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Research article

First evaluation of novel potential synergistic effects of glyphosate and arsenic mixture on *Rhinella arenarum* (Anura: Bufonidae) tadpoles

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ABSTRACT

The toxicity of glyphosate-based herbicide (GBH) and arsenite (As(III)) as individual toxicants and in mixture (50:50 v/v, GBH-As(III)) was determined in *Rhinella arenarum* tadpoles during acute (48 h) and chronic assays (22 days). In both types of assays, the levels of enzymatic activity [Acetylcholinesterase (AChE), Carboxylesterase (CbE), and Glutathione S-transferase (GST)] and the levels of thyroid hormones (triiodothyronine; T3 and thyroxine; T4) were examined. Additionally, the mitotic index (MI) of red blood cells (RBCs) and DNA damage index were calculated for the chronic assay. The results showed that the LC_{50} values at 48 h were 45.95 mg/L for GBH, 37.32 mg/L for As(III), and 30.31 mg/L for GBH-As(III) (with similar NOEC = 10 mg/L and LOEC = 20 mg/L between the three treatments). In the acute assay, Marking's additive index (S = 2.72) indicated synergistic toxicity for GBH-As(III). In larvae treated with GBH and As(III) at the NOEC-48h (10 mg/L), AChE activity increased by 36.25% and 33.05% respectively, CbE activity increased by 22.25% and 39.05 % respectively, and GST activity increased by 46.75% with the individual treatment with GBH and by 131.65 % with the GBH-As(III) mixture. Larvae exposed to the GBH-As(III) mixture also showed increased levels of T4 (25.67 %). In the chronic assay at NOEC-48h/8 (1.25 mg/L), As(III) and GBH-As(III) inhibited AChE activity (by 39.46 % and 35.65%, respectively), but did not alter CbE activity. In addition, As(III) highly increased (93.7 %) GST activity. GBH-As(III) increased T3 (97.34%) and T4 (540.93%) levels. Finally, GBH-As(III) increased the MI of RBCs and DNA damage. This study demonstrated strong synergistic toxicity of the GBH-As(III) mixture, negatively altering antioxidant systems and thyroid hormone levels, with consequences on RBC proliferation and DNA damage in treated *R. arenarum* tadpoles.

1. Introduction

Glyphosate is the active ingredient of Roundup®, the glyphosate-based herbicide (GBH) most used in Latin American agriculture, mainly in genetically modified crops (Roundup Ready® soybean; RR® soybean). Humans and wildlife have been exposed to GBH and its metabolites for many decades, and the estrogenic effects of these substances have been extensively documented (Mesnage et al., 2017). In addition, the continuous and increasing use of GBHs in agriculture can lead to an increase in the possible damaging effects on non-target organisms, such as amphibians (Berger et al., 2018). In the plain agroecosystems of the Argentine Pampa, Sasal et al. (2017) reported that, according with sampling periods, the majority glyphosate water samples were below 0.1 ug/L (4.7% of the data were above 240 ug/L) and a maximum reported level of reached 105 mg/L. Whereas in river surface waters in the northern Pampean region, Peruzzo et al. (2008) determined glyphosate concentrations between 0.10 and 0.70 mg/L. However, the field concentrations of glyphosate that may be particularly relevant to amphibian

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tadpoles are not only the ones observed in the environment, but also the ones directly related to amphibian habitats (ephemeral ponds). Mann and Bidwell (1999) considered a worst-case scenario for wild tadpoles of direct over-spraying with a GBH in a very shallow water body (5 cm in depth) at the highest authorized application rate, which would result in 21 mg/L of the whole product. In this respect, in ecotoxicological investigations, the use of native species is suitable because it provides ecological relevant information and may allow characterizing the potential risk to other sympatric species in freshwater systems. In accordance with the latter, amphibians are an emblematic group of vertebrates which have been widely used to study the effects of GBHs. Results of these studies have shown mortality or loss of osmotic stability, interference with gill morphology, lysis of gill epithelial cells, several sub-lethal histological effects, inhibition of B-esterases and detoxification enzymes, erythrocyte nuclear abnormalities, DNA damage, teratogenic effects by impairing retinoic acid signaling, and others (Mann and Bidwell, 1999; Lajmanovich et al., 2011, 2013; Relyea and Jones, 2009; Paganelli et al., 2010).

Arsenic (As) is a chemical element of great ecological significance due to its high toxicity, persistence and bioaccumulation, whose exposure represents an important health problem in many countries of the world (Majumder and Banik, 2018). In the environment, high doses of this ubiquitous and generally toxic element may cause severe damage to all living organisms (Wu et al., 2016). One of the regions most affected by As is Latin America, with total As concentrations in natural surface waters varying between 0.01 and 15 mg/L (Smedley and Kinniburgh, 2002). In Argentina, As concentrations range from 0.004 to 5.3 mg/L (Smedley et al., 2008). In the environment, As can be found either as arsenate (As(V)) or as arsenite (As(III)) (Smedley and Kinniburgh, 2002), and its valence state affects its toxicity and bioavailability, being As(V) less toxic to animals than As(III) (Ohki et al., 2002). In the ground water of the Argentine Chaco-Pampa Plain, As has revealed average percentages of 98.5 % for As(V) and 1.5 % for As(III) (Siegfried et al., 2015). Both As species are rapidly absorbed by the gastrointestinal tract of animals, and then As(V) is quickly reduced to As(III) and receives a methyl group (Cohen et al., 2013). While humans are exposed to As through food and water, aquatic animals are principally vulnerable to As in the environment because they can also take up this toxicant through the gills or skin (Ventura-Lima et al., 2011). Moreover, amphibians (tadpoles and adults) have been reported to be As bioaccumulators (Moriarty et al., 2013). Thus, several ecotoxicological studies on the effects of As exposure have been conducted in amphibian tadpoles. Chen et al. (2009), for example, found that chronic exposure to As(V) (1–1000 μg/L) reduced the swimming performance of leopard frog larvae (*Lithobates pipiens*), whereas Brodeur et al. (2009) found that acute and subchronic exposure to As(III) (10–80 mg/L) inhibited the growth of tadpoles of *Rhinella arenarum*. Likewise, Mardirosian et al. (2015, 2017) reported effects on oxidative stress, absorption and excretion in embryos and tadpoles of *R. arenarum* acutely and chronically exposed to As (III) (0.01–50 mg/L).

It is known that both GBHs and multiple heavy metals are frequently present in crop fields and surface waters such as those of lagoons, ponds and rivers (Jayasumana et al., 2015). However, the study of the additive, synergistic or antagonistic toxicity of a chemical mixture has been an enduring challenge in both environmental health and ecotoxicological studies (Olmstead and LeBlanc, 2005). In this context, Samsel and Seneff (2015) proposed a mechanism through which glyphosate greatly increases the toxicity of As through chelation, whereas Wang et al. (2017) demonstrated a synergistic effect of the glyphosate-As mixture on *Caenorhabditis elegans* in an acute toxicity assay. In addition, Defarge et al. (2017) pointed out that As could be present in the GBH formulation and that the two compounds may act synergistically, disrupting endocrine hormones.

In the Argentine Pampas, one of most extended plain regions in the world, RR® soybean cultivation and intense GBH use occur simultaneously, with high levels of As in both surface and ground waters. However, little is known about the toxicological interactions of GBH and As mixtures. Thus, the objectives of the current study were to determinate the individual toxicity of GBH and As(III), and to carry out a first and novel evaluation of their mixture, on tadpoles of the common South American toad, *Rhinella arenarum*. We hypothesized that the mixture of GBH and As(III) causes potential synergistic toxicity effects on enzymatic activities, thyroid hormone levels, proliferation of red blood cells (RBCs), and DNA damage. Considering that GBHs and As occur in surface and ground waters, these endpoints may allow evaluating and characterizing the potential synergistic interaction of glyphosate with arsenic in experimental and field biology studies.

2. Materials and methods

2.1. Experimental design

Tadpoles of the common South American toad *R. arenarum* (Anura: Bufonidae) were used as research organisms to study the effects of exposure to GBH and As(III). Premetamorphic tadpoles (Gosner stages, GS 26-30; Gosner, 1960) were collected from a site without agricultural activities and reduced As (Blettler et al., 2019) situated in the natural floodplain of the Paraná River (31° 39′ 45″ S, 60° 34′ 36″ W), Argentina, with collection permission of the Ministry of Environment of the Province of Santa Fe (EXP. N° 02101-0018518-1). *R. arenarum* has a wide-ranging geographic distribution and abundance in the Neotropical region (Argentina, Bolivia, Brazil, and Uruguay) and it is also cited as "not threatened" in the Red List of amphibians of Argentina (Vaira et al., 2012). *R. arenarum* was reported as one of the most sensitive amphibian species for toxicological tests due to its high sensitivity to water pollution (Lajmanovich et al., 2018). The tadpoles were acclimated under laboratory conditions during 48 h at 12-h light/dark cycle in flasks (with dechlorinated tap water, DTW, pH 7.4 ± 0.05; conductivity, 165 ± 12.5 μmhos/cm; dissolved oxygen concentration, 6.5 ± 1.5 mg/L; hardness, 52.5 mg/L of $CaCO_3$ at 22 ± 2 °C). The tadpoles were feed on boiled lettuce (*Lactuca sativa*) during all experiments according to previous studies with this species (e.g. Lajmanovich et al., 2011, 2013; 2014). The experiments with tadpoles followed the regulations of the ASIH (2004). Specimens were euthanized by immersion in a solution of 0.1% tricaine methanesulfonate (TMS, MS-222) buffered to pH 7.8 with $NaHCO_3$ following the protocol of the Animal Euthanasia Guide proposed by the Institutional Animal Care and Use Committee and the committee of bioethics of the Facultad de Bioquímica y Ciencias Biológicas of the Universidad Nacional del Litoral, Santa Fe, Argentina (Res. CD N°: 388/06).

2.2. Test substances

All assays were prepared using arsenic trioxide As_2O_3 99.5% (Biopack®; Zárate, Argentina). The GBH (74.7 % active ingredient [a.i.], N-(phosphonomethyl) glycine; Roundup Ultra-Max®, Monsanto Co., Argentina) was tested in commercial mixture because this is the form in which they are applied in cultivation fields and introduced into the environment (Relyea and Jones, 2009). Liquid chromatography tandem-mass spectrometry (LC-MS/MS) analyses using an Acquity UPLC® liquid chromatograph (Waters, Milford, MA, USA) coupled to a triple quadrupole mass spectrometer equipped with an ESI source able to operate in either the positive or negative-ion mode (TQD, Waters Micromass, UK), was used to confirm the concentration of glyphosate (a.i.) in the commercial formulation. Quantification procedure was performed using as a reference the calibration curve from 0.1 μg/L up to 100 μg/L, which was constructed in matrix (matrix-matched calibration). Recoveries were determined by analysing fortified samples at two levels of concentration (1 and 100 μg/L) in triplicate with the results ranging between 70 and 105% and an RSD <5% in all cases. LOD of 0.2 μg/L and LOQ 0.6 μg/L for glyphosate were determined using S/N ratio of 3 and 10 measurements, respectively, from 1 μg/L spiked samples. The error between nominal and measured concentrations did not exceed 5 %.

2.3. Toxicity assays

2.3.1. Acute toxicity assay (48 h)

For the acute short-term toxicity assay, glass flasks (12.5 cm in diameter and 13.5 cm high) with 1 L of DTW and ten tadpoles [GS 26-30; average size (snout-tail tip) 17 ± 0.25 mm and average weight 0.25 ± 0.05 g] were used in the experiments (N = 240 tadpoles per treatment). Tests were made at 22 ± 2 °C and at 12 h light/dark cycle. Negative controls with DTW were used. Larval mortality was monitored and dead larvae were removed every 24 h. The cumulative mortality in each treatment was calculated at 48-h of exposure. Median lethal concentration (LC_{50}) and no-, and lowest-observed-effect concentrations (NOEC and LOEC, respectively) were calculated. Both control and test solutions were made in triplicate. As a first approximation of toxicological interactions, the nominal GBH and As(III) concentrations used to test single toxicities were: 1.25, 2.5, 5, 10, 20, 40, and 80 mg/L (Brodeur et al., 2009; Lajmanovich et al., 2011). The toxicities of the two compounds in 50:50 v/v mixtures were estimated using the same nominal concentrations. Finally, a subsample of control tadpoles and a subsample of tadpoles treated with GBH, As(III), and GBH-As(III) (50:50 v/v) (N = 7–10, respectively) at the No-Observed-Effect Concentration (NOEC) at 48 h (10 mg/L) were used to measure acetylcholinesterase (AChE), carboxylesterase (CbE), and glutathione S-transferase (GST) activities, and thyroid hormone levels (triiodothyronine; T3 and thyroxine; T4).

2.3.2. Chronic toxicity assay (22 days)

Immediately after the acute assay, a chronic toxicity assay was carried out following the same methodology. Tadpoles (N = 40 per treatment) at the same GS range, weight and size as in the first assay were exposed to a nominal concentration of 1.25 mg/L of GBH, As(III), and GBH-As(III) 50:50 v/v mixture for 22 days. This concentration value was eight-fold lower than the NOEC obtained from the 48-h acute assay (i.e. NOEC-48h/8). All solutions were replaced every two days with freshly prepared solutions (or dechlorinated tap water in the case of controls) of equal concentrations. Subsamples of treated and control tadpoles were randomly taken to determine enzymatic activities (AChE, CbE and GST; N = 7–10), hormone levels (T3 and T4; N = 7–10), mitotic index (MI) (N = 7–10), and DNA damage (N = 7). The development stage (i.e. GS), growth, and survival percentage of tadpoles were also evaluated.

2.4. Biomarkers

2.4.1. Biological responses

To determine the levels of enzymatic activities and the levels of thyroid hormones, subsamples of treated tadpoles at 48 h and 22 days were weighed (g) and homogenized (1:10, w/v) in ice-cold 25 mM sucrose, 20 mM Tris-HCl buffer (pH = 7.4) containing 1 mM EDTA, using a polytron tissue grinder. The homogenates were centrifuged at 10,000 rpm for 15 min at 4 ± 1 °C, and stored at - 80 °C until biomarker endpoint analysis. The Biuret method was used for measurement of protein concentration in the supernatants (Kingsley, 1942) to analyze enzymatic activities.

2.4.2. Enzymatic activities (48 h and 22 days)

AChE activity was determined colorimetrically following Ellman et al. (1961). The reaction mixture (final volume [Final Volume = 930 μL]) consisted of 25 mM Tris-HCl containing 1 mM CaCl2 (pH = 7.6), 10 μL 20 mM acetylthiocholine iodide (AcSCh), and 50 μL DTNB (3×10-4 M, final concentration) and 20.0 μL of sample. The variation in optical density was recorded at 410 nm for 1 min at 25 °C using a JENWAY 6405 UV-VIS spectrophotometer. AChE activities were expressed as nmol min^{-1} mg^{-1} protein using a molar extinction coefficient of 13.6×10^3 M^{-1} cm^{-1}. CbE activity was measured by the method of Bunyan and Jennings (1968). The assay was carried out with 25 mmol/L Tris-HCl, 1 mmol/L $CaCl_2$ (pH = 7.6) and 20 μL of sample at 25 °C. The reaction was initiated by adding 50 μL of α-naphthyl acetate (1.04 mg/mL in acetone – α-NA) as substrate, and stopped after 10 min by addition of 500 μL of 2.5% SDS and subsequently 500 μL of 0.1% Fast Red ITR in 2.5% Triton X-100 in water (freshly prepared). Samples were left in darkness for 30 min and the complex absorbance was read at 530 nm. Hydrolysis of α-NA was expressed as nmol of substrate hydrolyzed $min^{-1}ml^{-1}$ using a molar extinction coefficient of 33.225×10^3 $M^{-1}cm^{-1}$). GST activity was determined spectrophotometrically using the method described by Habig et al. (1974) and adapted by Habdous et al. (2002) for mammal serum GST activity. The enzyme assay was performed at 340 nm in a mixture containing 910.0 μL of 100 mmol L^{-1} sodium–phosphate buffer (pH 6.5), 20.0 μL of 0.2 mmol L^{-1} 1-chloro-2, 4-dinitrobenzene, 50.0 μl of 5 mmol L^{-1} reduced glutathione, and 20.0μL of sample. Enzyme kinetics assays were performed at 25 °C and whole GST activity was expressed as nmol $min^{-1}mg^{-1}$protein using a molar extinction coefficient of 9.6 $\times10^3$L $mol^{-1}cm^{-1}$).

2.4.3. Thyroid hormone levels (48 h and 22 days)

Total thyroid hormone levels were measured using enzyme-linked electro-chemiluminescent immunoassay (ECLIA) kits (COBAS®, Roche Diagnostics, Indianapolis, IN, USA) following that previously described in Lajmanovich et al. (2019). The detection limits for T3 and T4 were 0.0001 ng/g and 2.1 ng/g, respectively. Due to difficulties related to sufficient amounts of blood collection because of the small size of the *R. arenarum* tadpoles, the whole-body was considered to thyroid hormones. Other authors (e.g. Gancedo et al., 1997; Li et al., 2016; Lajmanovich et al., 2019) have used this method.

2.4.4. Mitotic index (22 days)

Mitotic RBCs from treated tadpoles (N = 7–10) were used to determine the rate of cell proliferation or MI (Lajmanovich et al., 2014). Blood smears were prepared on clean slides, fixed, and stained by means of the May Grünwald/Giemsa method (Dacie and Lewis, 1984). The MI was determined in 1000 RBCs from each tadpole, with a microscope under 1000 × magnification.

2.4.5. DNA damage (22 days)

Treated tadpoles and controls (N = 7) were randomly selected to analyze DNA damage through the Alkaline Comet assay (in duplicate, and pH > 13), which was performed according to the method described by Singh et al. (1988), with the modifications by Curi et al. (2017). The blood of each tadpole was collected with a heparinized capillary tube of 50 μL. Blood samples were diluted 1:19 (v/v) with PBS medium and used immediately. Then, 2 μL of each diluted blood sample (approximately $4.0X10^3$ erythrocytes) was added to 100 μL of 1% low melting point agarose and a slide was prepared. To lyse the cellular and nuclear membranes of the embedded cells, the key-coded slides were directly immersed in freshly prepared ice-cold lysis solution (2.5 M NaCl, 100 mM Na_2 EDTA, 10 mM trizma base, 1% Triton X-100 and DMSO 10%; pH 10) and left at 4 °C overnight. The slides were then immersed in freshly prepared alkaline electrophoresis solution (300 mM NaOH and 1 mM Na_2 EDTA; pH > 13), first for unwinding (10 min) and then for electrophoresis (0.7–1 V cm^{-1}, 300 mAmp, 10 min at 4 °C). All the steps were carried out under conditions of minimum illumination and low temperature (on ice). Once electrophoresis has been concluded, the slides were neutralized and dehydrated with ethanol. Slides were stained with acridine orange at the time of analysis and 100 randomly selected comets from each tadpole were classified into five comet types according to tail size and intensity (0 = undamaged, I = low damage, II = medium damage, III = moderate damage, and IV = severe damage). Results are expressed as the DNA damage index (DI = n1 + 2.n2 + 3.n3 + 4.n4, where n1, n2, n3 and n4 were the number of cells in each class of damage, respectively) and it was quantified per treatment. Data are expressed as the DNA damage index (DI).

2.5. Statistics and data analyses

Lethal concentration (LC_{50}) values and their respective 95% confidence intervals (CI) were determined by the Trimmed Spearman-Karber procedure (Hamilton et al., 1977). The mortality data were assessed by ANOVA using Dunnett's post-hoc tests for multiple comparisons in order to determine the NOEC and the LOEC (U.S.EPA, 1989). The results of the enzyme activities (AChE, CbE and GST), hormone levels (T3 and T4), and DI were analyzed with ANOVA and Dunnett's test for post-hoc comparisons, whereas those of the MI of RBCs were analyzed using the binomial proportion test (Margolin et al., 1983). In the chronic assay, growth (average size and weight) was assessed by Kruskal-Wallis ANOVA, followed by Dunn's post-hoc test. Categorical data for the development stage (GS) were analyzed using the Chi-squared test. All data regarding biomarkers are reported as the mean ± SD. All these statistical analyses were performed using BioEstat software 5.0 (Ayres et al., 2008). A value of $p < 0.05$ was considered significant.

2.6. GBH and As(III) toxicological interactions

To determine whether the lethal effects of GBH and As(III) in a 50:50 v/v mixture were additive, synergistic, or antagonistic, we used the Marking's additive index (MAI; Marking, 1985). MAI was calculated using the equation: $Am/Ai + Bm/Bi = S$ where A and B were the individual toxicant, i and m were the individual and mixture LC_{50s}, respectively, and S was the sum of biological activity. The toxic compounds are considered antagonistic if $S < 1$, synergistic if $S > 1$, or additive is $S = 1$. Marking places an additive index value of 0 at $S = 1$, a sum of activity at which the mixture components would be equitoxic (a demonstration of additive toxicity). If $S \leq 1.0$, Additive Index (AI) $= 1/S - 1$. If for $S \geq 1.0$, $AI = -S + 1$. According to this scheme, when AI = 0, components are simply additive; negative and positive values indicate less than additivity and more than additivity, respectively. For the other biological endpoints, we used significant departures from additive toxicity to describe antagonistic and synergistic effects between compounds in mixtures (Hertzberg and MacDonell, 2002). The results regarding enzyme activities, thyroid hormones, MI, and DI were interpolated to estimate the median effective concentration (EC50) and 95% CI for exposure to a single toxicant (toxic potential) (EC50 TP) and for exposure to the binary mixture (EC50 BM). These endpoints were compared to determine whether the toxicological responses to binary mixtures were additive, antagonistic, or synergistic (Laetz et al., 2009). The criterion of non-overlapping 95% CI was used to establish the significant differences between EC_{50} values (Lajmanovich et al., 2013).

3. Results

3.1. Acute assay (48 h)

The results of the acute toxicity bioassays are summarized in Table 1. No mortality was observed in the control groups. The LC_{50} values at 48-h ranged from 30.31 mg/L for GBH-As (III) to 45.95 mg/L for GBH alone. According to the MAI, the mixture of both toxicants displayed synergistic toxicity, with a sum of activity of 2.72. Furthermore, the negative results of the AI indicated low additivity.

At the NOEC 48-h (10 mg/L), the mean value of AChE activity in control tadpoles was 21.85 ± 5.15 nmol min^{-1} mg^{-1} total protein, whereas that in tadpoles treated with GBH and As(III) was highly increased (by 36.25 and 33.05 %), respectively (ANOVA F = 3.27; $p < 0.01$) (Fig. 1A, 48 h). Regarding CbE activity, that in control tadpoles was 9.57 ± 1.96 nmol min^{-1} mg^{-1} total protein, whereas that in tadpoles treated with GBH and As(III) was increased (by 22.25 and 39.05 %) (ANOVA F = 8.62; $p < 0.01$) (Fig. 1B, 48 h). Regarding GST activity, that in control tadpoles was 117.65 ± 26.54 nmol $min^{-1}mg^{-1}$protein, whereas that in tadpoles treated with GBH and the GBH-As(III) mixture was significantly increased by 46.75% and 131.65% ($p < 0.01$) (Fig. 1C, 48 h).

Table 1
Summary of the results of the acute toxicity bioassay (48 h) of glyphosate-based herbicide (GBH) and arsenite (As(III)) in *Rhinella arenarum* tadpoles. GBH and As(III) were tested in a 50:50 v/v mixture.

Treatment	48-h LC_{50} (mg/L) (95% CI)	NOEC andLOEC (mg/L)	S‡	AI§
GBH	45.95 (37.59–56.17)	10 20	–	–
As(III)	37.32 (30.11–46.26)	10 20	–	–
GBH and As(III)	30.31 (26.58–34.58)	10 20	2.72	-1.72

(NOEC) No Observed Effect Concentration.
(LOEC) Lowest Observed Effect Concentration.
‡ (S) Sum of Biological Activity.
§ (*AI*) Additive Index.

The T3 and T4 mean values in premetamorphic tadpoles (GS 28-32) were 1.64 ng/g (±0.14) and 7.4 ng/g (±0.43), respectively (Fig. 1D–E, 48 h), and a significant increase was observed in T4 levels (25.67 %) in tadpoles exposed to the NOEC-48 h of GBH-As(III) (1.25 mg/L) (F = 3.95; $p < 0.05$) (Fig. 1E, 48 h).

3.2. Chronic toxicity assay (22 days)

Mortality in the chronic toxicity assay was lower than 1 %. The growth (average size and weight) and development stage (GS) of treated and control tadpoles were similar (Kruskal-Wallis KS = 3.65, Chi-squared = 1.84; $p > 0.05$, respectively). The AChE activity in control tadpoles was 26.24 ± 6.65 nmol $min^{-1}mg^{-1}$ protein, whereas that in tadpoles treated with As(III) and GBH-As(III) varied from 39.46 % (As (III)) to 35.65% (GBH-As (III)) (F = 7.91; $p < 0.01$; Fig. 1A, 22 days). The CbE activity in control tadpoles was 11.29 ± 1.98 nmol $min^{-1}mg^{-1}$ protein and the exposure to the toxicants tested did not affect the enzymatic activities at the NOEC/8 48-h (F = 1.42; $p < 0.05$; Fig. 1B, 22 days). GST activity in control tadpoles was 143.99 ± 24.76 nmol $min^{-1}mg^{-1}$ protein, whereas that in tadpoles exposed to 1.25 mg/L As(III) was highly increased (93.7 %; F = 24.38; $p < 0.01$; Fig. 1C, 22 days).

Both thyroid hormones showed a significant increase in the tadpoles exposed to the mixture, varying from 97.34% (T3; F = 4.91; $p < 0.01$; Fig. 1D, 22 days) to 540.93% (T4; F = 3.25; $p < 0.01$; Fig.1E, 22 days).

The MI at 22 days after As(III) and GBH-As(III) exposure increased to 619.35% (z = - 3.84; $p < 0.01$) (Fig. 2A). No statistically significant differences were found for GBH or As(III) individual exposures (Fig. 3). Normal DNA damage was observed in control tadpoles, with an average DI of 107.57 (±6.45) (Figs. 2B and 4). However, after As(III) and GBH-As(III) exposure, the DI (196.28 ± 29.7) increased significantly (by 73.45 %) as compared to that of controls (ANOVA F = 90.12; $p < 0.01$) (Figs. 2 C and 4).

Fig. 5 shows the relations between the EC_{50} values, which were estimated based on the results of the biomarkers studied to calculate the EC_{50} and 95 % CI for single toxic exposures (toxic potential) ($EC_{50\ TP}$) and for the binary mixture ($EC_{50\ BM}$). In the case of GST and T4 (acute exposure) as well as in the case of T3, T4, MI, and DI (chronic exposure), the toxicological responses to the binary mixture were synergistic. In contrast, for CbE (acute exposure) and GST (chronic exposure), the toxicological responses to the binary mixture were antagonistic. In all other cases, the interactions were additive.

4. Discussion

In the present study, we provide the first evidences that highlight the synergistic effects of GBH and As(III) on the enzymatic activities, levels of thyroid hormones, RBC proliferation and DNA damage in amphibian tadpoles. To mimic field exposures to these pollutants, we used an

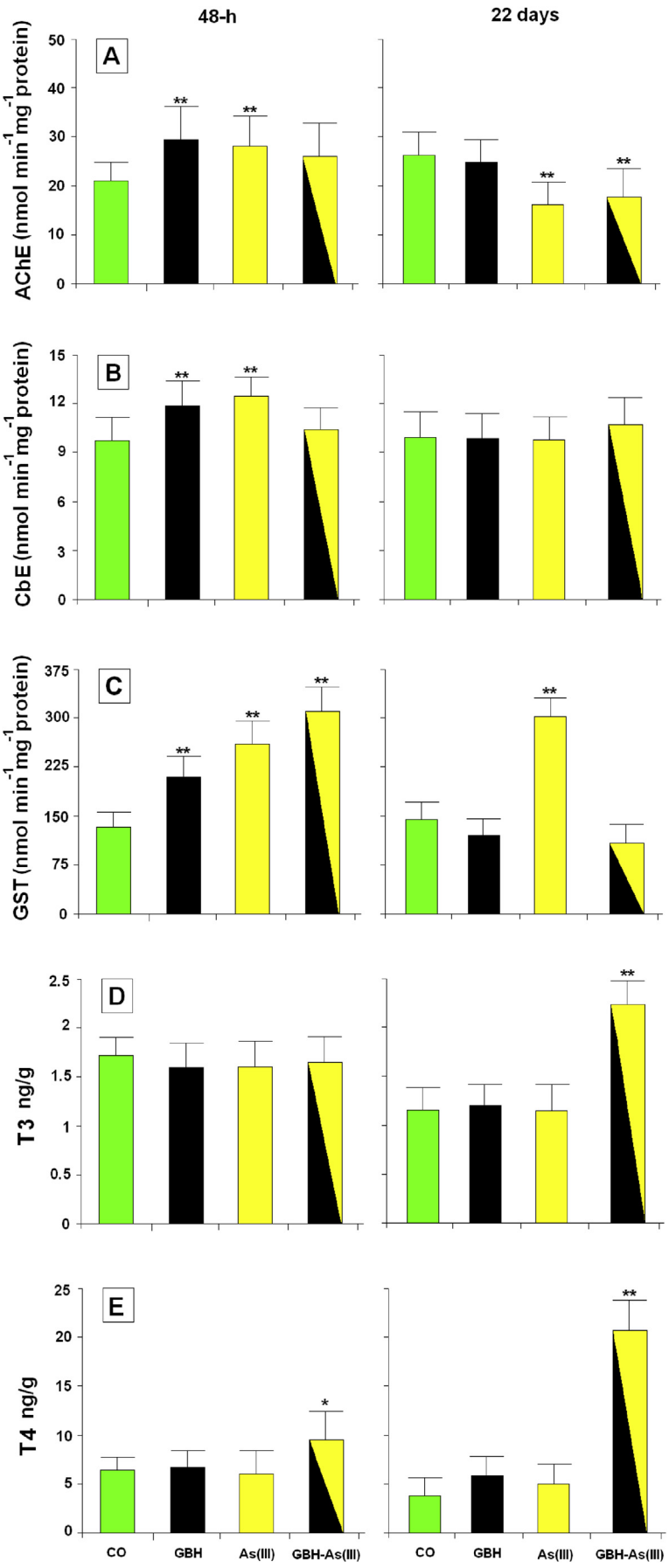

Fig. 1. Short-term (48-h) effect of the NOEC (10 mg/L) of glyphosate-based herbicide (GBH), arsenite (As(III)), and mixture of 50:50 v/v GBH-As(III) and chronic toxicity (22 days) of the NOEC-48h/8 (1.25 mg/L) of GBH and As(III), and the mixture of 50:50 v/v GBH-As(III) in *Rhinella arenarum* tadpoles. (A) Acetylcholinesterase (AChE) activity, (B) Carboxylesterase (CbE) activity, (C) Glutathione S-transferase (GST) activity, (D) Level of triiodothyronine (T3), and (E) Level of thyroxine (T4). Data are expressed as mean ± SD, $n = 7–10$. Significantly different from control (CO) (*$p < 0.05$; **$p < 0.01$; Dunnett's test).

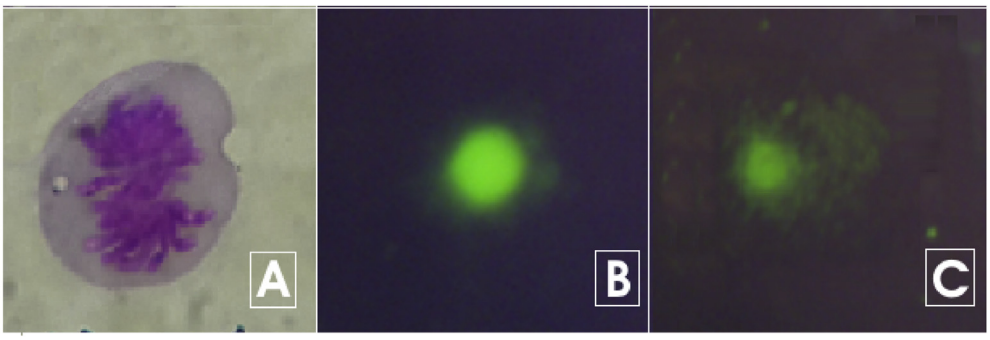

Fig. 2. Red blood cells (RBCs) of *Rhinella arenarum* tadpoles. (A) Mitotic RBC [May-Grünwald-Giemsa-stained blood smear (1000 x)]. DNA damage index (DI) (based on the Comet assay) at 22 days of exposure to the NOEC-48h/8 of glyphosate-based herbicide (GBH) and arsenite (As(III)), and the mixture of 50:50 v/v GBH-As(III); digitized images showing examples of undamaged (Type I comet) (B) and medium damage (Type III comet) RBCs (C) (400 x).

environmentally realistic concentration of their mixture. In this sense, chronic assays were performed at 1.25 mg/L of GBH-As(III) 50:50 mixture exposure, so the glyphosate concentration we tested was lower (0.625 mg/L) than those reported as of environmental relevance (i.e.700 ug/L; Peruzzo et al., 2008). However, in a first instance examination (acute assay) the obtained NOEC value (10 mg/L) is likely to represent the worst-case scenario exposure for wild tadpoles (i.e. 21 mg/L of GBH; Mann and Bidwell, 1999). In general, before studying chemical mixtures, it is essential to quantify the individual acute toxicity of each test compound. The toxicity values of GBHs depend considerably on the chemical structure of the surfactants used in the trademarks (Mann et al., 2009; Lajmanovich et al., 2011). In amphibian tadpoles, GBH toxicity, generally calculated for Roundup Original®, presents a wide range of LC_{50}, from 72.8 mg/L (Glifoglex®; Brodeur et al., 2014) to 1–5 mg/L (Relyea and Jones, 2009). In the present investigation, the LC_{50} 48-h value for premetamorphic tadpoles (GS 26-30) was 45.95 mg/L (for Roundup Ultra-Max®). It is important to note that, in previous studies, the LC_{50} 48-h value for prometamorphic *R. arenarum* tadpoles (GS 36-38) was 2.42 mg/L (Lajmanovich et al., 2011). Similarly, Candioti et al. (2010) revealed that *R. arenarum* prometamorphic tadpoles are more sensitive to pesticides than premetamorphic tadpoles. Our LC_{50} 48-h results (49.44 mg/L) are similar to those calculated for GBH (Roundup®) in Asian common toad (*Duttaphrynus melanostictus*) tadpoles (GS 25-26) (Jayawardena et al., 2011).

The LC_{50} 48-h value of 37.32 mg/L calculated for As(III) is consistent with the value previously determined by Mardirosian et al. (2015) for *R. arenarum* embryos (24.3 mg/L, Sodium (meta) arsenite). Similarly, Brodeur et al. (2009) obtained a LC_{50} 48-h value of 56.61 mg/L for the same anuran species (tadpoles in GS 25) exposed to As(III). Moreover, the NOEC 48-h value here determined (10 mg/L) was the same as that reported by Mardirosian et al. (2015) for sodium arsenite in *R. arenarum* embryos. Despite the widespread use of GBH and the impact of the environmental contamination of As on animal health, no studies have evaluated the toxicity of the mixture of both contaminants in amphibian tadpoles. Wang et al. (2017) proved that glyphosate and As showed synergistic toxicity in the nematode *Caenorhabditis elegans*. In this study, *R. arenarum* tadpoles exposed to a 50:50 mixture of GBH and As showed synergistic effect according to the MAI and low additivity according to the negative result of *AI*. This result highlights that single-compound assessments may underestimate the real risk for aquatic wildlife species in ponds where mixtures of GBHs and common forms of inorganic As species potentially occur.

AChE (a neural enzyme that catalyzes the hydrolysis of the neurotransmitter acetylcholine in the nervous system of animals) is a useful biomarker that has been used in studies of amphibian tadpoles exposed to

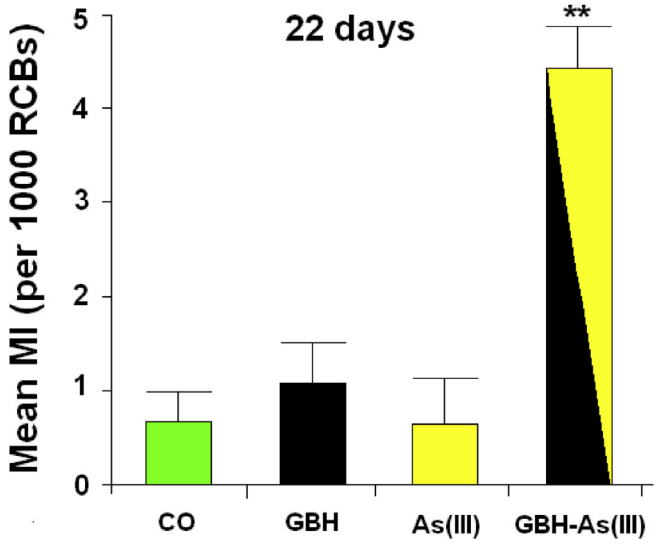

Fig. 3. Mitotic index (MI) over 1000 red blood cells (RBCs) in *Rhinella arenarum* tadpoles exposed to chronic toxicity (22 days) at the NOEC-48h/8 nominal value (1.25 mg/L) of glyphosate-based herbicide (GBH) and arsenite (As(III)), and the mixture of 50:50 v/v GBH-As(III). Data are expressed as mean ± SD, $n = 7–10$. Significantly different from control (CO) (**$p < 0.01$; Binomial Proportion's test).

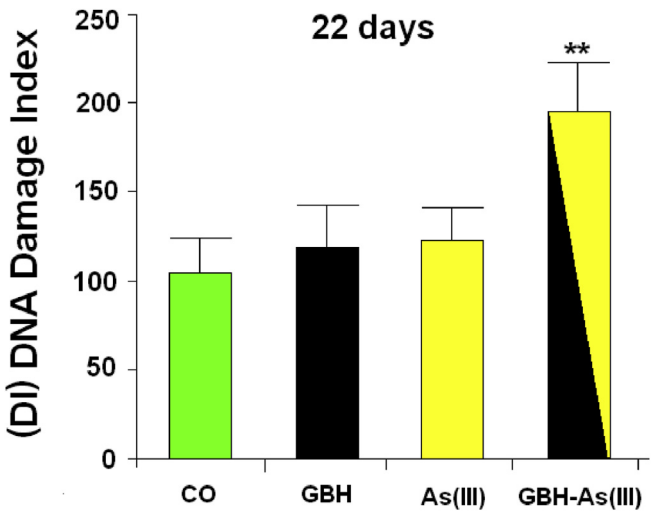

Fig. 4. Average DNA damage index (DI) (based on the Comet assay) in RBCs from *Rhinella arenarum* tadpoles exposed to chronic toxicity (22 days) at the NOEC-48h/8 nominal value (1.25 mg/L) of glyphosate-based herbicide (GBH) and arsenite (As(III)), and the mixture of 50:50 v/v GBH-As(III). Data are expressed as mean ± SD, $n = 7$. Significantly different from control (CO) (**$p < 0.01$; Dunnett's test).

pesticides (Freitas et al., 2017). In agreement with this, the NOEC 48-h of GBH increased AChE activity compared to controls. Samanta et al. (2014) found similar results in different tissues of teleostean fishes exposed to GBHs. In contrast, in our chronic toxicity assay at the NOEC/8 48-h, AChE activity was not affected. However, in a previous study of our lab, we found that exposure of *R. arenarum* tadpoles to a GBH inhibited AChE activity (Lajmanovich et al., 2011), corroborating the findings of several studies that have evaluated the exposure of different animal species (mussel, fish, rat) to GBHs (Sandrini et al., 2013). In the present study, the activity of AChE of *R. arenarum* tadpoles was induced at a high GBH concentration (NOEC 10 mg/L) and short-term exposure (48 h). In addition, after short exposure of tadpoles to the NOEC 48-h value of As (III), AChE activity (which is a good biomarker of As-induced neurotoxicity (Ali et al., 2010)), was increased. This increase may be due to a cholinergic imbalance, as demonstrated for exposure to metals, for example, Cd and Zn in rats (Carageorgiou et al., 2005). Similar findings have been reported in insects by Nath et al. (2015), in which acute exposure to As (96-h) increased AChE activity. In contrast, in the present study, chronic exposure to As(III) and GBH-As(III) (NOEC-48h/8, 1.25 mg/L) led to a decrease in AChE activity. Although the mechanisms that may explain how As decreases or increases AChE activity have not yet been well determined, they may involve the reaction between As and the free sulfhydryl groups of these enzymes (Ali et al., 2010). In the present study, the mixture of GBH and As(III) showed only additive effects for AChE activity. In this sense, several reports have suggested a decrease in AChE activity and severe damage to the nervous system associated with As chronic toxicity (Nagaraja and Desiraju, 1994). For example, in *Swiss albino* mice treated with As 136 ppm for 15 days, AChE was inhibited by

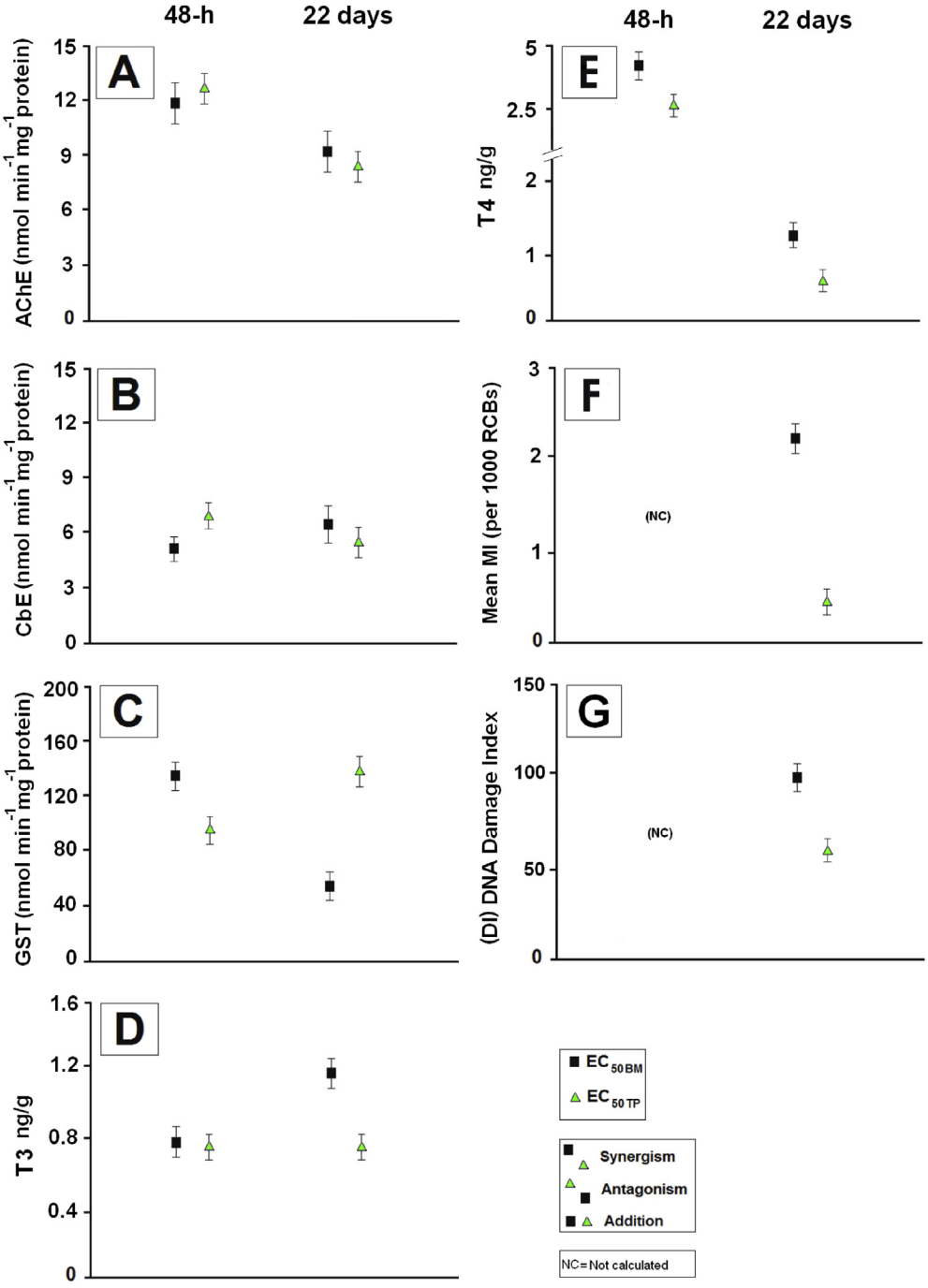

Fig. 5. Short-term (48-h) and chronic toxicities (22 days) of glyphosate-based herbicide (GBH), arsenite (A(III)), and the mixture of 50:50 v/v GBH-As(III) (see Fig. 1 for exposure values) on the activities of Acetylcholinesterase (AChE) (A), Carboxylesterase (CbE) (B), glutathione S-transferase (GST) (C), and levels of triiodothyronine (T3) (D), thyroxine (T4) (E), Mitotic Index (MI) (F), and DNA damage index (DI) (G) in *Rhinella arenarum* tadpoles. Median effective concentration calculated in binary mixture ($EC50_{BM}$) and median effective concentration (toxic potential) ($EC50_{TP}$). Each point represents a mean ($n = 7–10$), and the error bar indicates the 95 % CIs of the mean.

Fig. 6. Graphic summary that represents the synthesis of the novel potential geographical ecotoxicological risk that includes regions from Mexico to Argentine.

interruption of the acetylcholine cleavage activity (Sharma et al., 2018).

Another important B-esterase enzyme used to monitor aquatic fauna exposed to pesticides is CbE. This enzyme plays an important role in heavy metal detoxification (Stone et al., 2002). The present study showed that CbE activity increased at NOEC 48-h exposure to GBH and As(III). However, after exposure to the GBH-As mixture and chronic assays (NOEC-48h/8), CbE was similar to control values. The increase in CbE activity observed at short-term and higher concentrations (NOEC 48-h) suggests an increase in ester hydrolysis (Alyokhin et al., 2008). In amphibian tadpoles, CbE contributes to the detoxification of GBH, considering that CbE probably binds to the phosphate fraction (Lajmanovich et al., 2011). Possibly, the CbE detoxification response at chronic exposure (NOEC-48h/8) to As (III) may be compensated with the induction of GST activity (Lajmanovich et al., 2019). GST is part of the first line of cellular defense, and is also used as a biomarker of pesticide exposure. GST forms a family of multifunctional Phase II biotransformation enzymes (defense enzymes) that are mainly present in the cytosol of cells involved in the transport and elimination of reactive compounds in aquatic organisms (Livingstone, 2003). Thus, GST provides general protection against toxic electrophilic compounds such as As (Todorova et al., 2007). Regarding this issue, Greani et al. (2017) reported a considerable increase in GST activity in fish chronically exposed to As, and, in the present study, after 48-h exposure to GBH, As (III), and the mixture of both, GST activity increased in comparison with that of controls. Regarding GST activity, the mixture of both toxicants demonstrated to be synergistic at short time and antagonistic in chronic exposure. In accordance with that reported by Greulich and Pflugmacher (2004), our findings suggest that GST provides a first line of metabolic defense at short time of exposure. In chronic exposure to As, GST was induced, playing an important role in the detoxification process. This is probably due to the high bioaccumulation of As (III) in tadpole tissues (Chen et al., 2009). It should be noted that *R. areanarum* tadpoles actively bioaccumulate As (Mardirosian et al., 2017), and sublethal concentrations of this element cause a significant increase in GST activity in these organisms.

Thyroid hormones are essential for tadpole metamorphosis (Denver et al., 2002). These hormones provide information regarding *in vivo* mechanisms that occur throughout the developmental stages. Classical histochemical staining studies have shown that thyroid hormone treatment increases the mitotic activity in different tadpole organs (Weiss and Rossetti, 1951). In vertebrates, thyroid hormones are also crucial in metabolism and RBC proliferation (Dorgalaleh et al., 2013). The control mechanisms of RBC differentiation and hemoglobin synthesis during amphibian metamorphosis are thyroxine-induced. In this sense, Lanctôt et al. (2013) described the effects of GBHs on thyroid hormones in amphibian tadpoles, while Davey et al. (2008) determined As effects on the receptor of thyroid hormones. Several chemicals, including pesticides and metals, exert acute or chronic effects on thyroid hormones and an alteration in the thyroid hormone balance causes structural and functional changes in tadpole tissues and physiology (Miyata and Ose, 2012). In our study, after chronic exposure to the GBH-As (III) 50:50 v/v mixture, T3 and T4 levels were increased synergistically. These results are crucial considering that both toxicants are thyroid hormone disruptors (de Souza et al., 2017). However, to our knowledge, there is no published study on the synergistic effects of the GBH-As(III) mixture on the thyroid hormones of other vertebrates. The high levels of thyroid hormones reached could affect larval physiology and morphology, and indicate that endocrine-disrupting chemicals such as GBHs have high ecological significance (Miyata and Ose, 2012). Several chemicals (or their mixtures) have shown to have effects on the levels of thyroid hormones in vertebrates, and disruption of the thyroid axis has been recognized as an important point for the regulation of the use of chemicals (de Souza et al., 2017).

Thyroid hormone disorders are also linked to hematological abnormal parameters (Iddah et al., 2013). In the present study, the GBH-As (III) mixture increased the MI of RBCs. It should be noted that cytotoxic effects of GBH, including reduction of mitotic and proliferation indices on lymphocytes, have been previously reported (Siviková and Dianovský, 2006). Additionally, although As(III) has been found to induce abnormal results in mitotic cells and apoptosis in some cancer cell lines of normal human fibroblasts (Yih et al., 2012), studies regarding these effects are scarce. The thyroid hormone disorder caused by both pollutants in amphibians could be explained by some molecular pathways. According to de Souza et al. (2017), GBH exposure affects the expression of several genes, such as deiodinases (*Dio2*), thyroid-transporters (*Slco1c1*) (formerly *Oatp1c1*), thyroid-receptor (*Thra1* and *Thrb1*), (*Slc16a2*), and others, which are regulated by thyroid hormone metabolism. Similarly, Lanctôt et al. (2013) reported that GBH disrupts the expression of genes involved in the control of tadpole metamorphosis, specifically, thyroid-related (trβ, *Dio2*, and *Dio3*) and stress-related (*crf* and *grII*) genes. With respect to the effects of exposure to As on amphibian metamorphosis, Davey et al. (2008) reported that the expression of a transfected thyroid receptor (TR) response element-luciferase construct as well as that of the endogenous TR-regulated type I deiodinase (*Dio1*) gene are significantly altered.

Regarding DNA damage, the alkaline single-cell gel DNA electrophoresis (comet) assay is considered one of the most sensitive genotoxic tools to detect a broad spectrum of DNA break down in several species (Collins, 2004), and is an effective method to detect the DNA damage caused by As (Saleha Banu et al., 2001). In amphibian tadpoles, several studies have used the comet assay to determine the effect of several contaminants, including GBHs (Clements et al., 1997). In our present study, GBH did not induce DNA damage at NOEC-48h/8 (i.e. 1.25 mg/L). Similar findings were observed in *Lithobates catesbeianus* tadpoles exposed to Roundup® 1.69 mg/L in a chronic assay (Clements et al., 1997). On the other hand, in some freshwater aquatic species such as *Oreochromis mossambicus*, As induced DNA damage in a

concentration-response curve (Ahmed et al., 2011). In contrast, in the present study, *R. arenarum* tadpoles exposed to 1.25 mg/L As(III) showed no DNA damage in RBCs. Interestingly, a higher significant percentage of DNA damage was observed in tadpoles exposed to the GBH-As(III) mixture. This effect was found to be synergistic, as it was for thyroid hormone levels and the MI of RBCs. Previous studies using the comet assay have shown that high levels of thyroid hormones lead to an increase in DNA damage in human lymphocytes (Djelic and Anderson, 2003), suggesting that thyroid hormone endocrine disruption has genotoxic effects (Caballero-Gallardo et al., 2016).

Finally, our results highlight the hazardous association between GBHs and As(III), and provide more data and lines of evidences for the risk evaluation and characterization of these toxicants as observed in Andra Pradesh (India) and Central America (Jayasumana et al., 2014). The dispersion of As on surface and ground waters and the massive use of GBHs in Argentine territories reach more than 60 % and results presented here should be of concern to human, veterinary and wildlife health systems, being necessary to perform studies that determine the biochemical processes involved in the toxicological effects of GBH and As mixtures. Moreover, is important to emphasise that additional work at even more low environmentally-relevant concentrations would be needed to provide insight into the importance of this phenomenon in the environment and risks of effects on wild amphibians.

5. Conclusions

Wild amphibians are habitually simultaneously exposed to a mixture of toxic chemicals (e.g. pesticides, metals, emerging contaminants). In particular, the areas of soybean production in Argentina are similar to those where As is found in high concentrations. Thus, they provide a potential risk scenario for environmental health issues (see Fig. 6). The results of our study suggest that the mixture of the commercial formulation of GBH (Roundup Ultra-Max®) and As(III) has several toxic synergistic effects on *Rhinella arenarum*, a native toad species, altering its antioxidant system (GST), disrupting the expression of thyroid hormones (T3 and T4), inducing RBC proliferation, and causing DNA damage.

Declarations

Author contribution statement

Rafael Lajmanovich, Paola Peltzer: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Andres Attademo: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Candela Martinuzzi: Performed the experiments; Contributed reagents, materials, analysis tools or data.

María F. Simoniello, Carlina Colussi, Ana Cuzziol Boccioni, Mirna Sigrist: Contributed reagents, materials, analysis tools or data.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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