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Comparative assessment of individual and mixture chronic toxicity of glyphosate and glufosinate ammonium on amphibian tadpoles: A multibiomarker approach

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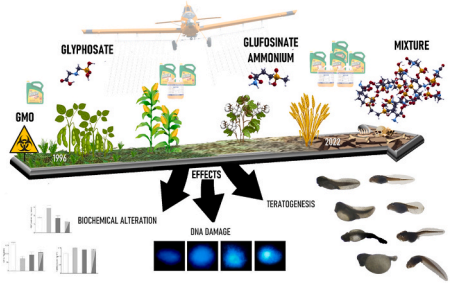
HIGHLIGHTS

- Glyphosate (GLY) and glufosinate (GA) sublethal effects differ in mixture.
- Mixture of both herbicides was antagonistic for most of analyzed biomarkers.
- GA-based herbicide has higher toxicity than GLY-based herbicide for tadpoles.

GRAPHICAL ABSTRACT



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ABSTRACT

The aim of the present study was to assess the ecotoxicity of glyphosate and glufosinate ammonium mixtures on amphibian tadpoles and the potential impact of mixture in aquatic ecosystems health. The bonding properties of the mixture based on computational chemistry and an experimental bioassay on morphology, DNA damage and biochemical biomarkers on tadpoles of the common toad Rhinella arenarum were studied. The results of the density functional theory analysis showed trends of the pesticides clustering to form exothermic mixtures, suggesting the likelihood of hot-spots of pesticides in real aquatic systems. In addition, biological effects of individual pesticides and the mixture were studied on tadpoles over 45 days-chronic bioassay. The bioassay consisted of four treatments: a negative control (CO), 2.5 mg L^{-1} of a glyphosate-based herbicide (GBH), 2.5 mg

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L^{-1} of a glufosinate ammonium-based herbicide (GABH) and their 50:50 (% v/v) mixture (GBH-GABH). Morphological abnormality rates were significantly higher in all herbicide treatments with respect to CO at 48 h of exposure. Abdominal edema was the most frequent type of abnormality recorded at 48 h, 10 and 45 days of exposure. DNA damage was recorded in all herbicides treatments. Thyroxin increased only in GABH treatment. Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) significantly increased in GBH treatment, indicating a GBH-neurotoxic effect. Glutathione S-transferase decreased in GABH and GBH-GABH treatments, while catalase decreased in individual GBH and GABH treatments. Overall, teratogenicity, DNA damage, hormonal disruption (T4), and oxidative stress were greater in GABH-treated tadpoles than GBH-treated tadpoles. This study also highlights the robust chemical interaction between the active ingredients of both herbicides, which is reflected on antagonisms in most of analyzed biomarkers, as well as potentiation and additivity in others. Based on our results, the GABH had a higher toxicity than GBH for amphibian tadpoles.

1. Introduction

The increase of chemical pesticides applied to genetically modified (GM) crops tolerant to herbicide glyphosate (GLY), has become one of the most threat for ecological conservation and public health worldwide (Landrigan and Benbrook, 2015). GM herbicide-tolerant crops account for 56% of glyphosate-based herbicides (GBH) use worldwide as pointed out by Duke (2018) for the United States (U.S.) context. In this sense, GBH use on GLY tolerant soybean crops hectares in 2014 was 315.4 million metric tons, with the U.S. (108 million metric tons), Brazil (94.5 million metric tons), and Argentina (56 millions metric tons) accounting for 82% of the global production (Benbrook, 2016). The average of GLY used into GM soybean crops since 1996 was 3 L/Ha. However, Argentine farmers use more than 12 L/Ha since 2013, usually mixed with other herbicides (Sly, 2017).

Moreover, glufosinate ammonium (GA)-based herbicides (GABH) are a broad-spectrum group of herbicides and their use are increasing worldwide to control GLY-resistant weeds (e.g., wheat, soybean, cotton and corn; Áy et al., 2012; Ayala et al., 2019). Concentrations of GLY and GA exceeding the European upper tolerable levels were found in rivers across the north-eastern Italy, (Masiol et al., 2018). Similarly, Geng et al. (2021) reported the wide spatial and seasonal distribution of GLY and GA in groundwater and surface water in agricultural watersheds from China. These authors higlighted the environmental risk of GLY and GA for aquatic organisms that occur in agroecosystems, and the potential contamination of drinking water. In addition, concentrations of GLY and GA were also reported a leats in 60% of water samples from open-reservoir tanks from Mid-eastern Argentina, which are located in the nearest of the agricultural fields and receive agrochemical by aereal derive (Demonte et al., 2018).

As mentioned above, GLY and GA are found together in the environment, however little is known about their interaction at the chemical level. A recent study indicates environmental risk of drinking water consumption and aquatic organisms safety due to GLY and GA exposure in surface waters (Yan et al., 2022). Thus, it is important to determine the molecular interactions between pesticide structures when these are combined, by studying of the thermochemical profiles and the trends to form mixtures (Dorofeeva and Moiseeva, 2006). Recently, the computational chemistry technique named Density Functional Theory (DFT) represents an excellent set of tools in order to study the thermochemistry of mixtures and interfaces, equilibrium properties and reactive mechanisms (Schleder et al., 2019; Li et al., 2021; Makkar and Ghosh, 2021; Mandal et al., 2021; Mo et al., 2021). In a previous study, mixture of contaminants interacted and produced synergistic effects (Lajmanovich et al., 2022). These effects included the exothermic adsorption energies and electronic density interaction for GLY and GA over polyethylene interfaces, showing the potential production of a new pollutant complex, which could affect the organisms and in the environment.

It is important to understand effects of pesticides alone and in mixture in the aquatic environment. A recent study in Argentina highlithed the effects of mixtures of GLY and GA that have been found in aquatic environments on sentinel organisms such as amphibian tadpoles, determining the importance of analyze the relevant concentrations (Peluso et al., 2019). Amphibian development and methamorphosis includes a series of organogenesis steps characterized by increase in metabolism and growth, reasons for why some authors considered amphibians more sensitive than mammals to contaminants (Quaranta et al., 2009). In addition, amphibian metamorphosis is similar to the development of other vertebrates, such as in the intestinal remodeling, brain development and bone differentiation (Buchholz, 2015). Amphibian metamorphosis is a significant parallel perinatal model to the endocrinology of mammalian birth (Wada, 2008). Therefore, thyroid regulated metamorphosis, is an ancestral feature of all chordates. This conservation of a regulatory network supports the homology of metamorphosis in the chordate lineage (Holzer and Laudet, 2013).

The teratogenic effects on vertebrates due to herbicides exposure were reported by several scientific studies (e.g. Alavanja, 2009; Araujo et al., 2016). Gly and GBH produces branchial and body axis abnormalities on *R. arenarum* embryos (Lajmanovich et al., 2003; Mottier et al., 2013; Bach et al., 2016; Babalola et al., 2019). Similarly, recorded field morphological abnormalities in amphibian embryos were linked to endogenous retinoic acid activity in amphibian in the mid-eastern of Argentina (Peltzer et al., 2011; Teglia et al., 2015). Environmental concentrations of GLY increased endogenous retinoic acid activity in amphibian larvae in aquatic systems of Argentina (Paganelli et al., 2010). In addittion, it has been demonstrated that maternal exposure to GBH produces intergenerational congenital anomalies (Milesi et al., 2018).

Similarly, in mice perinatal exposed to low doses of GABH induces changes in cell morphology, proliferation, apoptosis and alters the neurogenesis (Herzine et al., 2016). In addition, environmentally relevant concentrations of GABH produced embryotoxic and teratogenic effects in amphibian embryos (Babalola et al., 2021a), and causes a potential thyroids disrupting and larval development (Babalola et al., 2021b b). Effects of larval amphibians exposed to GABH also inhibited B-esterases and produced genotoxic damage (Peltzer et al., 2013; Lajmanovich et al., 2014). Moreover, both GLY and GA affected oxidative stress biomarkers such as glutathione-S-transferase, (GST) and catalase (CAT; Murussi et al., 2016; Moura et al., 2018; Zhang et al., 2019).

The aim of the present study was to compare and describe the interaction of GLY, GA and their combination by computational modelling. The study also aimed to assess the effects on morphology, DNA and biochemical biomarkers of those herbicides and their mixture on tadpoles of the common toad Rhinella arenarum.

2. Material and methods

2.1. Computational modeling of active ingredients of two herbicides

According to previous studies (Hu et al., 2021; Kaczmarek et al., 2021; Mesnage et al., 2019, 2021; Lajmanovich et al., 2022) the main actives principles GLY and GA were described as neutral species.

DFT calculations were performed using SIESTA code with basis set as described by Soler et al. (2002). Functional and algorithms used were described in Lajmanovich et al. (2022). All dynamics calculations were

performed under Born-Oppenheimer approximation during 10–15 ps, time afer which the final geometries were relaxed. The calculations included geometry relaxation of isolated molecules of the two herbicides; then seven molecules of each pesticides were placed as clusters. Once both clusters were optimized, the interaction between them to form a mixture was analyzed according to dynamics and relaxation (see supplementary data S1 for more detail).

The internal energy (ΔU) for the optimized geometries was calculated as thermodynamic state functions:

$$U_{clusterGA} = U_{clusterGA} - i.U_{moleculeGA} \quad (1)$$

$$\Delta U_{clusterGLY} = U_{clusterGLY} - i.U_{moleculeGLY} \quad (2)$$

$$\Delta U_{mixture} = U_{mixture\ -} \left(U_{clusterGly} + U_{clusterGA}\right) \quad (3)$$

where i is the number of isolated molecules which form the cluster. $U_{clusterGA}$, $U_{clusterGLY}$ and $U_{mixture}$, are the energies of the final optimized geometry for the cluster of GA, cluster of GLY and mixture GA-GLY respectively.

2.2. Test substances

The assay was performed with commercial formulations based on GLY and GA active ingredients, as this is the form in which these are introduced into the environment (Relyea and Jones, 2009). The commercial formulations chosen were: the GBH Mifos®, Chemotecnica Co., Argentina (48% active ingredient [a.i.] GLY, *N*-phosphonomethyl glycine) and the GABH Timón®, Red Surcos S.A., Argentina (20% a.i. GA, ammonium-dl-homoalanin-4-yl (methyl) phosphinate). The concentrations of the a.i. Of the commercial formulations were corroborated to ensure exact calculations for the preparation of the nominal concentrations of the solutions used in the test (Lajmanovich et al., 2019). To confirm the concentration of the a.i. in the, commercial formulations, the samples were diluted with distillated water and analyzed following, Demonte et al. (2018) procedure. A Liquid chromatography tandem-mass spectrometry (LC- MS/MS) using an Acquity UPLC® liquid chromatograph (Waters, Milford, MA, USA) coupled, to a triple quadrupole mass spectrometer equipped with an ESI source (TQD, Waters Micromass, UK) was used in the analysis. Quantification procedure was performed in triplicate using as a, reference the calibration curve from 0.1 μg L^{-1} up to 100 μg L^{-1}, which was constructed in matrix, (matrix-matched calibration). Pre-column derivatization steps, chromatographic system and, operating conditions are described in Supplementary data (S2). The full methodology was, validated as recommend at the European Commission guidance document on analytical quality control, and method validation procedure for pesticides residues (SANTE, 2021). The error between, nominal and measured test concentrations did not exceed 5% (samples of the GBH resulted in 45 ±, 5 of GLY and samples of GABH in 21 ± 2 of GA).

2.3. Experimental design

Surface egg strings of *R. arenarum* were collected from small temporary ponds situated in the natural floodplain of the Paraná River (31° 11′ 31″ S, 60° 9′ 29″ W). This site is considered free of pesticide contamination as an unpolluted site according to previuos studies (Peltzer et al., 2017; Fernández et al., 2020; Cuzziol Boccioni et al., 2021). The collection was allowed by the Ministerio de Ambiente of Santa Fe Province (EXP. N° 02101-0026248-0) from Argentina. Egg strings of several ovipositions (50 cm of each string) were immediately transported in dechlorinated tap water (DTW) to the laboratory. The eggs and hatching embryos were acclimated under laboratory conditions during 48 h:12 h light/dark cycle with DTW, pH 7.4 ± 0.05, conductivity 162 ± 10.5 μmhos cm^{-1}, dissolved oxygen concentration 6.5 ± 1.5 mg L^{-1}, and temperature of 24 ± 2 °C.

The bioassay consisted on four treatments. Nominal concentrations of GBH (2.5 mg.L^{-1}) and GABH (2.5 mg.L^{-1}) were tested individually and in mixture (GBH-GABH) with 50% of each stock solution with the same test (nominal) concentration as in the individual treatments. A negative control (CO) with dechlorinated tap water was also included. These individual concentrations were considered relevant in long term worst-case scenarios for natural lentic aquatic systems (Mann and Bidwell, 1999; Cuzziol Boccioni et al., 2021; Lajmanovich et al., 2022). The solutions were prepared with DTW that was used as a negative control. The solutions were totally renewed every 48 h. The experiment was maintained under the laboratory conditions mentioned above.

The bioassay was performed in triplicated, with a total of 12 tanks. For each tank, 30 ± 5 eggs (Gosner Stage, GS, 1–15, according to Gosner, 1960) were placed with 8 L of solution (meaning ≈100 eggs exposed to each treatmet). When the organisms reached GS 23–24, fed rations of approximately 0.2 g of boiled lettuce every 48 h were provided. At this time, the volume of each container was reduced by half (4 L). The chronic assay ended at 45 days, when most of the control individuals reached stages 31–34 (prometamorphosis).

The eggs and tadpoles used in the bioassays were treated in conformity with the standardized experimental laboratory guidelines of the American Society of Ichthyologists and Herpetologists (2004) and the Institutional Committee for the Care and Use of Animals (IACUC). Specimens were euthanized according to the Animal Euthanasia Guide proposed by the Bioethics Committee and Institutional Animal Care and Use Committee of the FBCB-UNL (Res. CD N: 388/06) using a solution of 0.1% (v/v) tricaine methane sulfonate (MS-222) buffered to pH 7.8 with $NaHCO_3$.

2.4. Morphological analysis

Morphological abnormalities were evaluated at 2, 10 and 45 days of exposure. Ten individuals per tank were randomly collected, euthanized and fixed in 10% (v/v) buffered formalin. Samples were analyzed with a stereoscopic Arcano® microscope, and photographed. Developmental stages were determined according to Gosner (1960). The abnormalities recorded included externally visible alterations in the whole body, as well as in specific organs, and followed Svartz et al. (2012) and Peltzer et al. (2013, 2019) classification. The occurrence of morphological abnormalities is reported as the percentage of individuals of each treatment presenting each type of abnormality.

In adittion, the frequency of morphological abnormalities during the first two days (48 h) of the bioassays was obtained, since early development is defined as the critical period when organogenesis and morphogenesis take place (Peltzer et al., 2017, 2019; Sandoval et al., 2022). In order to obtain the rate of morphological abnormalities, three photographs of each tank per treatment at 48 h of exposure were obtained in top view by a digital Moticam® camera. Photographs were evaluated using Image J software (free available in https://imagej.nih.gov/ij/) to quantify the rate of external morphological alterations in each treatment, identifying those with a normal appearance, and those with visible alteration.

2.5. DNA damage analysis: comet assay and comet assay modified for detection of oxidized bases (Endonuclease III)

At 45 days of exposure, eight larvae from each tank were randomly selected to analyze DNA damage through the Comet assay. The blood of each larva was taken by sectioning behind the operculum and collected with a heparinized capillary tube of 50 μL. The Alkaline Comet Assay was then performed according to the method described by Singh et al. (1988), with specifications previously standarized for amphibian tadpoles (Lajmanovich et al., 2015). Blood samples were diluted 1:19 (% v/v) with PBS medium and used immediately. Then, 2 μL of each diluted blood sample (approximately 4.0×10^3 erythrocytes) was added to 100 μL of 1% low melting point agarose and a slide was prepared. Negative (NC) and positive (PC) controls were included in each run, in both cases

using blood samples from invidious without any treatment. Negative control consisted of blood sample alone and positive control consisted of 100 μL of 25 μM of H_2O_2 added to blood sample slide. After 5 min of treatment on ice, each gel was washed twice in PBS, slides were immersed in lysis solution and the comet assay protocol was followed (Soler et al., 2002). After lysis, the slides were immersed in alkaline buffer (0.3 N NaOH, 1 mM EDTA, pH > 13) during 10 min for DNA unwinding, and electrophoresed in the same buffer. Electrophoresis conditions were: 10 min at 300 mA and 20 V (0.7 V/cm). Then, slides were neutralised in 0.4 M Tris buffer (pH 7.5) with three changes of 5 min each, dehydratated in methanol for 5 min and left to dry at room temperature. The slides were stained with ethidium bromide (0.02 μg/mL) and comet images were observed using a fluorescent microscope (Olympus® CX-40) equipped with an excitation filter (Olympus® U-RFLT 50).

The visual scoring system, a total of 50 comets on each gel (duplicated) were classified as belonging to one of five categories according to the tail and head intensity (different levels of damage). Each category was given a value between 0 and 4: type 0 = undamaged, no tail; I = low damage, II = medium damage, III = moderate damage, and IV = severe damage, almost all DNA in tail (Soler et al., 2002; Collins, 2014; Curi et al., 2017). Examples of each type of comet types obtained in this assay are shown in Fig. 3 C. An overall score, Damage Index (DI), was calculated for each gel by applying the following formula: (percentage of cells in class 0 × 0) + (percentage of cells in class 1 × 1) + (percentage of cells in class 2 × 2) + (percentage of cells in class 3 × 3) + (percentage of cells in class 4 × 4). Consequently, the total score was in the range from 0 to 400.

An increase in DI after incubation with the enzyme, compared with incubation with buffer alone, indicates the presence of oxidized bases. So, oxidative damage to DNA was calculated by subtracting breaks with buffer from breaks with Endonuclease III (ENDO III, obtained from Sigma®) as follows:

Endo III sites = DI with Endo III – DI with enzyme buffer alone (Collins, 2009).

2.6. Biochemical analysis

At the end of the bioassay, biochemical biomarkers were evaluated as additional parameters to the morphological and DNA biomarkers. Biochemical biomarkes included thyroid and cortisol hormones, neurotoxicity and oxidative stress enzymes. For these measurements, 10 tadpoles of each tank were collected, euthanized and individually homogenized at a temperature between 0 and 4 °C, with a Teflon-glass homogenizer and in ice-cold 25 mM sucrose, 20 mM Tris-HCl buffer (pH = 7.4) containing 1 mM EDTA, using a polytron® tissue grinder. During homogenization, the samples were kept cold with ice. Each homogenate sample was centrifuged at 14000 rpm for 10 min, and the resulting supernatant was used for measurements (Lajmanovich et al., 2019, 2022). The protein concentration of samples was determined in duplicate following the Bradford method (Babalola et al., 2019), using bovine serum albumin (BSA, Sigma ®) as standard. Reagents for enzyme determinations were obtained from Sigma. The absorbance was read at 595 nm.

2.6.1. Hormones

Thyroid hormone (thyroxine; T4) and cortisol (CRT) levels were measured using enzyme-linked electro-chemiluminescent immunoassay (ECLIA) kits (COBAS®, Roche Diagnostics, Indianapolis, IN, USA) following specifications for amphibians tadpoles previously set up in our lab (Lajmanovich et al., 2019; Cuzziol Boccioni et al., 2021). Detection limits were 0.42 μg. dl^{-1} for T4 and 0.02 μg. dl^{-1} for CRT.

2.6.2. Neurotoxicity biomarkers

Acetylcholinesterase (AChE) and Butyrylcholinesterase (BChE) activity was determined colorimetrically according to Ellman et al. (1961). For the reactions, specifications previously established for amphibian tadpoles were followed (Attademo et al., 2021; Lourido et al., 2022). Reactives were pucharged A molar extinction coefficient of 13.6×10^3 M^{-1} cm^{-1} was used for both enzymes. AChE and BChE activities were expressed as nmol min^{-1} mg^{-1} protein.

2.6.3. Oxidative stress biomarkers

Glutathione S-transferase (GST) activity was determined as described by Habig et al. (1974) and adapted by Habdous et al. (2002), following specifications for amphibian tadpoles previously standardized in our lab (Attademo et al., 2021; Lajmanovich et al., 2022). The GST activity was expressed as nmol min^{-1} mL^{-1} mg^{-1} protein using a molar extinction coefficient of 9.6×10^3 M^{-1} cm^{-1}.

Catalase (CAT) activity was determined by the rate of H_2O_2 (Biopack ®) decomposition at 240 nm according to Aebi (1984) and adapted for *R. arenarum* by Peluso et al. (2020). A molar extinction coefficient of 40 × M^{-1}·cm^{-1} was used and the activity was expressed as mmol min^{-1} mg^{-1} protein.

2.7. Interaction index

To determinate the type of interaction between the effects of both pesticides, the Interaction Index (II) was calculated for each biomarker according to Mansour et al. (2008): II= (M + C)/(A1+A2)

Where all components represent the mean values obtained for the biomarker: M for the mixture treatment (GBH-GABH), A1 and A2 for the individual treatments (GBH or GABH, respectively) and C for the control treatment. The values obtained for the II define the type of interaction. In case of positive effects (i.e. increase of biomarker in treatments respect control), if II > 1 interaction is defined as a potentiation, and II < 1 antagonism. Oppositely, in case of negative effects (decrease of biomarker in treatments respect to control), if II > 1 interaction is defined as antagonism, and II < 1 potentiation. In any case, if II = 1 the interaction is defined as additivity. Values between 0.95 and 1.05 were considered the limits of an addition, according to Mansour et al. (2008). For the analysis of the interactions, only mixture treatments that caused significant differences in the biomarkers were taken into account or when at least one of the individual concentrations caused significant differences respect to the control group (Bonifacio and Hued, 2019).

2.8. Data analysis

For all parameters, normality was tested by the Kolmogorov– Smirnov test and homogeneity of variances by Levene test. Morphological abnormalities rates were analyzed using the binomial proportion test (Margolin et al., 1983). The rate of morphological alteration is reported as the percentage of individuals with morphological alteration over the total of counted individuals.

DNA Damage index, oxidative damage to DNA, T4 level and enzyme activities (AChE, BChE, GST, CAT) are reported as the mean ± SD and were analyzed with ANOVA and Dunnett's test for post-hoc comparisons. Since CRT levels in some homogenate samples were below the detection limits, results were considered as % of samples over the detection limits. As CRT was expressed as % of detection, this biomarker was analyzed using a binomial test proportion. All these statistical analyses were performed using BioEstat software 5.0 (Ayres et al., 2003). A value of $P < 0.05$ was considered significant.

3. Results

3.1. Computational modeling of active ingredients

The thermochemical scheme of reaction and the molecular dynamic evolution are shown in Fig. 1 and Fig. S1 (Supplementary data), respectively. After the optimization of the single molecules of GLY and GA, the clusters were built by placing of seven molecules estranged 5 Å.

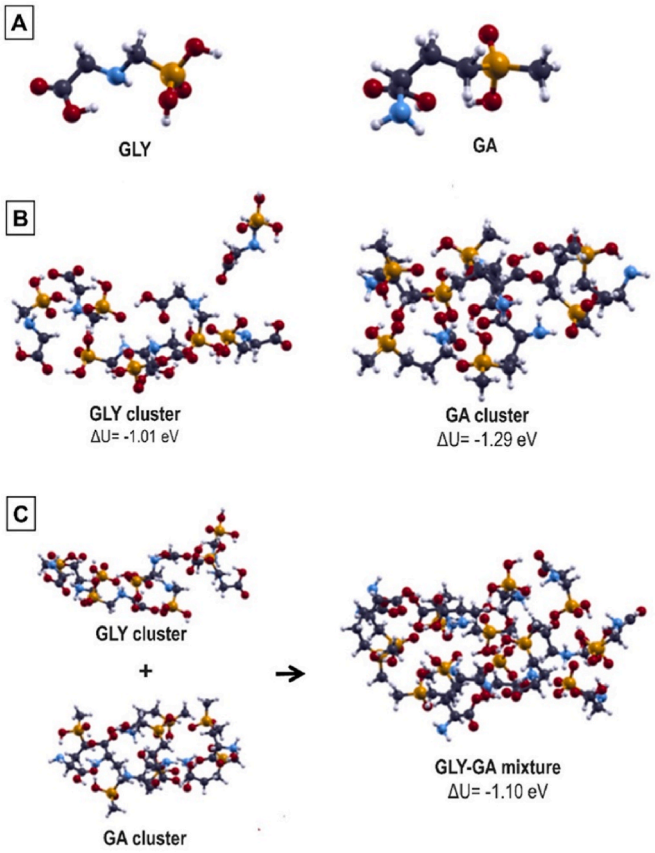

Fig. 1. Final geometries and energies of glyphosate (GLY) and glufosinate ammonium (GA) obtained by computational modeling (Density Functional Theory). Molecules of GLY and GLA (A) were clustered separately (B), and then both clustered were mixed (C).

The molecular dynamics simulation showed a trend to form a cluster for both active principles (see Figs. S1 and S2 of Supplementary data). The formation energies after relaxation for the clusters were −1.01 eV and −1.29 eV for GLY and GA respectively, indicating a considerable exothermic trend mainly in GA. The differential of electronic pseudo-densiti showed in Figs. S1 and S2 indicates that in both systems hydrogen bridge bonds managed the binding among the molecular structure.

Regarding to the reaction of clusters to obtain the mixture, the initial geometry was built placed the optimized geometries of the clusters at 8 Å each other (Fig. S2). The molecular dynamics simulation showed that the pure clusters tend to decouple, leading to a mixture. The differential pseudo electronic density shown in Figs. S3–e with hydrogen bridge bonds as dominant binding among the molecules generating a *wool ball* type particle.

Over the thermochemical reaction, the energy of mixture formation was −1.10 eV, showing that the trends of the pesticide clusters to form the mixture is highly exothermic. The temperature *versus* time plots in Figure S 1–3 showed that the temperature remained stable around 300 K during the whole simulations for every systems.

3.2. Morphological analysis

All treatment showed a higher morphological initial abnormalities rate than CO ($Z = -2.16$; $P = 0.01$ for GBH; $Z = -4.09$, $P < 0.01$ for GABH; $Z = -2.25$, $P = 0.01$ for GBH-GABH). The morphological abnormalities rates obtained at two days of exposure were 5.41% for CO, 9.04% for GBH treatment, 14.08% for GABH treatment, and 9.95% for GBH-GABH treatment. Abdominal edema, curvature of the dorsoventral axis, and bilateral asymmetry were recorded as the most common types of morphological abnormalities in all treatments upon 2 and 10 days of exposure. Fig. 2. Normal configuration (CO) and morphological abnormalities observed in *Rhinella arenarum* exposed to a glyphosate-based herbicide (GBH), glufosinate ammonium based herbicide (GABH) and their 50:50 (% v/v) mixture (GBH- GABH). The first two rows correspond to early development individuals exposed during first 2 days of the assay, the next two rows to premetamorphic tadpoles at 10 days of exposure, and the last rows to prometamorphic tadpoles with 45 days of exposure. References: BL: Bilateral Asimmetry; DV: dorsoventral axis curvature; ED: Abdominal edema; A: other abdominal alterations. Alteration in specific organs: E: Eyes (micro- or macrophtalmy), N: nose; OD: Oral disk, T: Tail, alteration. Escale bar: 2 mm.

Abnormal tail and abdomen were also frequently registered in treatments. Other types of abdominal anomalies, microphthalmia, alterations in the caudal fin and in the oral disc were also observed (Fig. 2).

At the end of the bioassay (45 days), edemas were the most common type of abnormality registered in GBH, while eyes abnormalities were the only type observed in GABH treatment. In GBH-GABH treatment, edemas, bilateral asymmetry and nose abnormalities were recorded.

3.3. DNA damage analysis

The DI obtained for negative and positive controls were 117.25 ± 8.61 and 230.25 ± 13.41, respectively. The DI obtained for CO treatment was 128 ± 15.27, whereas in herbicides treatments, it ranged between 179.5 ± 19.5 (GBH treatment); 179.55 ± 015.51 (GBH-GABH treatment) and 203.66 ± 25.59 (GABH treatment) (Fig. 3A). There was no significant difference between NC and the CO treatment. In contrast, DNA damage was significantly higher in PC and all herbicide treatments (79% in positive control, 40% in GBH and GBH-GABH treatments, and 59% in GABH treatment) respect to CO treatment ($F = 51.93$; $P < 0.01$).

The oxidative damage to DNA based on Endo III sites calculation was 6.42 ± 3.99 for CO. In herbicides treatments it varied between 29.87 ± 17.76 (GBH treatment), 22.33 ± 19.49 (GABH treatment) and 20.44 ± 18.27 (GBH-GABH treatment) (Fig. 3 B). The oxidative damage was significantly higher in GBH treatment respect to CO ($F = 2.65$, $P < 0.05$).

3.4. Biochemical biomarkers

3.4.1. Hormones activity

The T4 level in CO was 7.23 ± 0.35 ng g^{-1}, whereas the T4 values in herbicide treatments were 7.238 ± 0.38 ng g^{-1} (GBH treatment); 7.67 ± 0.71 ng g^{-1} (GABH treatment) and 7.52 ± 0.45 ng g^{-1} (GBH-GABH treatment) (Fig. 4A). A significant increase of 6.15% in GABH treatment was observed in CO ($F = 3.99$; $P = 0.01$).

Only the 20% of CO samples had CRT level over the detection limits, whereas 40% of GBH and GABH samples had CRT level over the detection limits (Fig. 4B). A significant higher percentage (60%) of GBH-GABH treatment samples had CRT level over the detection limits ($Z = -1.82$; $P < 0.05$) than CO samples.

3.4.2. Neurotoxicity biomarkers

The AChE activity in CO was 1.76 ± 0.57 nmol min^{-1} mg^{-1} protein, whereas the AChE activity in herbicides treatments were 2.87 ± 1.03 nmol min^{-1} mg^{-1} protein (GBH), 1.72 ± 0.82 nmol min^{-1} mg^{-1} protein (GABH) and 1.3 ± 0.23 nmol min^{-1} mg^{-1} protein (GBH-GABH) (Fig. 4C). A significant increase of 63% in GBH treatment respect to CO was recorded ($F = 6.71$, $P < 0.01$).

The BChE activity in CO was 0.115 ± 0.059 nmol min^{-1} mg^{-1} protein, whereas the BChE in herbicides treatments were 0.329 ± 0.232 nmol min^{-1} mg^{-1} protein (GBH), 0.128 ± 0.72 nmol min^{-1} mg^{-1} protein (GABH) and 0.297 ± 0.202 nmol min^{-1} mg^{-1} protein (GBH-GABH) (Fig. 4D). The increase of 186% and 158% in GBH and GBH-GABH treatments respect to CO was significant ($F = 4.84$, $P = 0.01$).

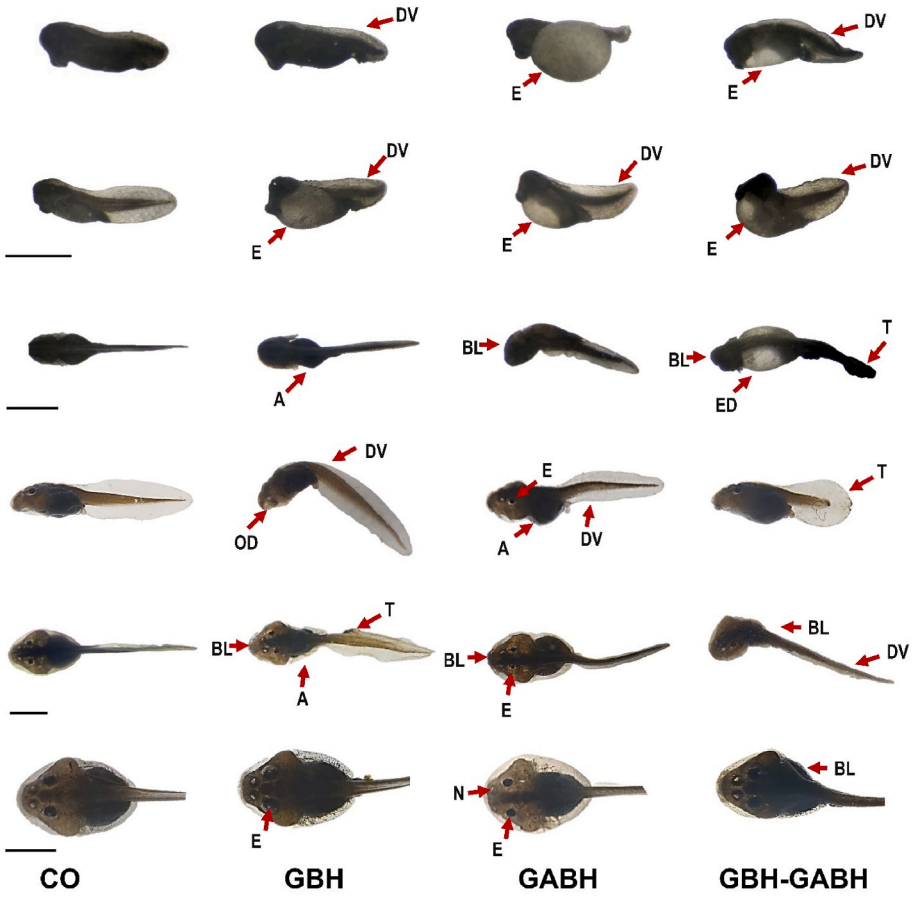

Fig. 2. Normal configuration (CO) and morphological abnormalities observed in Rhinella arenarum exposed to a glyphosate-based herbicide (GBH), glufosinate ammonium based herbicide (GABH) and their 50:50 (% v/v) mixture (GBH- GABH). The first two rows correspond to early development individuals exposed during the first 2 days of the assay, the next two rows to premetamorphic tadpoles at 10 days of exposure, and the last rows to prometamorphic tadpoles with 45 days of exposure. BL: bilateral asymmetry; DV: dorsoventral axis curvature; ED: abdominal edema; A: other abdominal alterations. Alteration in specific organs: E: eyes (micro- or macrophtalmy), N: nose; OD: oral disk, T: tail. Escale bar: 2 mm.

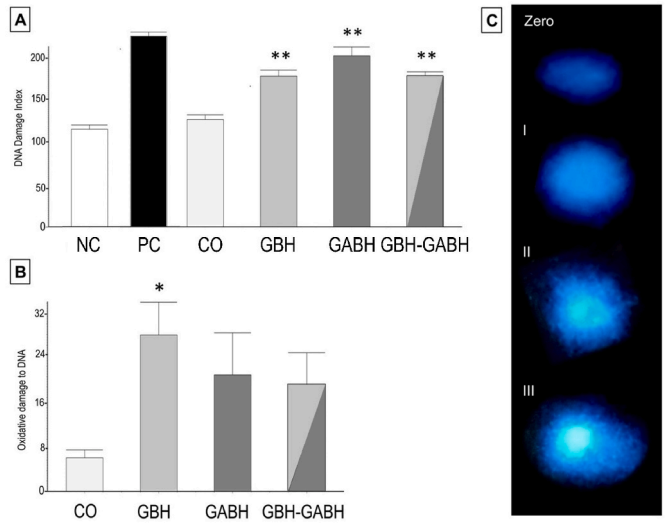

Fig. 3. Results of Comet assay for damage index (A) and oxidative damage to DNA based on detection of Oxidized Bases (Endonuclease III, B) obtained for *Rhinella arenarum* exposed to dechlorinate water (CO), a glyphosate-based herbicide (GBH), glufosinate ammonium based herbicide (GABH) and their 50:50 (% v/v) mixture (GBH- GABH). Negative and positive controls (NC and PC, respectively) for the comet assay are also showed. Examples of undamaged (Zero), Low damage (I), medium damage (II), and moderate damage (III) cells found in treatments are shown in C (magnification 400×).

3.4.3. *Oxidative stress biomarkers*

The GST activity in CO was 74.93 ± 16.05 nmol min $^{-1}$ mg^{-1} protein, whereas in the herbicides treatments were 71.03 ± 029.24 nmol min $^{-1}$ mg^{-1} protein (GBH); 58.65 ± 15.52 nmol min $^{-1}$ mg protein^{-1} (GABH) and 45.8 ± 10.8 nmol min $^{-1}$ mg protein^{-1} in GBH-GABH treatment (Fig. 4E). A significant decrease of 21.73% in GABH and 39% in GBH-GABH treatments respect to CO were recorded ($F = 3.78$, $P < 0.05$).

The CAT activity in CO was 192.26 ± 45.4 mmol min $^{-1}$ mg^{-1} protein, whereas in herbicides treatments were 88.86 ± 52.4 mmol min $^{-1}$ mg^{-1} protein (GBH); 115.22 ± 71.9 mmol min $^{-1}$ mg^{-1} protein (GABH) and 134.3 ± 60.1 mmol min $^{-1}$ mg^{-1} protein (GBH-GABH)treatment (Fig. 4F). A significant reduction of 54%, 40% and 30% in GBH, GABH and GBH-GABH treatments, respectively, respect to CO were recorded ($F = 7.092$; $P < 0.01$).

3.5. *Interaction index*

All of the nine quantitative biomarkers considered for the Interaction Index were significantly affected, by at least, one of the treatments assayed respect to CO. The most sensitive biomarkers were the morphological abnormalities rate, DI and CAT, which responded to both individual treatments (GBH and GABH) and the mixture (GBH-GABH). AChE only reported significant changes in GBH treatment, whereas T4 only showed changes in GABH treatment respect to CO. BChE showed changes in GBH and GBH-GABH treatments, whereas GST showed changes at GABH and GBH-GABH treatments. Interactions between the two herbicides were different according to the biomarker considered (Table 1). The combined action of herbicides was mainly antagonistic for most of the studied biomarkers, with the exception of additivity for T4 and CRT. Potentiation was only observed on GST biomarker.

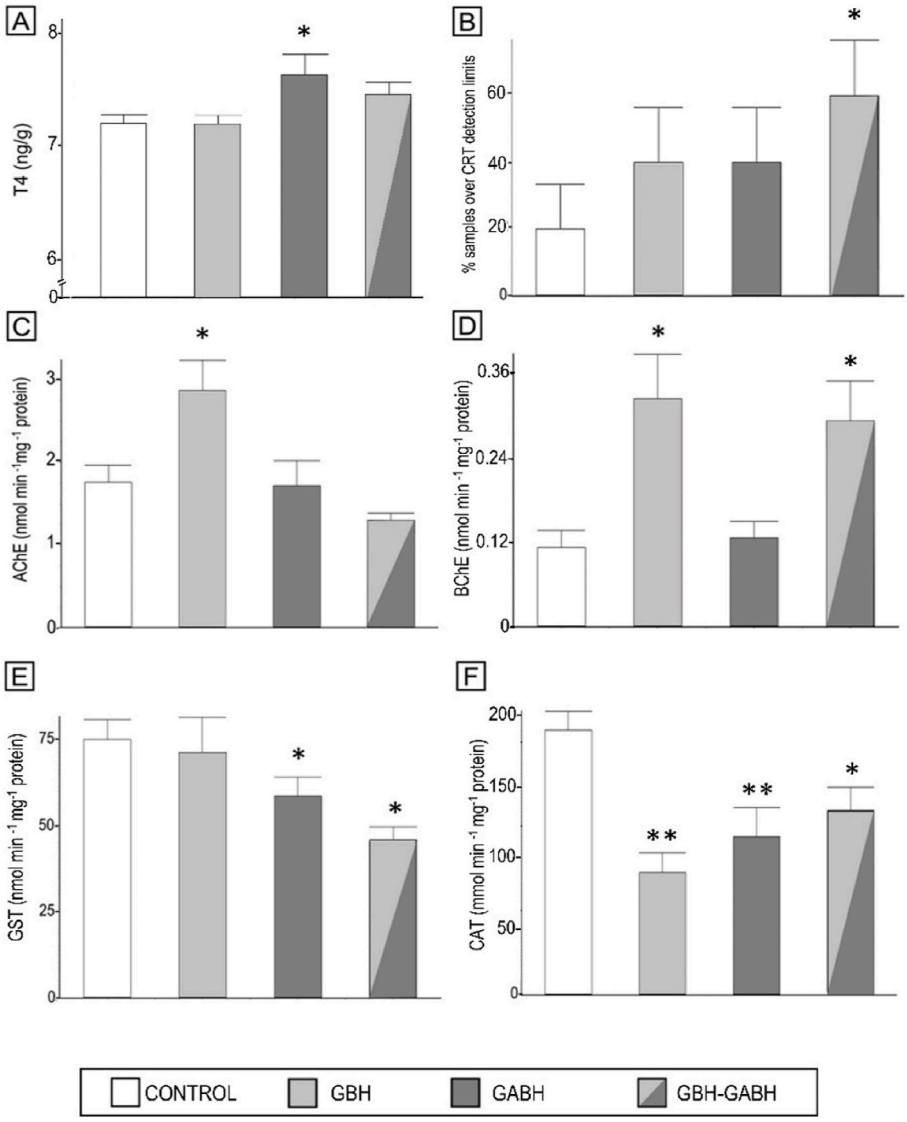

Fig. 4. Biochemical biomarkers analyzed on *Rhinella arenarum* exposed to a glyphosate-based herbicide (GBH), glufosinate ammonium based herbicide (GABH) and their 50:50 (% v/v) mixture (GBH- GABH): A. Thyroxine (T4); B.Cortisol (CRT); C. Acetylcholinesterase (AChE); D. Butyrylcholinesterase (BChE); E. Glutathione S-transferase (GST); F. Catalase (CAT); Asterisks indicates significant differences respect to control (*$P < 0.05$; **$P < 0.01$).

Table 1
Summary of results obtained for each biomarker in *Rhinella arenarum* tadpoles exposed to a glyphosate-based herbicide (GBH), glufosinate ammonium based herbicide (GABH) and their 50:50 (% v/v) mixture (GBH- GABH). The type of interaction of both herbicides in the mixture treatment (Potentiation, Antagonisms or Additivity) was defined according to the Interaction Index value (II) and the individual effects (↑ or ↓, considering treatments with significant increase or decrease respect to CO).

Biomarker	CO	GBH	GABH	GBH-GABH	II Individual effect		Type of interaction
Morphological abnormalities rate (%)	5.418	**9.04**	**14.08**	**9.95**	0.23↑		Antagonism
Damage index	128	**179.50**	**203.66**	**179.55**	0.80↑		Antagonism
Oxidative damage to DNA	6.43	**29.87**	22.33	20.44	0.22↑		Antagonism
T4 (ng g^{-1})	7.24	7.24	**7.67**	7.52	0.99↑		Additivity
Cortisol (% of samples over detection limits)	20	40	40	**60**	1↑ ↑		Additivity
AChE (nmol min^{-1} mg^{-1} protein)	1.76	**2.87**	1.72	**1.30**	0.38	↑	Antagonism
BChE (nmol min^{-1} mg^{-1} protein)	0.11	**0.329**	0.128	**0.29**	0.90	↑	Antagonism
CAT (mmol min^{-1} mg^{-1} protein)	192	**88**	**115**	**134**	1.60	↓	Antagonism
GST (nmol min^{-1} mg^{-1} protein)	74.94	71.04	**58.65**	**45.80**	0.93	↓	Potentiation

Values are expressed as mean obtained for each treatment, in the respective unit for each biomarker. Bold numbers indicate significant differences in the treatment respect to CO ($P < 0.05$).

4. Discussion

Glyphosate and glufosinate ammonium are the most employed herbicides in many countries around the world (Geng et al., 2021). According to the ArgenBio database (2022), there are currently 23 approved transgenic events for simultaneous tolerance to both for GLY

and GA herbicides, mainly for corn, soybean and cotton. However, the greatest environmental risk for the use of these "stacks event" which leads to the massive use of these two herbicides. For example, China has recently approved a transgenic combination event for drought, GLY and GA tolerant soybeans ArgenBio database, 2022). China is certainly the largest buyer of soybeans from Argentina (Sly, 2017) and this massive demands together with the mentioned event result in an increase of pesticide use, contamination and expansion of soybean crops. The soybean expansion toward semiarid ecosystems and wetlands is exponential, desvasting forests across Argentina (Grau et al., 2005; Asociación para la Promoción de la Cultura, 2020). These scenarios, similar to those in South America, contribute highly to climate change (Siyum, 2020).

There is numerous evidences that simultaneous presence of GLY and GA represent a risk for organisms in different environmental matrices (e.g., Demonte et al., 2018; Geng et al., 2021; Yan et al., 2022). However, potential interaction of both herbicides remain poorly studied and deserve more attention specially nowadays where trends to by applied together are improved.

The results of our study are the first worlwide that explains biological-toxicological interactions between GLY and GA-based herbicides that thermodynamically showed to form an exothermic and antagonistic mixture. Computational model results indicated that GLY and GA, as well as their cluster, can eventually interact to form mixtures with different electronic structure than the isolated molecules or clusters, which might change the toxicity effect. In addition, to the exothermic trend of the mixtures form, it is important to point out, that the final geometry of the clusters evolution showed that GLY and GA molecules are in a mixed (or non-ordered) distribution. Thus, the functional groups (mainly in the phosporous site) are now involved in a intramolecular stable interaction. This potential produce a change on the interfaces of the active principles (pesticides) and the active sites (biological) at some point in their mechanisms for every biomarker. The observed changes in geometry of the mixture might increment the surface/volume ratio, which usually increment the reactivity toward interfaces. Although a quantum mechanic molecular docking simulation for every systems should be necessary to get deeply insight the biomarker-mixture interaction, it is suggested that the strong interaction of the functional groups among the herbicide molecules would lead to a different electronic structure in the mixture is suggested. This could have an important role in the final activity and the different behaviors recorded on the toxicity testing (Zhan et al., 2013; Bhatt et al., 2021).

Development of amphibian embryos experience complex morphological changes that can be adversely affected by exposure to several types of pollutants (Vandenberg et al., 2012). In our study, high morphological abnormalities rates at early stages from all herbicides treatments indicate that GBH and GABH are teratogenic to amphibians. The morphological abnormalities rates recorded for control tadpoles (≈5%) were in accordance with the rates expected for amphibian wild populations, typically under 5% (Blaustein and Johnson, 2003). Previous reports from our lab, have shown teratogenic effects of sublethal concentrations of GBH on Scinax nasicus (mouth and craniofacial deformities, bent curved tails, and eye abnormalities; Lajmanovich et al., 2003), another common species that co-occur with R. arenarum tadpoles. A more recent study on the evaluation of GBH and ciprofloxacin exposure determined that GBH produced higher rates of edema, abnormal visceral organization, bilateral asymmetry and eyes abnormalities in R. arenarum tadpoles (Cuzziol Boccioni et al., 2021). One explanation may be linked to the increase of the endogenous retinoid activity in amphibian tadpoles that inhibits the expression of genes involved in multiple developmental processes such as left-right asymmetry, prechordal mesoderm, and notochord development and dorsal-ventral patterning of the neural tube, described by Paganelli et al. (2010) for GBH.

Moreover, teratogenic effects of GA were also previously reported for amphibian tadpoles as well as other vertebrate species. The teratogenic effects were more stronger in GABH at initial exposure (two days) than in the other herbicide treatments. Babalola et al. (2021b) studied morphological alterations of *Xenopus laevis* tadpoles exposed to a 1.6–3.0 mg L^{-1} GABH. In coincidence with our observations, these authors reported edema, gut abnormalities, axial, eye and head alterations as the most frequent observed types of abnormalities. Teratogenic effects of GA were also reported for zebrafish larvae and mouse embryos exposed to GA, producing tail curvature and head alterations, respectively (Watanabe and Iwase, 1996; Wang et al., 2016)

DNA damage of tadpoles samples exposed to GBH, GABH and GBH-GABH treatments were observed. GBHs have already been reported as genotoxic that may impact the genetic integrity of several amphibian species (e.g., Herek et al., 2021; Pavan et al., 2021). Genotoxicity of GABH on R. arenarum tadpoles has already been reported through the assessment of micronucleated erythrocytes and other erythrocyte nuclear abnormalities (Lajmanovich et al., 2014). Mouchet et al. (2006) suggested that the commet assays appears to be a sensitive and suitable method for detecting genotoxicity in amphibian. So, the increased DNA damage observed in our study for GABH exposed tadpoles confirms and reinforces the genotoxic effect of GABH as well as GBH on tadpoles of R. arenarum. There are several other studies that differ on the genotoxicity of the GA. According to some authors (Ebert et al., 1990; Schulte--Hermann et al., 2006), GA does not posed genotoxic risk, whereas more recent studies advised about the genotoxic effects of both GA and GABHs (Kanaya and Tsubokawa, 2007; Xiong et al., 2019).

Moreover, oxidative damage was verified by the modified comet assay only in GBH treated tadpoles. This result is in accordance with the studies of Odetti et al. (2020) and López González et al. (2022), both studies carried out on *Caiman latirostris* exposed to GLY, which oxidative damage was detected both with Endo III (oxidized pyrimidines) and FPG (oxidized purines) at both low and high GLY concentrations.

The chronic exposure with different herbicide treatments indicates that T4 levels increased only in GABH treatment. Babalola et al. (2021b) confirmed alteration of the thyroid axis of *Xenopus laevis* tadpoles exposed to a GABH. For aquatic wildlife species, thyroid disruption, therefore, could compromise health, fitness, and ultimately survival due to the various processes that the hormones mediate, including growth, development, and reproduction (Marlatt et al., 2013; Grott et al., 2022). Moreover, Cuzziol Boccioni et al. (2021) and Liu et al. (2021) suggested that alteration of T4 levels would be related to morphological alterations on tadpoles exposed to pesticides (e.g., GBH) in mixture with other contaminants of emergent concern (e.g. antibiotics).

The glucocorticoid cortisol plays a key role in the metabolic and ionic adjustments necessary to the stress response (Soso et al., 2007). Chronic exposure of R. arenarum larvae to the GBH and GABH mixture showed a significant increase in the percentage of CRT (60%, compared to 20% of CO). Similarly, high CRT is observed in amphibian tadpoles when exposed to herbicides such as atrazine and GLY (Burraco and Gomez-Mestre, 2016; McMahon et al., 2017). In this sense, during tadpoles chronic exposure to stressors (i.e. Roundup™), the hypothalamic-pituitary interrenal (HPI) axis may become disrupted, and the effects of increased CRT become immunosuppressive (Gabor et al., 2019).

The result of our study regarding to neurotoxicity, suggest a possible colinesteric effect GBH on the increase activity of AChE and BChE. Both AChE and BChE are considered essential components of cholinergic synapses in the nervous systems (Franjesevic et al., 2019). BChE is recognized to be more sensitive to pesticide exposure than AChE, since BChE is less specific in terms of substrates and therefore has a more important role than AChE in the biotransformation of xenobiotics (Sanchez-Hernandez, 2007; Peluso et al., 2021). A significant increase in the BChE was also recorded in GBH-GABH treatment. Its increased activity leads to increased neurotransmitter release in cholinergic synaptic clefts and the consequently over-stimulation of postsynaptic receptors. Therefore, it is probable assuming that such an increase is related to the effects of oxidative stress induced by the GBH (Malafaia et al., 2021). Although the specific mechanisms through the increase of both enzymes

occurred in GBH presence in chronic exposure, the disruption in the nervous system by alteration of these enzymes can lead to behavioral changes that represents a risk for organisms survival (Sanchez-Hernandez, 2007).

AChE nor BChE were significantly affected by exposure to 2.5 mg L^{-1} GA. In contrast, previous acute assays from our lab showed significant inhibition of both enzymes activities in *Boana pulchellus* tadpoles exposed to 15 mg L^{-1} GA, and inhibition of AChE in *Scinax squalirostris* tadpoles exposed to 6.25 mg L^{-1} GA (Peltzer et al., 2013; Lajmanovich et al., 2022). These differences would suggest a dintinctive response according to species-specific action, time of exposure or effects at higher concentrations than those tested in the present study. However, the known effect of GA on the nervous system should not be underestimated. Several studies warned about consequences of GA exposure on nervous systems, including structural alteration of the tissue (Calas et al., 2008; Lee and Kang, 2021; Yeon et al., 2022) and other parameters related to the nervous functioning as locomotor performance and muscular function (Kutlesa and Caveney, 2001; Peltzer et al., 2013).

The GST plays a key role on phase II of the biotransformation process that protects cells from contaminants (Van der Oost et al., 2003). It is expected that GST increases in detoxification processes, since it contributes to protecting tissues from oxidative stress (Livingstone, 2001). Several studies reported induction of GST, when animals were acutely exposed to individual and mixed contaminants (Lajmanovich et al., 2014; Güngördü et al., 2016; Freitas et al., 2017). However, in the present study, GST activity was decreased in GABH and GBH-GABH chronic exposure. The activation of GST activity in response to acute exposure in contrast to variety of response in long chronic exposure depending on treatments (the absence of changes in GBH treatmen, and decrease in GABH and GBH-GABH treatments), suggest a time and compound-dependent action. This chronic response of GST was also observed in a previous study with the same tadpole species (Cuzziol Boccioni et al., 2021). This pattern may be related to the inactivation of GST by toxicants; to depletion of glutathione conjugation, leading GST to lose its activity (Ensibi et al., 2012), or to a physiological adaptation of these organisms to the pollutants. The decrease of GST in GABH treatment may be related to the well known interference of GA in the biosynthesis of gluthatione due to structural similarity to glutamate and consequently interference of its role (Suter and Sachsse, 1986; Ebert et al., 1990).

The CAT activity is related to the reactive oxygen species (ROS) elimination in the bioactivation process of xenobiotic (Sk and Bhattacharya, 2006), thus the induction of CAT provides a first line of defense against ROS (Costa et al., 2008). In the present study, the decrease of CAT activity in all treatments might suggest an impairment in the response towards increased ROS generation. A decrease in CAT activity has been already described for amphibian exposed to different pesticides including GLY and GA (Sotomayor et al., 2015; Ferrari et al., 2011; Reichert et al., 2022). Peluso et al. (2020) reported differential response of CAT on embryos and tadpoles of *R. arenarum* exposed to GLY solution. The temporary change in CAT activity that ended in a decrease in tadpoles may indicate exposure to substances that generate oxidative stress as observed here in tadpoles exposed to GBH, GABH and GBH-GABH. Overall, the studied enzymes of the antioxidant defense mechanisms needed for the protection and bioactivation processes were altered, suggesting an impairment on the normal response of CAT on GBH, GABH and GBH-GABH mixture exposure.

The GBH-GABH mixture had an antagonistic response in most of the evaluated biomarkers, whereas only hormonal response showed additivity and GST potentiation. The diversity of combined effects of contaminants on amphibians has been well explained from different bioecological aspects. Some authors concluded that the combined effect depends on the concentration at which the contaminants are studied, producing antagonism at low concentrations and synergy at higher concentrations (Kunz and Fent, 2009; Ma et al., 2016). Other authors consider the proportion of each compound on the mixture as these informed a synergistic effect of non-equitoxic mixture unlike the equitoxic mixture (Peluso et al., 2022). Other authors also evaluated the changes in the mixture components interaction at different exposure times, and found antagonism in acute exposures and synergy in chronic exposures to mixtures of xenobiotics were observed (Svartz et al., 2012). Besides, the biomarkers are distributed in different places in organisms, which increase the complexity of the system when evaluating biological mechanisms on active sites and behaviors. Moreover, toxicity of mixtures herbicides on *R. arenarum* varying from additive to antagonist were also reported by Ruiz Arcaute et al. (2020) when evaluate the interaction of GLY-dicamba and GLY-flurochloridone. These authors also highlighted the considerations of unknown excipients in commercial formulations additional to the active ingredients in mixture bioassays to have a realistic conclusion.

Coalova et al. (2014) proposed that toxicity of co-adyuvants can be additive to the toxicity of the active ingredient and that therefore, these substances play an important role in the effect that the pesticides comercial formulation might have on the organisms. It is important to note that the fact that the biomarkers studied here showed a non-additive effect reveals the high difficulty to assign an expected toxicity, which might be different in the diversity of the environmental matrices.

Overall, the analysis of biomarkers from different levels of organization provides precise information on different aspects of larval development, which may allow a better understanding of thesub-lethal effects of these stressors on declines in amphibian populations (Van der Oost et al., 2003; Peltzer et al., 2017) and accuracy of environmental risk assessments (ERAs). Several reviews on ERAs for GBH and GABH in aquatic environments have minimized the risk of these pesticides for amphibians based on mortality endpoints (e.g., Soso et al., 2007; Güngördü et al., 2016; Geng et al., 2021; Babalola et al., 2021a) have minimized the risk of these pesticides for amphibians. However, due to their unique physiology and dependence on the aquatic environment during early development or during more time for some species, and to the fact that the timing of reproduction occurs concurrently with pesticides applications and that their distribution broadly coincides with GM crops, amphibians are highly sensitive to environmental exposure to both herbicides as suggested by Lajmanovich et al. (2003). In their study from Central-Eastern region of Argentina, these authors described teratological effects in wild amphibians, that togheter with other factors, could have a strong association with the intensive use of agrochemicals that characterize the region. Authors therefore managed to compile and characterize the first catalog of malformations related to agricultural sites for South America (Peltzer et al., 2011). Also in peri-rural areas in the north of Entre Reichert et al. (2022) reported the first evidence in the country on the effect of agricultural drift on target organs (testicles) as indicative of endocrine disruption. Therefore, it is more than clear that sublethal effects deserve fundamental consideration in environmental risk assessments for pesticides and other pollutants, but we also highlight that evaluation of the joint effect of pollutant mixtures is needed to be taken into account. As shown in this and previous studies (Cuzziol Boccioni et al., 2021; Lajmanovich et al., 2022), the effects of individual pollutants may differ in complex mixtures, which is the most likely form that these are found in real environmental conditions.

5. Conclusions

The higher toxicity and ecological implication of GABH was determined in a previous study (Lajmanovich et al., 2022), where GABH was five hundred percent more toxic than GBH. In this same line of evidence, the present investigation showed that there is a strong chemical interaction between the active ingredients of both herbicides, which is demonstrated mainly as antagonistic in most of the analyzed biomarkers. In this study, the trend of higher toxicity of GABH compared to GBH was also observed mainly regarding teratogenic effect, DNA damage, hormonal disruption (T4), and oxidative stress. Indeed, the greater toxicity of GABH and the mixture with GBH increased the known

ecotoxicological risk for aquatic organisms and aquatic system health. Finally, an urgent stop of the constinously approval of GM crops resistant to herbicides, such as GA, that lack of bioethical evaluations and avals at multidisciplinary scientific and populational levels, is needed.

Author contribution statement

Ana P. Cuzziol Boccioni: Methodology, Formal analysis, Investigation, Writing original draft, Germán Lener: Methodology, Investigation, Writing-Review, Julieta Peluso: Methodology, Review & Editing, Paola M. Peltzer: Formal analysis, Investigation, Writing-Review & Editing, Andrés M. Attademo: Supervision. Methodology, Review, Carolina Aronzon: Methodology, Review, María F. Simoniello: Methodology, Review, Luisina D. Demonte: Methodology, María R. Repetti: Methodology, Rafael C. Lajmanovich: Writing-Review & Editing, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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This work is dedicated to the memory of Carlos Vicente, (Grain NGO and UCCSNAL founder), a referent in the fight for free seeds and food sovereignty. His peasant and ecological struggles have promoted communities care for biodiversity and the defense of their territories and subsistence, and have motivated scientists committed to society and nature.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2022.136554.

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