotoxicity of the 48% GLY-based herbicide (GBH) Credit® and the 10.59% IMZT-based herbicide (IBH) Pivot® H commercial products, used alone and as a binary mixture in *R. arenarum* tadpoles using a semi-static renewal test. The potential interactions resulting from the mixture of these herbicides, such as additivity, synergism, or antagonism, were evaluated.

## Material and methods

### Chemicals

Credit® and Pivot® H were employed for testing GLY [*N*-(phosphonomethyl) glycine; CAS1071-83-6] and IMZT [5-ethyl-2-(4-isopropyl-4-methyl-5-oxo-4,5-dihydroimidazol-1H-2-yl) nicotinic acid; CAS 81335-77-5], respectively. Credit® is a GBH commercial formulation containing 48% of isopropylamine salt (Dow AgroSciences Argentina S.A., Argentina). Pivot® H is a IBH trade product containing 10.59% of IMZT (BASF Argentina S.A., Argentina). The two-herbicide formulations used contained proprietary adjuvants of unknown identity, as the manufacturers did not provide this information. The concentrations informed throughout the study represent the nominal concentrations of GLY or IMZT contained in the formulations Credit® and Pivot® H, respectively. Cyclophosphamide (CP; CAS 6055-19-2) and all other analytical grade compounds were provided by Sigma-Aldrich Co. (St. Louis, MO), except $K_2Cr_2O_7$ [Cr(VI); CAS 7778-50-9] which was purchased from Merck KGaA (Darmstadt, Germany).

### Quality control

Concentration analyses of GLY and IMZT in the test solutions were verified by QV Chem Laboratory (La Plata, Buenos Aires, Argentina) employing HPLC following the OSHA Analytical Method PV2067 and U.S. Geological Survey Report 01-4134 (Furlong et al. 2011), respectively. Concentrations of analytes in 5 and 10% $LC50_{96\ h}$ test solutions were determined after preparation and at 24 h

thereafter. Detection limits were 0.2 mg/L and 0.5 µg/L for GLY and IMZT, respectively.

## Anuran tadpoles

*Rhinella arenarum* has a wide distribution in the Neotropical region, including the Southern cone of America. Tadpoles were collected from a temporary, uncontaminated pond near La Plata city (35°10′S, 57°51′W, Buenos Aires Province, Argentina) at stage 9 of development (GS9) (Gosner 1960). Hatches were collected with the permission of the Flora and Fauna Direction from the Buenos Aires Province (Buenos Aires, Argentina) (code 22500-22339/13) and the Ethical Committee from the National University of La Plata (code 11/N699, 11/N746, 11/N817 and 11/N847). Hatches were acclimated to laboratory conditions during at least 20 days in a 16/8 light-dark cycle with dechlorinated tap water until the beginning of the experiment as reported previously (Nikoloff et al. 2014b; Soloneski et al. 2016). Water physicochemical parameters (mean ± SE) were: 20 ± 1 °C; pH, 7.55 ± 0.1; 6.3 ± 0.3 mg/L dissolved oxygen; <0.2 mg/L $NH4^+$; 143 ± 23.5 mg/L $CaCO_3$. Individuals were cared following the recommendations reported for native species by Cei (1980), Kehr (1987, 1989) and Natale et al. (2006). Once tadpoles reached GS36 (range 35–37), acute lethal and sublethal tests were conducted to determine mortality and DNA single-strand breaks.

## Determination of LC50 values

LC50 values for GBH and IBH commercial formulations Credit® and Pivot® H, respectively, were determined after 24, 48, 72, and 96 h of exposure by Probit analyses.

Acute toxicity of IBH was calculated according standardised methods (USEPA 1975, 1982, 1989, 2002) with minor adaptations previously reported (Ruiz de Arcaute et al. 2014; Soloneski et al. 2016; Vera-Candioti et al. 2010). Experiments were performed using 10 tadpoles at GS36 (range 35–37) for each experimental point, maintained in a 1 L glass container as reported elsewhere (Natale et al. 2018; Nikoloff et al. 2014b; Pérez-Iglesias et al. 2018; Soloneski et al. 2016), and exposed to eight different concentrations of IBH ranging from 0.50 to 4.00 mg/L IMZT during 96 h equivalent to 0.50, 0.75, 1.00, 1.50, 3.00, 3.50, 3.75 and 4.00 mg/L. Negative (dechlorinated tap water; pH 7.5 ± 0.1; hardness, 143 ± 23.5 mg/L $CaCO_3$) and positive controls [23 mg/L Cr(VI)-treated tadpoles] were conducted and run simultaneously with IBH-exposed tadpoles. All test solutions were prepared immediately before use and replaced every 24 h. Tadpoles were not fed throughout the experiment. Dead tadpoles were removed and survival was evaluated daily by visual observation. 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.

## Single-cell gel electrophoresis assay

Experiments were performed using 10 larvae at GS36 (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, tadpoles were exposed to Credit® at 3.91 and 7.82 mg/L and Pivot® H at 0.05 and 0.10 mg/L, respectively (see Section 2.4). Dechlorinated tap water and 40 mg/L CP were employed as negative and positive control, respectively, and run in parallel with the GBH- and IBH-exposed tadpoles. After 96 h of exposure, tadpoles were immersed in ice water (ASIH 2004), and an aliquot of blood was obtained by sectioning behind the operculum. The alkaline SCGE assay was carried out as suggested by Singh (1996), with modifications for anuran larvae reported elsewhere (Pérez-Iglesias et al. 2014, 2015, 2017, 2018; Soloneski et al. 2016). 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 (Çavaş 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 represents the nucleoid type, and $N_0 - N_{IV}$ represent the total number of nucleoids scored. Experiments were performed in triplicate and run simultaneously for each experimental point.

## Statistical analysis

A *t*-test was performed for comparisons in chemical analyses. Mortality data were analyzed using the U.S. EPA Probit Analysis statistical software, version 1.5 (USEPA 2002), based on Finney's method (Finney 1971). Statistica software version 7.0 (StatSoft, OK) was employed for other statistical analyses. The proportion of individuals affected per test chamber was calculated for lethal and sublethal endpoints (mortality, damaged cells and GDI). Each proportion was angular transformed and a one-way ANOVA with Dunnett's test was performed (Zar 2010), whereas a one-way ANOVA with Tukey's test was performed for

**Table 1** Analysis of DNA damage measured by comet assay in peripheral blood erythrocytes of *Rhinella arenarum* cells exposed to GBH formulation Credit® and IBH formulation Pivot® H[a]

| Chemicals | Number of animals observed | Number of cells analysed | Nucleoids Categories % ± SE | | | | % of damaged cells (II + III + IV) |
|---|---|---|---|---|---|---|---|
| | | | Type 0 + I | Type II | Type III | Type IV | |
| Negative control | 10 | 1012 | 84.49 ± 9.32 | 10.47 ± 1.47 | 2.87 ± 0.59 | 2.17 ± 0.72 | 15.51 ± 1.56 |
| GBH | | | | | | | |
| 5% $LC50_{96\ h}$ | 10 | 1114 | 43.90 ± 7.22***## | 41.74 ± 10.01*** | 8.26 ± 1.81### | 6.10 ± 2.12### | 56.10 ± 8.23***## |
| 10% $LC50_{96\ h}$ | 10 | 1250 | 32.00 ± 7.59***## | 54.32 ± 4.36***## | 8.00 ± 1.66# | 5.68 ± 1.76### | 68.00 ± 6.52***## |
| IBH | | | | | | | |
| 5% $LC50_{96\ h}$ | 10 | 1154 | 31.98 ± 7.41*** | 39.51 ± 5.54*** | 17.94 ± 3.56** | 10.57 ± 5.15## | 68.02 ± 9.42*** |
| 10% $LC50_{96\ h}$ | 10 | 920 | 18.59 ± 8.10*** | 25.76 ± 6.96*# | 27.93 ± 6.29*** | 27.72 ± 10.31**## | 81.41 ± 7.78*** |
| Mixture | | | | | | | |
| 5% $LC50_{96\ h}$ GBH + 5% $LC50_{96\ h}$ IBH | 10 | 1079 | 14.37 ± 3.28*** | 27.53 ± 4.36* | 29.00 ± 5.27*** | 29.10 ± 6.92*** | 85.63 ± 5.43*** |
| 10% $LC50_{96\ h}$ GBH + 10% $LC50_{96\ h}$ IBH | 10 | 1066 | 2.35 ± 1.35*** | 8.16 ± 1.41 | 24.20 ± 4.81*** | 65.29 ± 6.21*** | 97.65 ± 9.37*** |
| Positive control[b] | 10 | 1230 | 19.76 ± 5.37*** | 37.80 ± 9.53** | 24.23 ± 4.89*** | 18.21 ± 8.32 | 80.24 ± 8.22*** |

[a]GBH, Glyphosate-based herbicide; IBH, Imazethapyr-based herbicide

[b]Cyclophosphamide (CP, 40 mg/L) was used as positive control

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; significant differences with respect to negative control values

#$P < 0.05$; ##$P < 0.01$; ###$P < 0.001$; significant differences with respect to herbicide alone exposed tadpole values

comparison between negative control data. ANOVA assumptions were corroborated with Barlett's test for homogeneity of variances and a $\chi^2$ test for normality. In cases which did not perform the assumptions of normality a Kruskal-Wallis test was made. The relationships between time and GDI data were evaluated by simple linear regression and correlation analyses. Concentration-response (C-R) curves at 96 h were estimated with their 95% confidence limits. Regression and correlation coefficients were calculated for each C-R curve. The relationship between concentration and GDI data was analysed using simple linear regression and correlation analyses. For all tests, the criterion for significance was 0.05.

## Results

### Chemical analysis

No significant variation was observed between concentrations of the pure compound ($P = 0.94$; $t$-value = 0.14) in the test solutions after 24 h (concentration range 97 ± 5% recovery) indicating the stability of the analyte over evaluated period.

### Lethal end-points

Acute toxicity of the GBH Credit® formulation on *R. arenarum* has been reported previously by Soloneski et al. (2016). In mortality experiments, LC50 values for GLY of 89.44 mg/L (82.68–96.36 mg/L), 85.96 mg/L (65.02–113.66 mg/L), 82.08 mg/L (80.16–92.20 mg/L) and 78.18 mg/L (75.77–81.22 mg/L) were reported after 24, 48, 72 and 96 h of exposure, respectively. Results revealed a significant time-dependent increase in lethality when the time of exposure increased from 24 to 96 h ($r = -0.99$; $P < 0.001$) (Soloneski et al. 2016).

Mortality data for IBH showed the same LC50 value of 1.0 mg/L IMZT (0.91–1.12 mg/L) after 24–72 h of exposure, whereas the LC50 value after 96 h of exposure was 0.99 mg/L IMZT (0.91–1.08 mg/L). Finally, the LC50 was found to be time-independent, ($r = -0.77$; $P > 0.05$).

### Sublethal end-points: DNA damage

Exposure to CP (Positive control) increased the frequency of damaged nucleoids ($P = 0.00$; F = 21.79) as well as the GDI value in regard to negative control values ($P = 0.00$; F = 17.49; Table 1; Fig. 1). The increase in DNA

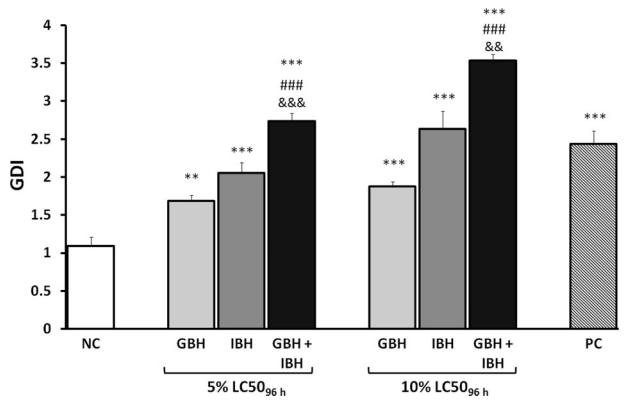


**Fig. 1** DNA damage induced by the GBH Credit® (light grey bars) and the IBH Pivot® H (dark grey bars), detected by the single cell gel electrophoresis assay in *R. arenarum* tadpoles exposed under laboratory conditions. Tadpoles were exposed for 96 h to either GBH or IBH at 5 and 10% of each $LC50_{96\ h}$ concentration and to binary mixtures (black bars) of GBH-IBH at 5% of each $LC50_{96\ h}$ concentration and 10% of each $LC50_{96\ h}$ concentration. Results are expressed as pooled values of genetic damage indexes from three independent experiments. NC: negative controls (white bar) and PC: positive controls (stripped bar). $^{**}P < 0.01$; $^{***}P < 0.001$ (significant differences compared to negative control values). $^{\#\#\#}P < 0.001$ (significant differences compared to GBH-alone values). $^{\&\&}P < 0.01$; $^{\&\&\&}P < 0.001$ (significant differences compared to IBH-alone values)

damage was due to an enhanced frequency of type II ($P = 0.01$; $F = 11.13$) and III nucleoids ($P = 0.00$; $F = 9.01$) and a decrease of type 0 + I nucleoids ($P = 0.00$; $F = 17.49$; Table 1).

### Glyphosate-based herbicide-exposed larvae

After 96 h of exposure to GBH, GDI values in larvae exposed both to 5% ($P = 0.01$; $F = 26.78$) and to 10% ($P = 0.001$; $F = 26.78$) of the GBH $LC50_{96\ h}$ concentration were augmented compared to negative controls (Fig. 1). This increase resulted from an increased frequency of type II nucleoids ($P = 0.00$; $F = 11.13$) and a simultaneous decrease in the frequency of type 0 + I nucleoids ($P = 0.00$; $F = 19.74$) for tadpoles exposed to both 5 and 10% of the GBH $LC50_{96\ h}$ concentration (Table 1).

### Imazethapyr-based herbicide-exposed larvae

In tadpoles exposed to IBH, enhanced GDI values were reported when tadpoles were exposed to both 5 and 10% of the $LC50_{96\ h}$ concentration ($P = 0.00$; $F = 26.78$; Fig. 1). The increment was due to an enhanced frequency of type II ($P = 0.00$; $F = 11.13$) and III nucleoids ($P = 0.00$; $F = 9.90$) and a simultaneous decreased frequency of type 0 + I nucleoids ($P = 0.00$; $F = 19.74$) when tadpoles were exposed to 5% of the $LC50_{96\ h}$ concentration (Table 1). Similarly, a significant increase in the frequency of type II ($P = 0.04$; $F = 11.13$), III ($P = 0.00$; $F = 9.90$) and IV nucleoids ($P = 0.01$; $F = 12.60$) and a decreased frequency of type 0 + I nucleoids ($P = 0.00$; $F = 19.74$) was observed in tadpoles exposed to 10% of the IBH $LC50_{96\ h}$ concentration (Table 1).

### Glyphosate- plus imazethapyr-based herbicide-treated larvae

The combination of GBH and IBH increased the GDI in regard to negative control ($P = 0.00$; $F = 26.78$; Fig.1) and to specimens exposed to GBH ($0.01 > P < 0.001$; Fig. 1) or IBH only ($P < 0.001$; Fig.1). For both mixture concentrations, such an effect was due to an increased frequency of type III ($P = 0.00$; $F = 9.90$) and IV nucleoids ($P = 0.00$: $F = 12.60$) and a decrease of type 0 + I nucleoids ($P = 0.00$; $F = 19.74$; Table 1). In addition, an increase of type II nucleoids was observed in larvae exposed to GBH-IBH at 5% $LC50_{96\ h}$ concentration ($P = 0.02$; $F = 11.13$), but not with GBH-IBH at 10% $LC50_{96\ h}$ concentrations ($P = 0.85$; $F = 11.13$) (Table 1). Besides, significant differences were observed between the GDI in tadpoles exposed to GBH-IBH at 5% $LC50_{96\ h}$ concentrations and those exposed to GBH-IBH at 10% $LC50_{96\ h}$ concentrations ($P = 0.00$; $t$-value = 5.26). Overall, treatment with GBH-IBH at 5% $LC50_{96\ h}$ concentrations induced 1.63- and 1.33-fold increases in the GDI in regard to those induced by 5% GBH $LC50_{96\ h}$ ($P = 0.0001$; $F = 20.54$) and 5% IBH $LC50_{96\ h}$ ($P = 0.001$; $F = 20.54$) treatments, respectively. Similarly, 1.89- and 1.34-fold increases in the GDI occurred in larvae exposed to the mixture comprising GBH-IBH at 10% $LC50_{96\ h}$ concentrations over those induced by 10% GBH $LC50_{96\ h}$ ($P = 0.0001$; $F = 18.84$) and 10% IBH $LC50_{96\ h}$ ($P = 0.011$; $F = 18.84$) concentrations, respectively (Fig. 1).

## DISCUSSION

Our results showed $LC50_{96\ h}$ values of 78.18 mg/L GLY and 0.99 mg/L IMZT for GBH Credit® and IBH Pivot® H, respectively. Concerning the acute lethality of the agrochemicals obtained in this study and according to the directives of the U.S. EPA for aquatic organisms (USEPA 2017), GLY and IMZT could be ranked as slightly and highly toxic compounds, respectively. Besides, GLY and IMZT can be classified as harmful compounds for aquatic biota (Category III) according to criterion established by the United Nations directives (UN 2011). Additionally, whereas GLY should be considered as a harmful agrochemical, IMZT represents a chemical that may cause long-term adverse effects in the aquatic environment, following the hazard classification categories contained in European Union regulations (Mazzatorta et al. 2002).

The current observations showed a $LC50_{96\ h}$ value for IBH in *R. arenarum* larvae equivalent to 0.99 mg/L IMZT

(confidence limits 0.91–1.08 mg/L). To the best of our knowledge, acute toxicity for IMZT in this species has not been yet studied. Thus, the current result constitutes the first experimental data on acute lethality induced by the IBH Pivot® H in *R. arenarum* tadpoles. Previous studies reported $LC50_{96\ h}$ values of 1000 mg/L IMZT for the water flea *Daphnia magna* (Cladocera, Daphniidae), 240 mg/L IMZT for the channel catfish *Ictalurus punctatus* (Siluriformes, Ictaluridae), 340 mg/L IMZT for the rainbow trout *Oncorhynchus mykiss* (Salmoniformes, Salmonidae) and 420 mg/L IMZT for the bluegill *Lepomis macrochirus* (Perciformes, Centrarchidae) (TOXNET 2017). Accordingly, it is evident that *R. arenarum* tadpoles are approximately 1010, 424, 343 and 242 times more sensitive to IMZT than *D. magna*, *L. macrochirus*, *O. mykiss* and *I. punctatus*, respectively. Recently, in a study of another native anuran species, the Montevideo tree frog *Boana pulchella* (formerly named *Hypsiboas pulchellus*) larvae, a $LC50_{96\ h}$ value for IBH of 1.55 mg/L IMZT (confidence limits, 1.51–1.60) was determined for the same IBH commercial product, namely Pivot® H (Pérez-Iglesias et al. 2015). It seems evident then that *B. pulchella* is nearly 1.56 times less sensitive to IBH than *R. arenarum* at the same premetamorphic developmental stage. So far, acute lethality data of IMZT among aquatic organisms have been reported for a scarce number of species. Thus, we cannot ensure that the observed pattern of *R. arenarum* being more sensitive to IBH than *B. pulchella* would be repeated with other environmental stressors. Whether the higher sensitivity to IBH that we observed in *R. arenarum* premetamorphic tadpoles compared to *B. pulchella* larvae is restricted to one of the herbicides under study or could be extended to other xenobiotics remains an open question. Further studies are required to analyse this concept using a battery of different xenobiotics, e.g., including several other herbicides in this Neotropical anuran species.

Our results agree with the widely accepted concept that the SCGE assay is a useful and versatile laboratory methodology for monitoring amphibians to xenobiotic exposure, including pesticides (Feng et al. 2004; Maselli et al. 2010; Mouchet et al. 2007; Pérez-Iglesias et al. 2014, 2015, 2018; Yin et al. 2008). Consistent with this concept and addressing the lack of information on the harmful effects exerted by a reduced number of pesticides in Argentinean amphibian species, we analyzed in *R. arenarum* late-stage larvae the lethal and sublethal toxicity of the active ingredient flurochloridone present in two commercially available products namely Twin Pack Gold® and Rainbow® (Nikoloff et al. 2014b). *B. pulchella* tadpoles have been used as a non-conventional biotic matrix for the evaluation of the same toxic effects exerted by the neonicotinoid insecticide imidacloprid and its formulated product Glacoxan Imida (Pérez-Iglesias et al. 2014; Ruiz de Arcaute et al. 2014), and Pivot® H, the IBH commercial product (Pérez-Iglesias et al. 2015, 2017, 2018). Consequently, our current observations represent the first in vivo experiment employing SCGE as a technique for detecting and quantifying DNA single-strand breaks caused by the IBH Pivot® H in peripheral blood cells of *R. arenarum* late-stage larvae.

Nowadays, due to the modern agricultural practices, pesticides are often applied as mixtures of at least two different agrochemicals, in which they may interact with each other, resulting in diverging effects on biota, both target and non-target organisms (Baas et al. 2010; Belden and Lydy 2000; Rodney et al. 2013). Briefly, the interaction may cause an additive, synergistic, or antagonistic effects (Blouin et al. 2010). For a mixture, when a larger response is observed than the effects of each component applied singly, this is referred to as a synergistic pattern. In contrast, an antagonistic effect is when the interaction results in a reduction of the effect caused by any of the individual components. Finally, although rare, an additive consequence is achieved when the resulting effect is equal or similar to the overall of the effects caused by each constituent (Blouin et al. 2010).

Our results clearly demonstrate that the binary mixtures of GBH-IBH at 5% $LC50_{96\ h}$ concentrations, as well as GBH-IBH at 10% $LC50_{96\ h}$ concentrations increased the GDI beyond the values obtained when tadpoles were exposed only to GBH or IBH, regardless of the analyte concentration present in the mixture. Consequently, a synergistic effect could be observed for the mixture of GBH and IBH when the SGCE assay was employed to detect genotoxicity in *R. arenarum* tadpoles. Toxicity caused by binary combinations of the herbicides GLY and IMZT has not been reported so far. Reports are available of investigations of mixtures containing GLY and other agrochemicals in different biotic matrices, and synergistic effects were reported for most of them (Bielecki et al. 2004; Brodeur et al. 2014; Olszyk et al. 2015; Santos et al. 2011; Tatum et al. 2012). On the other hand, in an earlier study where the common wheat *Triticum aestivum* was exposed to GLY in binary combinations with aminoalkane and aminofluorene phosphonates at concentrations which approximated the respective EC50 values, either antagonistic or additive responses were reported (Bielecki et al. 2004). Similar toxicity pattern was also reported by Brodeur et al. (2016) who demonstrated antagonistic toxicities of commercial formulations containing GLY and cypermethrin in the teleost *Cnesterodon decemmaculatus*.

In a small number of studies, *R. arenarum* larvae have been employed as biotic matrix to study possible interaction of pesticide mixtures. In a previous study, equitoxic and nonequitoxic combinations of GBH, present in the commercial formulations Glifosato Atanor® and Glifolex®, and the cypermethrin-based insecticide, contained in the

commercial formulations Xiper® and Glextrin®, demonstrated a synergistic effect that was able to influence the survival of *R. arenarum* tadpoles, regardless of the combinations (Brodeur et al. 2014). Recently, we employed the SCGE assay to evaluate genotoxicity caused by the interaction of the GBH Credit® and the dicamba-based herbicide Banvel® when tadpoles of the species were acutely exposed for 96 h (Soloneski et al. 2016). Results demonstrated that both herbicide formulated products exerted DNA damage in circulating blood cells. Additionally, both herbicides when applied as mixtures raised the levels of DNA damage to higher frequencies than those observed for each individual herbicide, thus demonstrating that GBH Credit® and the dicamba-based herbicide Banvel® act synergistically when applied together (Soloneski et al. 2016).

It is worth mentioning that in the present study the lowest concentration equivalent to 5% $LC50_{96\ h}$ (3.91 mg/L GLY) tested when Credit® formulation was assayed might be considered nearly environmental realistic. Previous reports indicate that GLY was detected in water bodies near agricultural crops at concentrations up to 3.7 mg/L (Giesy et al. 2000). However, specifically for Argentina, Peruzzo et al. (2008) found GLY values between 0.10 to 0.70 mg/L in Pampasic water streams. In addition, Castro Berman et al. (2018) reported maximum concentrations of GLY of 4.52 µg/L for surface water, 0.13 µg/L for suspended particulate matter and 20.34 µg/kg for sediment samples, respectively. In relation to IBH, we demonstrated a concentration of 0.99 mg/L IMZT (0.91–1.08 mg/L) as the $LC50_{96\ h}$ value for the premetamorphic tadpoles. Thus, this concentration range represents a relatively higher value than the 14 µg/L IMZT reported for water streams from Argentinean Pampasic habitats (Peluso et al. 2008) where an application field ratio of 100–150 g a.i/ha is commonly employed (Bindraban et al. 2009; CASAFE 2017). Northwardly, the IMZT concentrations reported for Argentinean productive areas is nearly 7.6 times higher than the higher concentration detected for surface water in USA (Mattice et al. 2012). Thus, IMZT concentrations assayed in this study are unusual in the environment, perhaps only registered when specific events would occur, e.g., a direct application adjacent to surface waters in creeks, ponds and drainage ditches by accidental discharge, among others.

Our results highlight that the SCGE assay provides a powerful method for evaluating the genotoxicity exerted by mixtures of pesticides on a biotic matrix, specifically in anuran larvae. Furthermore, they could allow us to suggest that the DNA lesions induced by the combination of GBH Credit® and IBH Pivot® H may increase genomic instability and have a deleterious effect on several biological processes in exposed organisms, including development, behavior, survival outcome, reproduction and population fitness (Beebee 2005; Jones et al. 2009). Finally, it is essential to note that several commercial applications of GBH-IBH mixtures are widely used in agricultural systems worldwide. Available information demonstrates that 12 commercial pesticides containing a mixture of GLY and IMZT as active ingredients have been registered in Argentina (CASAFE 2017). Based on our observations, mixtures of GBH and IBH formulations could put at risk amphibian populations by producing synergistic DNA damage that they could magnify the harmful effects on non-target species inhabiting aquatic agroecosystems. Years ago, the USEPA (1982) highlighted that the toxic effects of a pure compound can differ from that of the commercial product carrying the active ingredient. The presence within commercial formulations of additive compounds, also called inerts, the identity of which is often kept confidential by the manufacturing companies, may cause different toxicity to that of the active ingredient and appears to be a distinctive attribute in the pesticide toxicology (Belden et al. 2010; Brühl et al. 2011; Grisolia et al. 2004; Mann and Bidwell 1999; Nikoloff et al. 2014a; Soloneski et al. 2007; Soloneski and Larramendy 2010). Therefore, attention should be taken with the real significance of pesticide binary mixtures when assayed in toxicological studies.

Acute GLY toxicity is considered to be very low by the World Health Organization (WHO-FAO 1997) depending on the coadjuvants, such as surfactants, humectants, and dispersants, always included within the formulations (Mann et al. 2009; Wagner et al. 2013). Recently, the International Agency for Research on Cancer classified GLY as "probably carcinogenic in humans" (Category 2A) (IARC 2017). Reports agree in demonstrating that commercial GBH formulations are more 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 GBH formulations containing trimethylsulfonium salt of GLY are more toxic than those in which GLY is present as an isopropylamine salt (Pettersson and Ekelund 2006). Furthermore, laboratory studies demonstrated for the latter that the toxicity is largely due the presence of the surfactant polyethoxylated tallow amine (POEA). Several reports demonstrate that POEA may have a toxicity several times higher than GLY itself, making the formulated mixture of greater toxicity than both the active ingredient and GBH formulations not containing POEA (Bolognesi et al. 1997; Moore et al. 2012; Tsui and Chu 2003, 2008). In agreement with these observations, *R. arenarum* tadpoles exposed to several POEA-free GBH formulations showed LC50 values 20–26 times greater than the values reported in tadpoles exposed to Roundup Original® (Brodeur et al. 2014; Lajmanovich et al. 2011). Furthermore, the $LC50_{96\ h}$ value of 78.18 mg/L calculated for the GBH formulation Credit® (Soloneski et al. 2016) is consistent with LC50 values reported previously for the

same anuran species and POEA-free GBH formulations (Brodeur et al. 2014; Lajmanovich et al. 2011). In the case of IMZT, the herbicide has been classified as a slightly toxic compound (Class III) by the U.S. EPA (PPDB 2014) and reported as a harmful irritant for the respiratory track, skin, and eyes, as well as classified as a dangerous compound for the environment by the European Union (PPDB 2014). Overall, data available about the toxicology and ecotoxicology of IMZT are scarce. When IMZT was administered orally, low or moderate acute toxicity was reported in rats. Using algae and aquatic invertebrates, low levels of toxicity have been reported. On the other hand, when aquatic plants were employed as targets, high acute levels of toxicity were observed. Among terrestrial invertebrates, insects such as honeybees and annelids such as earthworms have been reported to have extremely high sensitivity and low sensitivity to IMZT, respectively. So far, the levels of acute toxicity exerted by the herbicide were found not to be acutely toxic for fish, including *I. punctatus*, *L. macrochirus* and *O. mykiss* (PPDB 2014). Although, Moraes et al. (2011) reported disorders in oxidative stress parameters in the common carp *Cyprinus carpio* (Cypriniformes, Cyprinidae) after exposure to IBH formulations. Furthermore, we recently demonstrated acute genotoxic effects, including the induction of micronuclei and primary DNA lesions in circulating erythrocytes from *B. pulchella* tadpoles exposed to IBH formulation under laboratory conditions (Pérez-Iglesias et al. 2015).

Accordingly, caution should be taken with the concept of the binary mixtures tested in the current study since they represent a much more complex framework as they involved two biocide formulations which, by themselves, represent mixtures of several unknown constituents according the Argentinean Administration. We cannot assume whether the proportion of each constituent of the mixtures we used follow the same or a similar profile in an environmental aquatic systems as the different components of the formulations can suffer different transformation or degradation patterns in the environment. Thus, different pattern of occurrence in the water matrix could be produced diverging from that we tested. Additional studies are necessary to determine whether the toxicity caused by the mixtures of GBH Credit® and IBH Pivot® H in the *R. arenarum* tadpoles is attributable to the active ingredients by themselves or result from the presence of additive compounds included in the herbicide formulated products.

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## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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