Research article

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

Rafael C. Lajmanovich\textsuperscript{a, d,*}, Paola M. Peltzer\textsuperscript{a, d}, Andrés M. Attademo\textsuperscript{a, d}, Candela S. Martinuzzi\textsuperscript{a, d}, María F. Simioniello\textsuperscript{b}, Carla L. Colussi\textsuperscript{a}, Ana P. Cuzziol Boccioni\textsuperscript{a, d}, Mirna Sigrist\textsuperscript{c}

\textsuperscript{a} Laboratorio de Ecotoxicología, Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral (FBCB-UNL), Casilla de Correo 242, Santa Fe, 3000, Argentina

\textsuperscript{b} Cátedra de Toxicología, Farmacología y Bioquímica Legal. Facultad de Bioquímica y Ciencias Biológicas (FBCB), Universidad Nacional del Litoral (UNL), Santa Fe, Argentina

\textsuperscript{c} Programa de Investigación y Análisis de Residuos y Contaminantes Químicos (PRINARC), Facultad de Ingeniería Química, FIQ-UNL, Santa Fe, Argentina

\textsuperscript{d} Consejo Nacional de Investigaciones Científicas Técnicas (CONICET), Buenos Aires, C1033AAJ, Argentina

**ARTICLE INFO**

Keywords: Environmental science, Toxicology, Amphibians, Glyphosate, Arsenite, Biomarkers, DNA

**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 LC50 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 \(= 10 \text{ mg/L} \) and LOEC \(= 20 \text{ mg/L} \) 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). In the chronic assay, 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, Saxal et al. (2017) reported that, according with sampling periods, the majority glyphosate water samples were below 0.1 \(\mu\)g/L (4.7\% of the data were above 240 \(\mu\)g/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...
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 sympatic 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-estersases 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 Kinniburn, 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 Kinniburn, 2002), and its valence state affects its toxicity and bioavailability, being As(V) less toxic to animals than As(III) (Okhi 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 R. arenarum. Likewise, Mardiroian 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 (Olimstead and LeBlanc, 2005). In this context, Samel 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 Cae. norwidiiis 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 determine 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 tanks (with dechlorinated tap water, DTW, pH 7.4 ± 0.05; conductivity, 165 ± 12.5 μhos/cm; dissolved oxygen concentration, 6.5 ± 1.5 mg/L; hardness, 52.5 mg/L of CaCO3 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 ASH (2004). Specimens were euthanized by immersion in a solution of 0.1% tricaine methanesulfonate (TMS, MS-222) buffered to pH 7.8 with NaHCO3 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 As2O3 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 (LC50) and NO2- 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 μg/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) mixture for 22 days. This concentration value was to a nominal concentration of 1.25 mg/L of GBH, As(III), and GBH-As(III) (50:50 v/v mixture). The assay was carried out with 25 mmol/L Tris-HCl, 1 mmol/L CaCl2 (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 · 10⁻⁴ 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 mammalian serum GST activity. The enzyme assay was performed at 340 nm in a mixture containing 910.0 μL of 100 mM L⁻¹ sodium-phosphate buffer (pH 6.5), 20.0 μL of 0.2 mM L⁻¹ 1-chloro-2, 4-dinitrobenzene, 50.0 μL of 5 mM L⁻¹ reduced glutathione, and 20.0 μL 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 · 10⁻⁵ M⁻¹ cm⁻¹).

2.4. Enzymatic activities (48 h and 22 days)

Total thyroid hormone levels were measured using enzyme-linked electro-chemiluminescent immunonassay (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 4.0X10⁵ 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 Na₂EDTA, 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 Na₂EDTA; 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 comet from each tadpole were classified into five comet types according to tail size and intensity (0 = undamaged, 1 = low damage, 2 = medium damage, 3 = moderate damage, and 4 = severe damage). Results are expressed as the DNA damage index (DI = n1 + 2n2 + 3n3 + 4n4, 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 (LC50) 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 post-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: \( \text{MAI} = \frac{B_m/B_i}{A_m/A_i} \) where \( A_m \) and \( B_m \) were the individual toxicant, \( A_i \) and \( B_i \) were the individual and mixture LC50s, respectively, and \( S \) was the sum of biological activity. The toxic compounds are considered antagonistic if \( S < 1 \), synergistic if \( S > 1 \), or additive if \( S = 1 \). 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 \( S < 1 \), Additive Index (AI) = \( 1/S - 1 \). If \( S > 1 \), 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 EC50 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 LC50 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 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

| Treatment | 48-h LC50 (mg/L) (95% CI) | NOEC and LOEC (mg/L) | S' | AI |
|-----------|--------------------------|----------------------|---|---|
| GBH       | 45.95 (37.59–56.17) 10 | 0.15 – 1.95 | 20 |
| As(III)   | 37.32 (30.11–46.26) 10 | 0.15 – 1.95 | 20 |
| GBH and As(III) | 30.31 (26.58–34.58) 10 | 0.15 – 1.95 | 20 |

(NEC) No Observed Effect Concentration. (LOEC) Lowest Observed Effect Concentration. (S) Sum of Biological Activity. (AI) Additive Index.

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).
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 LC50, from 72.8 mg/L (Glifoglex®; Brodeur et al., 2014) to 1–5 mg/L (Releya and Jones, 2009). In the present investigation, the LC50 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 LC50 48-h value for metamorphic R. arenarum tadpoles (GS 36-38) was 2.42 mg/L (Lajmanovich et al., 2011). Similarly, Candioti et al. (2010) revealed that R. arenarum metamorphic tadpoles are more sensitive to pesticides than premetamorphic tadpoles. Our LC50 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 LC50 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 LC50 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...
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...
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 metabolitic 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. areanum 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, Lanctot 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 (Sivková 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 (Sclcl1) (formerly Oatplc1), thyroid-receptor (Thra1 and Thrb1), (Scl16a2), and others, which are regulated by thyroid hormone metabolism. Similarly, Lanctot et al. (2013) reported that GBH disrupts the expression of genes involved in the control of tadpole metamorphosis, specifically, thyroid-related (tr4, Dio2, and Dio3) and stress-related (cre and grf) genes. With respect to the effects of exposure to As on amphibian metamorphosis, Davey et al. (2008) reported that the expression of a translocated 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 Lthobates catatbatan 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 (Caballerò-Gallardo et al., 2016).

Finally, our results highlight the association between GBHs and As(III), and provide more data and lines of evidence 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 GBHSs 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, it is important to emphasize 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 antioxidative 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, Carla Colussi, Ana Cuzzol Bocconii, Mirna Sigrist: Contributed reagents, materials, analysis tools or data.

**Funding statement**

This study was supported in part by National Agency for Promotion of Science and Technology (ANPCyT FONCYT PICT, N° 1069), and Course of Action for Research and Science Promotion (CAI + D-UNL, PIC N° 100004LI).

**Competing interest statement**

The authors declare no conflict of interest.

**Additional information**

No additional information is available for this paper.

Acknowledgements

This work is dedicated to the memory of Prof. Dr. Andres Carrasco, University of Buenos Aires, Argentina, an outstanding scientist and a motivation to those concerned with stopping the social and environmental impact of transnational corporations and the governmental establishment that legalizes the use of harmful pesticides. We thank Mario Milocco for the artwork in the graphical abstract and Agustín Bassó for laboratory assistance. We also thank María Victoria Gonzalez Eusevi (Scientific English Service) for proofreading the manuscript and correcting grammar and spelling mistakes. Finally, we thank the anonymous reviewers for their constructive criticism and suggestions.

**References**

Ahmed, M.K., Habibullah-Al-Mamun, M., Hossain, M.A., Arif, M., Parvin, E., Akter, M.S., Khan, M.S., Islam, M.M., 2011. Assessing the genotoxic potentials of arsenic in tilapia (*Oreochromis mossambicus*) using alkaline comet assay and micronucleus test. Chemosphere 84, 143–149.

Ali, N., Hoque, M.A., Haqu, A., Salam, K.A., Karim, M.R., Rahman, A., Islam, K., Saud, Z.A., Khalek, M.A., Akhond, A.A., Hossain, M., Mandal, A., Karim, M.R., Miyataka, H., Himeno, S., Hossain, K., 2010. Association between arsenic exposure and plasma cholinesterase activity: a population based study in Bangladesh. Environ. Health 9, 36.

Alyokhin, A., Baker, M., Mota-Sanchez, D., Dively, G., Graffis, E., 2008. Colorado potato beetle resistance to insecticides. Am. J. Potato Res. 85, 395–413.

ASHI-American Society of Ichthyologists and Herpetologists, 2004. Guidelines for Use of Live Amphibians and Reptiles in Field and Laboratory Research. Herpetological Animal Care and Use Committee (HACCC), Washington DC.

Ayres Jr., M., Ayres, D., Santos, A., 2008. Bioisotati, Versoöto 0. Sociedade Civil Mamirauá. MCT-CNpg, Belem, Brazil.

Balter, G., Gref, F., Pallut, B., Hoffmann, J., Brühl, C.A., Wagner, N., 2018. How does changing pesticide usage over time affect migrating amphibians: a case study on the use of Glyphosate-Based Herbicides in German agriculture over 20 years. Front. Environ. Sci. 6, 6.

Blettler, M.C., Oberholster, P.J., Madlala, T., Eberle, E.G., Amser, M.L., De Klerk, A.R., Truter, J.C., Marchese, M.R., Latoisinos, F.G., Szputyni, R., 2019. Habitat characteristics, hydrology and anthropogenic pollution as important factors for distribution of biota in the middle Parana River, Argentina. Esophyly. Hydrob. 19, 296–306.

Brodeur, J.C., Assrey, C.M., Sutrum, A., Herkovits, J., 2009. Acute and subchronic toxicity of arsenic and zinc to tadpoles of *Rhinella arenarum* both alone and in combination. J. Toxicol. Environ. Health 72, 884–890.

Brodeur, J.C., Poliseri, M.R., D’Andrea, M.F., Sánchez, M., 2014. Synergy between glyphosate- and cypermethrin-based pesticides during acute exposures in tadpoles of the common South American toad *Rhinella arenarum*. Chemosphere 11, 70–76.

Bunyan, P.J., Jennings, D.M., 1968. Organophosphorus poisoning: some properties of beetle resistance to insecticides. Am. J. Potato Res. 85, 395–413.

Caballerò-Gallardo, K., Olivero-Velber, J., Freeman, J.L., 2016. Toxicogenomics to evaluate endocrine disrupting effects of environmental chemicals using the zebrafish model. Curr. Genom. 17, 515–527.

Candioti, J.V., Natalie, G.S., Soloneski, S., Ronco, A.E., Larramendy, M.L., 2010. Sublethal and lethal effects on Rhinella arenarum (Anura, Bufonidae) tadpoles exerted by the primicarb-containing technical formulation insecticide Alfacida ®. Chemosphere 78, 249–255.

Carageorgios, H., Tzotzis, V., Sideris, A., Zarros, A., Tsakiris, S., 2005. Cadmium effects on brain Acetylcholinesterase activity and antioxidant status of adult rats: modulation by Zinc, Calcium and L-Cysteine co-administration. Basic Clin. Pharmacol. Toxicol. 97, 320–324.

Chen, T.H., Gross, J.A., Karasov, W.H., 2009. Chronic exposure to pentavalent arsenic of tadpoles exposed to the GBH-As(III) mixture. Bioaccumulation and reduced swimming performance. Ecotoxicology 18, 587–593.

Clements, C., Ralph, S., Petras, M., 1997. Genotoxicity of select herbicides in *Rana catesbeiana* tadpoles using the alkaline single-cell gel DNA electrophoresis (comet) assay. Environ. Mol. Mutagen. 29, 277–288.

Cohen, S., Arnold, L., Beck, B., Lewis, A., Eldan, M., 2013. Evaluation of the carcinogenicity of inorganic arsenic. Crit. Rev. Toxicol. 43, 711–752.

Collins, A.R., 2004. The comet assay for DNA damage and repair: principles, applications, and limitations. Mol. Biotechnol. 26, 249–261.

Curti, L.M., Peltzer, P.M., Martinuzzi, C., Attademo, M.A., Seib, S., Simonelli, M.F., Lajmanovich, R.C., 2017. Altered development, oxidative stress and DNA damage in *Lepadictys chaquensis* (Anura: Lepidicidae) larvae exposed to the pirimicarb-containing technical formulation insecticide Alfacida ®. Chemosphere 78, 249–255.

Davey, J.C., Nomikos, A.P., Wungirionum, M., Sherman, J.R., Ingram, L., Bati, C., Lariviere, J.P., Hamilton, J.W., 2008. Arsenic as an endocrine disrupter: arsenic disrupts retinoic acid receptor-and thyroid hormone receptor-mediated gene regulation and thyroid hormone-mediated amphibian tail metamorphosis. Environ. Health Perspect. 116, 165–172.
Denver, R.J., Boone, G.C., Glenneimer, K.A., 2002. Endocrinology of complex life cycles: amphibians. In: Pfaff, D., Arnold, A., Egen, A., Fahrbach, S., Moss, R., Rubin, R., editors, Hormones, Brain and Behavior. 2. Academic Press, San Diego, 468–413.

de Souza, J.S., Kirys, M.M., da Conceição, R.R., Glebicki, G., Romanò, R.M., Ortiga- Carvalho, T.M., Giannocca, G., da Silva, I.D., Dias da Silva, M.R., Romanò, M.A., Chimamia, M.I., 2017. Perinatal exposure to glyphosate-based herbicide alters the thermoregulatory axis in male rats. Toxicology 377, 25–37.

djelic, N., Anderson, D., 2003. The effect of the antioxidant catalase on oestrogen, triiodothyronine and noradrenaline in the Comet assay. Teratog. Carcinog. Mutagen. 23, 69–81.

Dorgaleah, A., Mahmoodi, M., Varmaghami, B., Kiani Node, F., Saeedi Kia, O., Alizadeh, S.H., Tabibian, Sh., Barmed, T., Momeri, M., Abbasian, S., Khashi Khoti, Z., 2013. Effect of pesticide dysfunctions on blood cell count and red blood cell indices. Iran J. Pediatr. Hormon. Oncol. 3, 73–77.

Ellman, G.L., Courtney, K.D., Andresr, Jr., Vaughan, J., Featherstone, R.M., 1961. A new and rapid colorimetric determination of cholinesterase activity. Biochem. Pharmacol. 7, 88–95.

Freitas, J.S., Felício, A.A., Teresa, F.B., Alves de Almeida, E., 2017. Combined effects of temperature and clozomane (Gamit®) on oxidative stress responses and B esterase activity of Physalaemus notaretir (Leiuperidae) and Rhinella schneideri (Bufonidae) tadpoles. Chemosphere 185, 58–562.

Gancedo, B., Alonso-Gómez, A.L., de Pedro, N., Delgado, M.J., Alonso-Redate, M., 1997. Changes in thyroid hormone concentrations and total contents through ontogeny in three anuran species: evidence for daily cycles. Gen. Comp. Endocrinol. 107, 240–250.

Goerner, K.J., 1960. A simplified table for staging anuran embryos and larvae with notes on identification. Herpetologica 16, 183–190.

Greani, S., Lourkisti, R., Berti, L., Marchand, B., Giannettini, J., Santini, J., Quilichini, Y., 2017. Estimation of thyroid hormones in anuran embryos and tadpoles. J. Toxicol. Pathol. 25, 1

Habdous, M., Vincent-Viry, M., Visvikis, S., Romanò, G., 2002. Uptake and effects on detoxication enzymes of glyphosate-based herbicide (Roundup) on oxidative stress responses and B esterase activity of Physalaemus nattereri (Leiuperidae) and Rhinella schneideri (Bufonidae) tadpoles. Chemosphere 185, 58–562.

Hertzberg, R., MacDonell, M., 2002. Synergy and other ineffective mixture risk assessments. Food. Chem. Toxicol. 108 (Pt A), 30–42.

Hadi, M.A., Kato, O., 2012. Thyroid hormone-disrupting effects and the amphibian metamorphosis assay. J. Toxicol. Pathol. 25, 1–9.

Hosn, R., Khodaei, D., Rezaei, S.H., Jamali, M., 2018. Effect of tissue culture medium on the formation of retinoic acid in anuran embryos. Int. J. Biological Sciences 14, 176.

Jayawardena, U.A., Navaratne, A.N., Amerasinghe, P.H., Rajakaruna, R.S., 2011. A simplification of the Greulich–Pyle table for staging anuran embryos and larvae with notes on identification. Gen. Comp. Endocrinol. 107, 190.

Kingsley, G.R., 1942. The direct biuret method for the determination of serum proteins as an index of liver disease. J. Biol. Chem. 249, 7130–7139.

Khatib, Z., 2013. Effect of thyroid dysfunctions on blood cell count and red blood cell indices. Iran J. Hematol. Oncol. 11, 714–719.

Kujawa, K., Hanakata, R., Akaike, K., Utsunomiya, T., Ishii, K., 2014. Effect of the glyphosate-based herbicide (Roundup) on oxidative stress responses and B esterase activity of Physalaemus nattereri (Leiuperidae) and Rhinella schneideri (Bufonidae) tadpoles. Chemosphere 185, 58–562.

Lajmanovich, R.C., Lajmanovich et al. Heliyon 5 (2019) e02601

Paravani, E.V., Demonte, L., Repetti, M.R., Bedendo, D.J., Medero, S.L., Goette, J.J., 2013. Changes in thyroid hormone concentrations and total contents through ontogeny in three anuran species: evidence for daily cycles. Gen. Comp. Endocrinol. 107, 190.

Paganelli, A., Gnazzo, V., Acosta, H., Lomaz, A.L., de Pedro, N., Delgado, M.J., Alonso-Bedate, M., 1997. Effect of tissue culture medium on the formation of retinoic acid in anuran embryos. Int. J. Biological Sciences 14, 176.

Pegas, P., Andrade, M.I., 2017. Induction of micronuclei and nuclear abnormalities in lymphocytes from human volunteers exposed to glyphosate-based herbicide (Roundup) in the environmental area of the town of Rhinella arenarum. Environ. Toxicol. Chem. 36, 2009–2014.

Qi, X., Chen, X., Wang, Y., Wang, H., 2018. Effects of glyphosate on cholinesterase activity of grasshopper. Am. Eurasian J. Toxicol. Sci. 7, 173–176.

Platts, K.E., LeBlanc, G.A., 2005. Toxicity assessment of environ- mentally relevant pollutant mixtures using a heuristic model. Integr. Environ. Assess. Manag. 1, 122–127.

Proctor, E.M., Vaz, P., 2012. Thyroid hormones and hematological indices in liver disorders patients at a hospital in Eritrea. J. Biol. Chem. 249, 7130–7139.

Reddy, R.B., Jones, D.K., 2000. The synergistic toxic effect of mercuric acid formation. J. Biol. Chem. 269, 107,013–107,019.

Rocha, R.R., Glebocki, G., Romano, R.M., Ortiguero, J.I., 2015. Effect of tissue culture medium on the formation of retinoic acid in anuran embryos. Int. J. Biological Sciences 14, 176.

Sanchez, M.I., 2017. Effects of tissue culture medium on the formation of retinoic acid in anuran embryos. Int. J. Biological Sciences 14, 176.

Sharma, A., Kshetrimayum, C., Sadhu, P.: in vitro studies. Aquat. Toxicol. 173, 1–17.

Singh, N.P., McCoy, M.T., Tice, R.R., Schneider, E.L., 1988. A simple technique for quantification of low levels of DNA damage in individual cells. Exp. Cell Res. 175, 292–297.

Sivková, K., Dánovský, V., 2006. Cytoprotective effect of tissue culture medium on cultivated bovine peripheral lymphocytes. Int. J. Hyg Environ Health. 209 (1), 15–20.

Sivková, K., Dánovský, V., 2006. Cytoprotective effect of tissue culture medium on cultivated bovine peripheral lymphocytes. Int. J. Hyg Environ Health. 209 (1), 15–20.

Sivková, K., Dánovský, V., 2006. Cytoprotective effect of tissue culture medium on cultivated bovine peripheral lymphocytes. Int. J. Hyg Environ Health. 209 (1), 15–20.
Argentina) using the ARSOlux biosensor. Int. J. Environ. Res. Public Health 12 (5), 5465–5482.

Smedley, P., Kinniburgh, D., 2002. A review of the source, behavior and distribution of arsenic in natural waters. Appl. Geochem. 17, 517–568.

Smedley, P.L., Nicoll, H.B., Macdonald, D.M.J., Kinniburgh, D.G., 2008. Arsenic in groundwater and sediments from La Pampa Province, Argentina. In: Bundschuh, J., Armienta, M.A., Birkle, P., Bhattacharya, P., Matuschill, J., Mukherjee, A.B. (Eds.), Natural Arsenic in Groundwaters of Latin America. Taylor & Francis, pp. 35–45.

Stone, D., Jepson, P., Laskowski, R., 2002. Trends in detoxification enzymes and heavy metal accumulation in ground beetles (Coleoptera: Carabidae) inhabiting a gradient of pollution. Comp. Biochem. Physiol. Part C: Pharmacol. Toxicol. 132, 105–112.

Todorova, T., Vuilleumier, S., Kujumdzieva, A., 2007. Role of glutathione S transferases and glutathione in arsenic and peroxide resistance in Saccharomyces cerevisiae: a reverse genetic analysis approach. Biotechnol. Biotechnol. Equip. 21, 348–352.

U.S. Environmental Protection Agency, 1989. Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Fresh Water Organisms. Environmental Protection Agency, Cincinnati.

Vaira, M., Akmentins, M., Attademo, M., Baldó, D., Barrasso, D.A., Barrionuevo, S., et al., 2012. Categorización del estado de conservación de los anfibios de la República Argentina. Cuad. Herpetol. 26, 131–159.

Ventura-Lima, J., Bogo, M.R., Monserrat, J.M., 2011. Arsenic toxicity in mammals and aquatic animals: a comparative biochemical approach. Ecotoxicol. Environ. Saf. 74, 211–218.

Wang, Y., Ezemaduka, A.N., Li, Z., Chen, Z., Song, C., 2017. Joint toxicity of arsenic, copper and glyphosate on behavior, reproduction and heat shock protein response in Caenorhabditis elegans. Bull. Environ. Contam. Toxicol. 98, 465–471.

Weiss, P., Rossetti, F., 1951. Growth responses of opposite sign among different neuron types exposed to thyroid hormone. Proc. Natl. Acad. Sci. U.S.A. 37, 540–556.

Wu, X., Cobbina, S.J., Mao, G., Xu, H., Zhang, Z., Yang, L., 2016. A review of toxicity and mechanisms of individual and mixtures of heavy metals in the environment. Environ. Sci. Pollut. Res. 23, 8244–8259.

Yih, L.H., Wu, Y.C., Hsu, N.C., Kuo, J.H., 2012. Arsenic trioxide induces abnormal mitotic spindles through a Pip4kIIγ/Rho pathway. Toxicol. Sci. 128, 115–125.