Environmental Science and Pollution Research
https://doi.org/10.1007/s11356-021-16784-0

RESEARCH ARTICLE

Synergistic effects of glyphosate- and 2,4-D-based pesticides mixtures on *Rhinella arenarum* larvae

Julieta Peluso [1,2] · **Agustina Furió Lanuza** [1] · **Cristina S. Pérez Coll** [1,2] · **Carolina M. Aronzon** [1,2]

Received: 7 May 2021 / Accepted: 23 September 2021

Abstract

Glyphosate and 2,4-D are two herbicides commonly used together. Since there is little information about the interactions between these pesticides, the aim of this study was to evaluate the single and joint lethal toxicity of the glyphosate-based herbicide (GBH) ATANOR® (43.8% of glyphosate, isopropylamine salt) and the 2,4-D-based herbicide (2,4-DBH) Así Max 50® (602000 mg/L of 2,4-D) on *Rhinella arenarum* larvae. Equitoxic and non-equitoxic mixtures were prepared according to the recommendation for their combination and analyzed with a fixed ratio design at different exposure times and levels of lethality (LC10, LC50, and LC90). GBH (504h-LC50=38.67 mg ae/L) was significantly more toxic than 2,4-DBH (504h-LC50=250.31 mg ae/L) and their toxicity was time-dependent. At 48h, the equitoxic mixture toxicity was additive and from the 96h was antagonistic at LC10 and LC50 effect level. The non-equitoxic mixture toxicity was additive at LC10 effect level from the 48h to the 168h, and synergistic from the 240h. At LC50 and LC90 effect level, the mixture interaction resulted synergistic for all exposure times. This is the first study to report the synergistic interactions between GBH and 2,4-DBH on amphibians, alerting about its negative impact on aquatic ecosystems.

Keywords Glyphosate · 2,4-D · Mixture toxicity · Synergism · Commercial formulations · Herbicides · Amphibians

Introduction

Agriculture is the main economic activity in Argentina. In the last decades, the agricultural development model was based on the expansion of the agricultural barrier and the intensification of production through the use of genetically modified crops (Pengue 2004). This model implies a greater consumption of land and agrochemicals. In consequence, it increases the pollution in agricultural regions by mixtures of diverse pesticides (De Gerónimo et al. 2014; Etchegoyen et al. 2017). In particular, the main transgenic crops (soybean, corn, and wheat) are designed to tolerate glyphosate, a broad-spectrum non-selective post-emergent herbicide that inhibits 5-enolpyruvylshikimate-3-phosphate synthase, an essential enzyme for the production of aromatic amino acids in plants and a few microbial species (Achary et al. 2020). Despite that animals obtain these aromatic amino acids through diet and do not have this enzyme, glyphosate has been classified as a harmful compound for aquatic organisms (category III) following the classification criteria proposed by the European Union directives (UN 2011). Recently, it was classified as "probably carcinogenic to humans" (group 2A), by the IARC (2015). In water bodies of agroecosystems of Argentina, the concentrations of glyphosate varied between 0.0001 and 105 mg/L(Peluso et al. 2020b; Sasal et al. 2017). Particularly, one of the most applied commercial formulations is ATANOR® (48% glyphosate as isopropylamine salt) (Romero et al. 2011).

On the other hand, 2,4-dichlorophenoxyacetic acid (2,4-D) is also one of the most employed pesticides in Argentina. Between 2013 and 2015, 2,4-D was the third most imported agrochemical in the country (SENASA 2017). Moreover, its use has increased due to its implementation in mixtures tanks as a result of the emergence of glyphosate resistant weeds (Leiva and Picapietra 2012). 2,4-D is a systemic herbicide used mainly in agriculture, in genetically modified corn and

Responsible Editor: Bruno Nunes

✉ Carolina M. Aronzon
caronzon@unsam.edu.ar

[1] Instituto de Investigación e Ingeniería Ambiental, IIIA, UNSAM-CONICET, 3iA, Campus Miguelete, 25 de Mayo y Francia, C.P. 1650 San Martín, Buenos Aires, Argentina

[2] Consejo Nacional de Investigaciones Científicas y Tecnológicas (CONICET), Buenos Aires, Argentina

soybean crops (Cappello et al. 2013; Merini et al. 2008). Additionally, it can be applied in pastures, forest management, and gardens to control broadleaf weeds and aquatic vegetation (Islam et al. 2018). 2,4-D is a stable and persistent auxin-like substance involved in plant growth and development. The exposure to this compound results in an overstimulation of growth, which leads to plant death (Zimdahl 2018). It has been classified by the WHO (1990) as moderately hazardous (Class II) and by the IARC (2015) as "possibly carcinogenic to humans" (group 2B). There is little information about the environmental concentrations of 2,4-D in Argentina (Ruiz de Arcaute et al. 2016); Peluso et al. (2020a) informed an environmental concentration between the limit of detection and quantification (1–4 μg/L) in a river of Buenos Aires province.

Glyphosate and 2,4-D present different environmental behavior and can enter ephemeral ponds or aquatic systems close to fumigated lands. On the one hand, glyphosate and AMPA (its metabolite) bind strongly to topsoil particles and therefore reach water bodies, ponds, and lakes through water erosion events (Bento et al. 2019). On the other hand, 2,4-D present a high mobility and can reach aquatic ecosystems by runoff (Health Canada Pest Management Regulatory Agency 2016). Additionally, both pesticides may enter aquatic systems from washing fumigation machinery.

When pesticides are applied, the most affected environments are the shallow freshwater aquatic/estuarine systems and even pools that are often associated with agricultural areas, which are common habitats for amphibians (Solomon and Thompson 2003). Additionally, most amphibian species have seasonal reproduction, coinciding the breeding and the embryo-larval development with the application of pesticides and fertilizers during spring-summer(Lenhardt et al. 2015). In the last years, a decline in amphibian populations has been registered (Green et al. 2020), and it was related to habitat loss and increased environmental degradation mainly by agriculture (Davidson and Knapp 2007; Peltzer et al. 2011). Particularly, *Rhinella arenarum* is a representative species of the Argentine herpetofauna, and may inhabit water bodies of agroecosystems (Peltzer et al. 2011). Despite that it is classified as a least concern species by the IUCN (Kwet et al. 2004), several studies warn about the vulnerability of this species to chemical contaminants in agroecosystems, which led to an increased incidence of malformations and populations declines (Bionda et al. 2013; Peltzer et al. 2011).

Several laboratory studies have shown that different commercial formulations of glyphosate are moderately toxic to amphibian (Bernal et al. 2009; Chen et al. 2004; Edginton et al. 2004; Fuentes et al. 2011; Howe et al. 2004; Lajmanovich et al. 2003; Mann and Bidwell 1999; Relyea and Jones 2009; Sing Yadav et al. 2013). Moreover, the acute toxicity of glyphosate-based pesticides, as Atanor®, Credit®, Glifloglex®, and Roundup Ultra Max®, was informed in the range of 19.4 mg/L and 78.18 mg/L(Brodeur et al. 2014; Soloneski et al. 2016). It has been previously demonstrated that glyphosate causes oxidative stress, inhibits the action of key enzymes for regulation of cell cycle, and produces genotoxic damage on amphibian larvae (Carvalho et al. 2020; Lima et al. 2020). Toxicity studies of 2,4-D on amphibians have shown that the herbicide inhibits oocyte maturation (LaChapelle et al. 2007; Stebbins-Boaz et al. 2004) and has teratogenic effects on *Xenopus laevis*(Morgan et al. 1996) and *Rhinella arenarum*, one of the most susceptible amphibian species during the embryo-larval period (Aronzon et al. 2011). In *Physalaemus albonotatus* larvae, exposure to 2,4-D caused histological alterations in the liver and morphological abnormalities (Curi et al. 2019). Also, in *Lithobates catesbeianus*, *Leptodactylus fuscus*, and *Physalaemus nattereri* larvae, altered total hepatic lipids, protein, and carbohydrate contents were observed. Also, the respiration rates and swimming speed were affected (Freitas et al. 2019). Moreover, 2,4-D increased GST activity and DNA damage by dermal exposure of *R. arenarum* adults (Lajmanovich et al. 2015).

When *Boana faber* and *Leptodactylus latrans* larvae were exposed to a combination of glyphosate and 2,4-D, growth, and swimming activity were altered, an increase in morphological abnormalities was observed and erythrocytes showed micronuclei and other nuclear abnormalities (Pavan et al. 2021). However, there is no information about the lethal toxicological interactions in mixtures of glyphosate and 2,4-D on amphibians, so the aim of the current study was to evaluate the lethal toxicity of equitoxic and non-equitoxic binary mixtures of these pesticides on larvae of the common South American toad, *Rhinella arenarum.* It is noteworthy the importance of assessing the toxicity of complex commercial formulations, since these are the ones that are actually applied in the fields. Moreover, non-equitoxic mixtures are more environmentally realistic, and in this study, the mixture was prepared according to the combination recommended for their use in fields present in the pesticide bottle labels.

Materials and methods

Substances and test solutions

The commercial formulations "Atanor®" and "Así Max 50®" were used for testing single and joint toxicity of glyphosate (GBH) and 2,4-D (2,4-DBH) based herbicides, respectively. "Atanor®" is a commercial formulation of glyphosate (*N*-(phosphonomethyl) glycine; CAS No. 1071-83-6) containing 43.8% of glyphosate in the form of monopotasic salt which corresponds to 35.6% of glyphosate acid equivalent (ae). "Así Max 50®" is a commercial formulation of the dimethylamine salt of the 2,4-dichlorophenoxyacetic acid (CAS No. 2008-39-1) containing 602000 mg/L of 2,4-D which corresponds to 500000 mg/L of 2,4-D ae. Both herbicide formulations included undisclosed proprietary additives, surfactants, or emulsifiers. The glyphosate and 2,4-D concentrations in test solutions were checked. For the glyphosate determination, the stock solution and the sample were derivatized with 100 µL of 0.5 mM FMOC and 100 µL of Borate Buffer pH 9. They were allowed to react at room temperature for 30 min. Then, samples were filtered through 0.45 µm nylon filters and quantified by HPLC (Agilent 1100 Series equipment) with an ODS (C18) reversed phase column and a fluorescence detector. The injection volume was 5 µL and the flow was 0.5 mL/min. The 2,4-D concentration was analyzed by direct injection of the sample against a standard solution with a concentration similar to the expected in the sample. The mobile phase was acetonitrile:acetic acid 4% (55:45). An Agilent 1200 HPLC system with a LiChrospher RP-18 (250×4 mm; 5 µm) and a DAD detector was employed. Both standards were purchased at Sigma-Aldrich (99.9% purity). The herbicide concentrations employed during this study represent the nominal concentration of ae of the active principle contained in the formulation. The error between the nominal and measured concentrations did not exceed 10%.

Single test solutions

For the single toxicity assay, GBH and 2,4-DBH stock solutions of 1000 mg ae/L and 25000 mg ae/L were prepared in deionized water, respectively. The test range was determined based on a preliminary bioassay and previous data on the effects of the herbicides in other related species. Ten test solutions were prepared with AMPHITOX solution (AS: Na^+ 14.75; Cl^- 22.71; K^+ 0.26; Ca_2^+ 0.36 and HCO_3^- 1.45 mg/L), in concentrations ranging between 5 and 80 mg ae/L for glyphosate (5, 10, 20, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, and 80 mg ae/L) and 50–1250 mg ae/L for 2,4-D (50, 75, 100, 150, 200, 300, 350, 400, 500, 600, 800, 1000, 1250 mg ae/L). Test solutions were completely renewed every other day in order to ensure the physicochemical conditions of the AS solution (hardness lower than<2 mg/L CaCO3, conductivity of 59.4 µS/cm, a pH between 6.86-7.45), the levels of dissolved oxygen (>7 mg/L) and the herbicide concentrations. Every time, new stock solutions were prepared.

Mixture test solutions

GBH and 2,4-DBH mixture toxicity was evaluated using a fixed ratio design according to the protocols described by Aronzon et al. (2016). Stock solutions of equitoxic and non-equitoxic mixtures were prepared by dissolving the corresponding volume of the commercial formulation of GBH and 2,4-DBH in AS. Equitoxic mixture was prepared based on the LC50-168 h for each compound, to combine both herbicides in equal proportions according to their respective toxicity (Table 1). For that purpose, preliminary bioassays were performed with single GBH and 2,4-DBH to obtain the LC50-168 h, which were 48.18 (43.74–51.40) mg/L and 419 (404–430) mg/L, respectively. Equitoxic relation was maintained as there were no significant difference with the LC50 obtained in the simultaneous single exposure of this study.

Non-equitoxic mixture was prepared according to the glyphosate label recommendation for the combination with the amine salt of 2,4-D (Table 1). Besides, both pesticides were combined in a ratio based on a commercial herbicide formulation increasingly used in Argentina (Mestizo®, from Atanor®, Argentina) (Lozano et al. 2018). Test solutions of equitoxic and non-equitoxic mixtures were prepared by diluting the corresponding volume of each stock solution in AS, in order to maintain the proportion of compounds. Solutions were completely renewed every other day as in the single bioassays.

Acquisition of *Rhinella arenarum* larvae

Two mating pairs of *Rhinella arenarum* adults weighing approximately 200–250 g per animal were acquired in a non-impacted site of Buenos Aires province, Argentina (34° 49′ 55.7″ S 58° 07′ 11.3″ W). The maintenance and care of toads and the acquisition, maintenance, and selection of embryos were conducted according to methods described in the AMPHITOX protocols (Pérez Coll et al. 2017). Ovulation of female toads was induced by means of an intraperitoneal injection of 5000 IU of human chorionic gonadotropin (Gonacor® 5000) (Mann and Bidwell 2000). Oocytes were fertilized in vitro using a 10% spermatozoid suspension in AS. Sperm viability was evaluated by analyzing the morphology and motility of sperm under optical microscopy (Olympus CX41, 400× magnification). Fertility was considered acceptable with rates greater than 75%. Embryos were kept in AS at 20 ± 2°C, alternating 12-hlight/dark cycles, until they reach the complete operculum stage (S.25), defined according to Del Conte and Sirlin (1951). All experiments were conducted according to the international standards on animal welfare (Beaupre et al. 2004) and controlled and approved by the Institutional committee for the care and use of animals in experimentation (CICUAE) of the National University of San Martín (UNSAM) (Res. No. 14/2016).

Experimental protocols

Rhinella arenarum larvae at complete operculum stage (S.25) were continuously exposed to the single GBH and 2,4-DBH solutions for 504 h and to the mixtures for 240 h. For each experimental condition, 10 larvae were placed in covered 10-cm-diameter glass Petri dishes with 40 mL of AS (controls) or test solutions. Test solutions were entirely replaced every 48 h, and temperature was maintained between 20 ± 2°C,

Table 1. Concentration and composition of glyphosate (GBH) and 2,4-D (2,4-DBH) based herbicides on equitoxic and non-equitoxic mixture solutions.

Mixture stock solution	Total exposure concentrations (mg/L)	GBH in exposure solution (mg/L)	2,4-DBH in exposure solution (mg/L)
Equitoxic	936	96	840
	748.8	76.8	672
	655.2	67.2	588
	561.6	57.6	504
	468	48	420
	421.2	43.2	378
	374.4	38.4	336
	327.6	33.6	294
	280.8	28.8	252
	187.2	19.2	168
	93.6	9.6	84
Non-equitoxic	220	150	70
	132	90	42
	110	75	35
	88	60	28
	77	52.5	24.5
	66	45	21
	55	37.5	17.5
	44	30	14

alternating 12-hlight/dark cycles, throughout the exposure. Dissolved oxygen (Lutron PDO-519 oximeter) and pH (Adwa AD-12 pH meter) were measured during the exposure time to ensure the conditions. Dead individuals were removed, and survival was evaluated every day. Larvae were fed with 6 ± 0.5 mg of balanced fish food TetraColor® every other day. Toxicity bioassays were repeated two times with different clutches, so besides the preliminary single bioassays, we performed eight toxicity bioassays.

Data analysis

Lethality data were statistically analyzed by the USEPA PROBIT Program (EPA 1988). LC10, 50, and 90s were obtained for each chemical. The significance level of mean separation ($p < 0.05$) of two LC50 values was based on the lack of overlap between the 95% confidence limits (Yu et al. 2019).

Mixture results were analyzed using the median-effect/Combination Index (CI) developed by Chou (2017). This method is based on the median-effect principle (mass-action law)(EPA 1988), which demonstrates that there is an univocal relationship between concentration and effects, independently of the number of substances and mechanisms of action or inhibition. The Compusyn program (Sprague 1970) was used for the calculation of CI values at different effect levels (Fa= proportion of individuals affected), when $CI < 1$, $CI = 1$, and $CI > 1$ indicate synergism, additivity, and antagonism, respectively.

Results

Chronic toxicity of single GBH- and 2,4-D-BH

The survival of larvae in the control groups was between 90 and 100%. GBH and 2,4-DBH concentrations that cause 10%, 50%, and 90% mortality of larvae throughout the 504 h of exposure are shown in Figure 1. GBH was significantly more toxic than 2,4-DBH, as the LC50s of 2,4-DBH were between 12 and six times higher than the ones of GBH. The toxicity of both pesticides was time-dependent since it significantly increased almost two and five times through the exposure time for GBH and 2,4-DBH, respectively. The LC50 for GBH decreased from a value of 64.02 (60.69–68.60) mg ae/L at 24 h to 59.91 (55.76–64.56) mg ae/L and 38.67 (32.22–44.015) mg ae/L at 96 and 504 h, respectively. On the other hand, for 2,4-DBH, the LC50 decreased from 1420 (2949–1168) mg ae/L at 24 h to 746 (787.56–705.43) mg ae/L and 250.31 (284.03–218.55) mg ae/L at 96h and 504 h, respectively.

Chronic toxicity of equitoxic and non-equitoxic binary mixtures of GBH and 2,4-DBH

The LC50s for single GBH, 2,4-DBH, and the $LC50_M$s for GBH and 2,4-DBH in the equitoxic and non-equitoxic mixture are shown in Figures 2 A and B. It is noteworthy that in both mixture proportions, the $LC50_M$ of both pesticides are

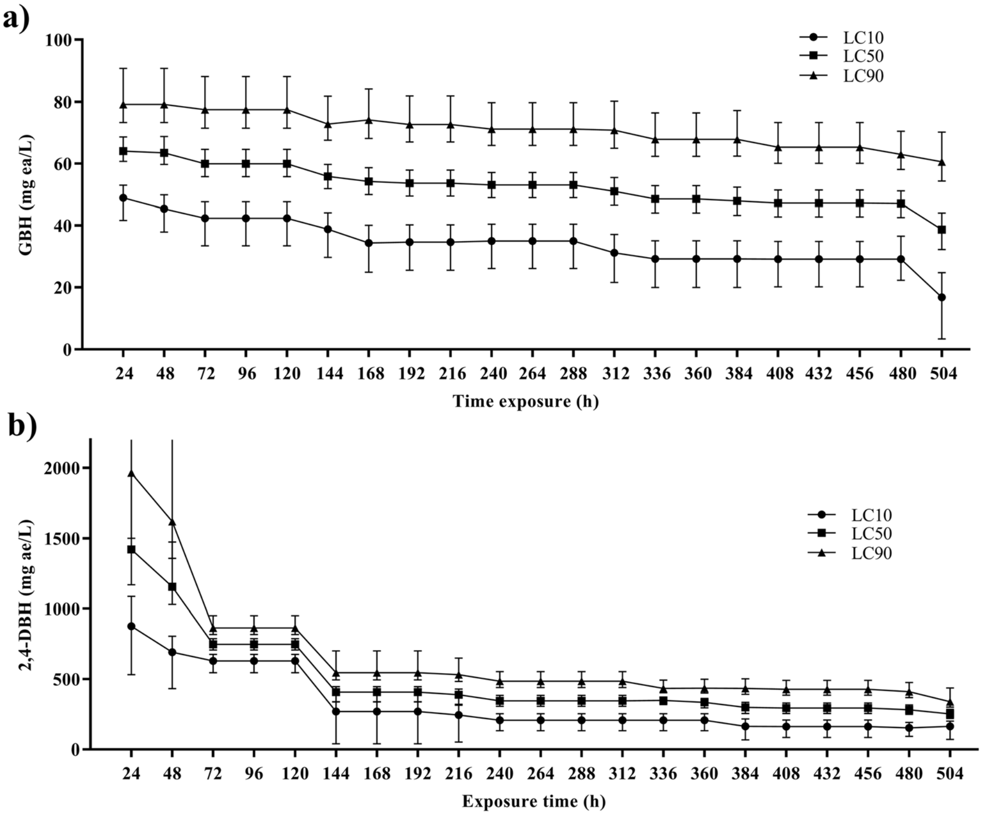


Fig. 1 Glyphosate (GBH)- and 2,4-D (GBH) based herbicide concentrations that cause the 10% (Lethal concentration 10, LC10), 50% (Lethal concentration 50, LC50), and 90% (Lethal concentration 90, LC90) mortality of *Rhinella arenarum* larvae, with 95% of confidence intervals, at different exposure times

always lower than their corresponding single LC50. Moreover, the $LC50_M$ of the pesticides are in relation of its contribution in the mixture proportion. This is particularly noticeable in the $LC50_M$ of 2,4-DBH in the non-equitoxic mixture. In both cases, the GBH seems to modulate the joint toxicity curve. However, in order to understand the nature of the combination toxicity, a joint toxicological analysis is required. In that sense, the CI values of equitoxic and non-equitoxic binary mixture of GBH and 2,4-DBH are shown in Table 2. The equitoxic mixture presented an additive interaction in the acute exposure time of 48 h at all effect levels (0.1, 0.5, and 0.9). The interactions turned to antagonistic at the 0.1 and 0.5 effect levels from the 96 h to the 240 h. The same change in interactions was observed at the 0.9 effect level from the 168 h. On the other hand, the non-equitoxic mixture showed an additive response at the 0.1 effect level from the 48 h to the 168 h, turning to synergistic at the 240 h. Indeed, the mixture interaction resulted synergistic for all exposure times at the 0.5 and 0.9 effect levels.

Figure 3 illustrates the results obtained for equitoxic and non-equitoxic mixtures at the 0.5 effect level. The diagonal isobole linking the values of 1 Toxic Unit (TU) on the y(2,4-DBH) and x(GBH) axes is the line of concentration addition. The TU arbitrarily assigns a value of 1 TU to a concentration of toxicant that elicits a particular response; in this case, it represents the 50% mortality (LC50)(Sprague 1970). The equitoxic mixture lies above and to the right of the additivity line from the 96 h of exposure, which confirms the antagonistic interaction. However, the non-equitoxic mixture lies below and to the left of the additivity line, which shows the synergic interactions at all exposure times (Figure 3).

Joint toxicity could not be calculated beyond 240 h due to the high mortality observed during the exposure to mixtures.

Discussion

We comparatively evaluated the chronic lethal toxicity and prevailing interactions in equitoxic and non-equitoxic mixtures of two different commercial herbicide formulations on *R. arenarum* larvae. Both pesticide formulations exerted lethal effects on *R. arenarum* larvae. The observed lethal effects may be due to the ability of both herbicides to induce oxidative stress, morphological and histological alterations, DNA damage, and altered swimming activity as observed in larvae of different amphibian species (Curi et al. 2019; Freitas et al. 2019; Pavan et al. 2021). Also, when two substances have different mechanisms of action, they can affect different biological endpoints, so their behavior may differ from the expected additive effects (Deneer 2000). In this case, as the pesticides have some similar but also different endpoints, it is difficult to predict the effects of the mixture. Thus, joint toxicity evaluations are important to assess the change in the behavior at different proportions. GBH resulted up to 18 times

Table 2. Combination Index (CI) values, at different effect levels, with 95% confidence intervals for *Rhinella arenarum* larvae exposed to equitoxic and non-equitoxic mixtures of glyphosate (GBH) and 2,4-D (2,4-DBH) based herbicides from 48 h to 240 h.

		Combination Index (CI)					
Mixture stock solution	Exposure Time (h)	Effect level (0.1)	Interaction	Effect level (0.5)	Interaction	Effect level (0.9)	Interaction
Equitoxic	48	1.46 +/− 0.52	Additive	1.16 +/−0.22	Additive	0.97 +/− 0.30	Additive
	96	1.90 +/− 0.22	Antagonism	1.21 +/− 0.12	Antagonism	0.90 +/− 0.10	Additive
	168	1.83 +/− 0.56	Antagonism	1.56 +/− 0.27	Antagonism	1.37 +/− 0.16	Antagonism
	240	1.96 +/− 0.55	Antagonism	1.66 +/− 0.26	Antagonism	1.46 +/− 0.10	Antagonism
Non−equitoxic	48	0.85 +/− 0.17	Additive	0.82 +/− 0.04	Synergism	0.82 +/− 0.14	Synergism
	96	0.10 +/− 0.09	Additive	0.87 +/− 0.03	Synergism	0.77 +/− 0.07	Synergism
	168	0.89 +/− 0.15	Additive	0.80 +/− 0.09	Synergism	0.74 +/− 0.18	Synergism
	240	0.78 +/− 0.16	Synergism	0.77 +/− 0.09	Synergism	0.79 +/− 0.19	Synergism

more toxic to larvae than 2,4-DBH. This fact becomes very relevant since the first one is the most applied herbicide in Argentina and is commonly found in the environment (Lupi et al. 2015). The observed time-dependent toxicity of both pesticides highlights the relevance of assessing toxicity in chronic periods. In particular, the GBH concentration that produced toxicity on larvae at chronic exposure is in the range of previously informed environmental concentrations in Argentine water bodies from the distribution area of *R. arenarum* (0.0001–105 mg/L) (Berkovic et al. 2006; Sasal et al. 2017). Even though there is no previous information about 2,4-D concentrations in agroecosystems of Argentina, the estimated range of environmental concentrations in freshwater bodies is 0.004–0.024 mg/L, while in agricultural fields, its concentration may reach up to 4 mg/L(Islam et al. 2018).

Commercial formulations contain different substances that enhance the performance of the active ingredient. For most pesticides of Argentina, these substances are unknown (CASAFE 2001), and generally, they are not considered to play an active role in the toxicity on non-target organisms (WHO 1990). However, their harmful effects may exceed the ones caused by the active ingredients and alter the toxicity of the product (WHO 1990). Particularly, the toxicity of glyphosate is highly dependent on the kind of surfactant used in the different brands (Lajmanovich et al. 2011; Mann et al. 2009). In comparison with the results informed in previous studies on *R. arenarum* larvae exposed to other commercial formulations, the one assessed in the present study, “Atanor®,” resulted more toxic than Glifoglex and Credit® with LC50-96 h of 72.8 mg ae/ L and 96 h 78.18 mg/L, respectively (Brodeur et al. 2014; Howe et al. 2004), but less toxic than Roundup Ultra-MaX®, with a LC50-48 h of 45.95 mg/L(Lajmanovich et al. 2019), and a previous version of Atanor ® with a LC50-96h of 19.4 mg/L(Brodeur et al. 2014).

On the other hand, in comparison with the results informed in a previous study on *R. arenarum* larvae exposed to the butyl ester of 2,4-D, the commercial formulation of the amine salt evaluated in this study resulted significantly less toxic. Thus, the LC50-168 h of larvae exposed to the butyl ester of 2,4-D was 13.4 mg/L(Aronzon et al. 2011) while the LC50-168 h informed in the present work for the amine salt of 2,4-DBH was 406.42 (445.77-338.32) mg/L. A lower toxicity of the amine salt was also seen in the exposure to the commercial formulation of the dimethylamine salt of 2,4-D, Zamba®, which elicited a similar lethal toxicity on *Physalaemus albonotatus* larvae with a LC50-96 h of 350 mg/L(Curi et al. 2019). However, in mixture studies, it is more relevant to assess the toxicity of the amine salt since it is the most commonly employed in tanks and binary mixtures of the formulations available in the market (Lozano et al. 2018).

In the present study, the evaluated equitoxic mixture of the herbicides suggested mainly an antagonistic interaction. However, the non-equitoxic mixtures of GBH and 2,4-DBH indicated the presence of synergism for all exposure times at the 0.5 and 0.9 effect levels, and for chronic exposure times at the 0.1 effect level (Table 2 and Figure 3). These results show the existence of different types of interactions that depend on the proportion of the compounds in the mixture. Also, the interactions may vary according to the to the compound form since Carvalho et al. (2020) found that the combination of glyphosate with 2,4-D or 2,4-D DMA showed a synergistic pattern whereas the combination of glyphosate with 2,4-D BE was antagonistic. This fact highlights the need to assess mixture toxicity not only at different proportions of each compound and at different effect level but also at chronic exposure times since they are more environmentally accurate. The assessment of the toxicity of non-equitoxic mixtures provides a better understanding of the behavior of the mixture in comparison to evaluating only the equitoxic mixture, so these results become more significant as the non-equitoxic mixture is

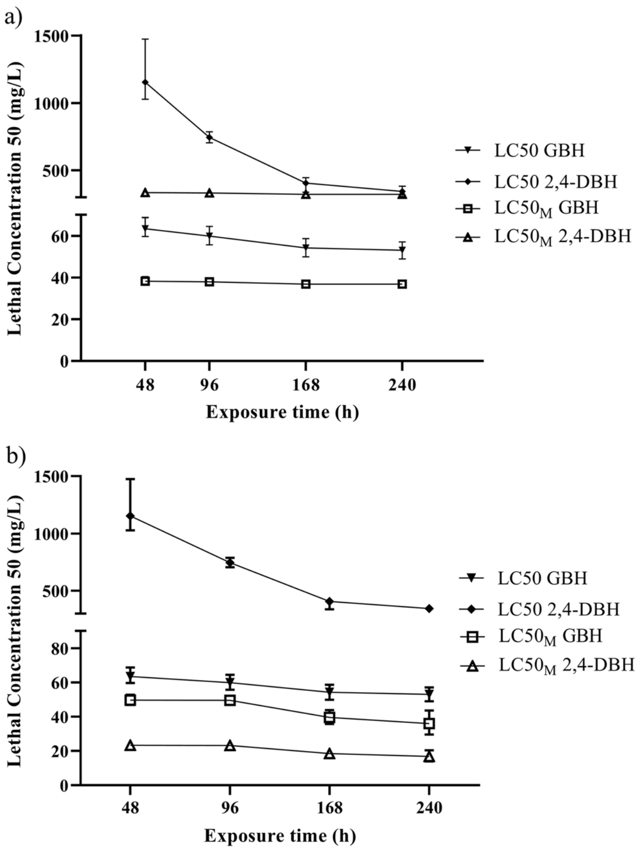
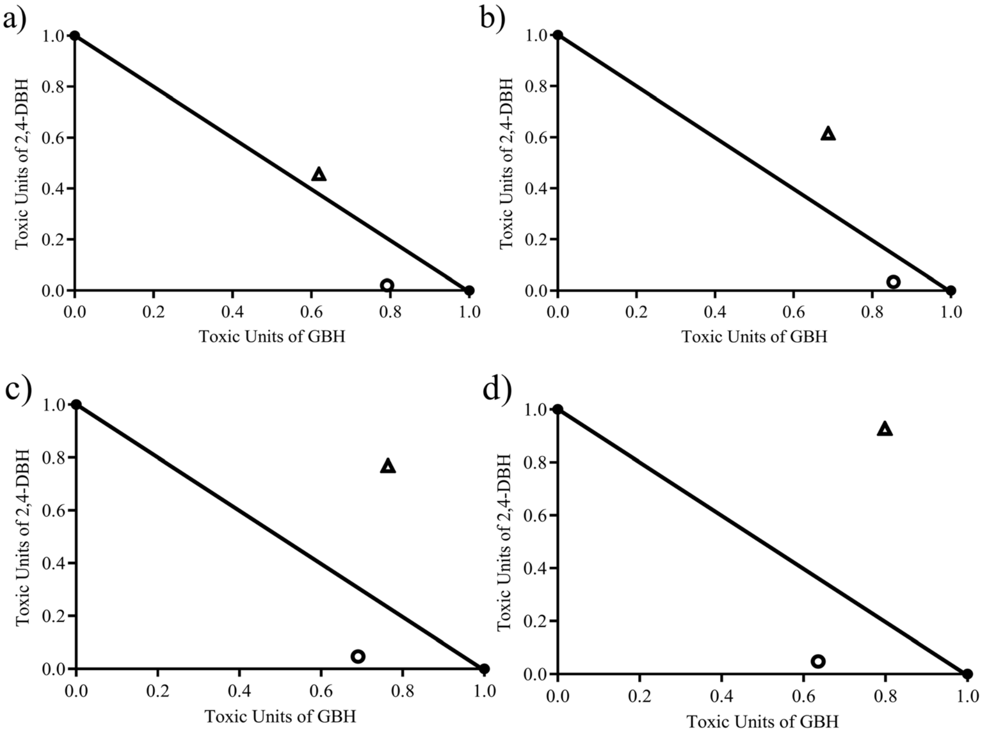


Fig. 2 Lethal concentrations 50 of glyphosate (GBH) and 2,4-D (2,4-DGH) based herbicides, and of equitoxic (**a**) and non-equitoxic (**b**) mixtures on *Rhinella arenarum* larvae. LC50: lethal concentration 50 for single GBH and 2,4-DBH; LC50$_M$: lethal concentration 50for GBH and 2,4-DBH in the equitoxic and non-equitoxic mixtures

more environmentally realistic since in the environment is highly unlikely that the mixture of chemicals occurs in an equitoxic way (Brodeur et al. 2014). Also, a synergic effect is expected in mixtures of these pesticides at any concentration in a non-equitoxic relation, which includes concentrations that may be present in freshwater bodies. In this case, the assessed non-equitoxic mixture was prepared according to the recommendation for the combination with the amine salt of 2,4-D present on the label of glyphosate and of a commercial herbicide formulation increasingly used in Argentina (Lozano et al. 2018).

A recent study about the toxicity of mixtures of glyphosate and 2,4-D based herbicides proved their genotoxic, morphological and behavioral effects on *Boana faber* and *Leptodactylus latrans* tadpoles (Pavan et al. 2021). However, our results are, up to our knowledge, the first to clearly demonstrate the presence of synergistic toxicological interaction on lethal effects between these two herbicides. Also, they highlight that single-compound assessments may underestimate the real risk for aquatic wildlife species that are exposed to complex mixtures of substances. It must be emphasized that the herbicides combined in our study are commercial formulations. Since each one is a combination of several components, the resulting mixtures are more complex systems than simple binary mixtures. Further studies will be required to explain the results of the present study from a mechanistic concept. Despite that synergistic interactions represent a minority of the reported cases (WHO 1990), glyphosate has previously shown synergistic interaction with cypermethrin and arsenic on *R. arenarum* larvae (Brodeur

Fig. 3 Isobolograms showing the composition of the equitoxic (Δ) and non-equitoxic (○) mixtures of glyphosate (GBH) and 2,4-D (2,4-DGH) based herbicides that cause 50% mortality of *Rhinella arenarum* larvae (lethal concentration 50, LC50) at 48 (**a**), 96 h (**b**), 168 h (**c**), and 240 h (**d**) of exposure



et al. 2014; Lajmanovich et al. 2019). Moreover, these results are relevant because the use of a local species provides ecological pertinent information and might allow characterizing the potential risk on the native fauna (Lajmanovich et al. 2019).

Conclusions

The environmentally realistic non-equitoxic mixture of GBH and 2,4-DBH showed synergic effect at different effect levels of lethality on the larvae of the native amphibian specie *Rhinella arenarum*. The present findings result relevant from a regulatory point of view given the widely use of the herbicides, the environmental relevance of the assayed concentrations, and the need for amphibian conservation around the world.

Acknowledgements We thank Ferring Pharmaceuticals for providing the human chorionic gonadotropin.

Author contribution Julieta Peluso: data curation, writing original draft, and writing reviewing and editing. Agustina Furió Lanuza: data curation, formal analysis, investigation, validation. Cristina S. Pérez Coll: conceptualization, methodology, resources. Carolina M. Aronzon: data curation, formal analysis, investigation, conceptualization, methodology, investigation, resources, validation, writing original draft and reviewing and editing, funding acquisition.

Funding This study was supported by a research grant from Consejo Nacional de Investigaciones Científicas y Técnicas (PIP 112-201301-00140).

Declarations

Ethics approval and consent to participate Not applicable.

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

References

Achary VMM, Sheri V, Manna M, Panditi V, Borphukan B, Ram B, Agarwal A, Fartyal D, Teotia D, Masakapalli SK (2020) Overexpression of improved EPSPS gene results in field level glyphosate tolerance and higher grain yield in rice. Plant Biotechnol J 18:2504–2519

Aronzon C, Sandoval M, Herkovits J, Pérez-Coll C (2011)Stage-dependent toxicity of 2,4-dichlorophenoxyacetic on the embryonic development of a South American toad, *Rhinella arenarum*. Environ Toxicol 26:373–381

Aronzon CM, Svartz GV, Pérez Coll CS (2016) Synergy between diazinon and nonylphenol in toxicity during the early development of the *Rhinella arenarum* toad. Water Air Soil Pollut 227:1–10

Beaupre SJ, Jacobson ER, Lillywhite HB, Zamudio K (2004): Guidelines for use of live amphibians and reptiles in field and laboratory research. Herpetological Animal Care and Use Committee (HACC) of the American Society of Ichthyologists and Herpetologists

Bento CP, van der Hoeven S, Yang X, Riksen MM, Mol HG, Ritsema CJ, Geissen V (2019) Dynamics of glyphosate and AMPA in the soil surface layer of glyphosate-resistant crop cultivations in the loess Pampas of Argentina. Environ Pollut 244:323–331

Berkovic A, Marino DJ, Lespade P, Ronco A (2006) Análisis de pesticidas asociados al cultivo de soja en aguas y sedimentos de un sector productivo de la pampa ondulada. Dissertation. . XXVI Congreso Argentino de Química, Facultad de Química, Bioquímica y Farmacia. Universidad Nacional de San Luis, Argentina.

Bernal MH, Solomon KR, Carrasquilla G (2009) Toxicity of formulated glyphosate (Glyphos) and Cosmo-flux to larval colombian frogs 1. Laboratory acutetoxicity. J Toxicol Environ Health 72A:961–965

Bionda C, Lajmanovich R, Salas N, Martino A, di Tada I (2013) Population demography in *Rhinella arenarum* (Anura: Bufonidae) and *Physalaemus biligonigerus* (Anura: Leiuperidae) in agroecosystems in the province of Córdoba, Argentina. Rev Biol Trop 61:1389–1400

Brodeur JC, Poliserpi MB, Sánchez M (2014) Synergy between glyphosate-and cypermethrin-based pesticides during acute exposures in tadpoles of the common South American Toad *Rhinella arenarum*. Chemosphere 112:70–76

Cappello V, Fortunato N, Tangorra M, Vergara AR (2013) Plaguicidas en el territorio bonaerense: información toxicológica, ecotoxicológica y comportamiento ambiental. Dirección Provincial de Recursos Naturales. Programa Gestión Ambiental En Agroecosistemas. Buenos Aires 1

Carvalho WF, de Arcaute CR, Torres L, e Silva DdM, Soloneski S, Larramendy ML (2020) Genotoxicity of mixtures of glyphosate with 2, 4-dichlorophenoxyacetic acid chemical forms towards *Cnesterodon decemmaculatus* (Pisces, Poeciliidae). Environ Sci Pollut Res 27:6515–6525

CASAFE (2001) Guía de productos fitosanitarios para la República Argentina. Ed. Cámara de Sanidad Agropecuaria y Fertilizantes Buenos Aires

Chen CY, Hathaway KM, Folt CL (2004) Multiple stress effects of Visionherbicide, pH, and food on zooplankton and larval amphibian species fromforest wetlands. Environ Toxicol Chem 23:823–831

Curi LM, Peltzer PM, Sandoval MT, Lajmanovich RC (2019) Acute toxicity and sublethal effects caused by a commercial herbicide formulated with 2, 4-D on *Physalaemus albonotatus* Tadpoles. Water Air Soil Pollut 230:22

Davidson C, Knapp RA (2007) Multiple stressors and amphibian declines: dual impacts of pesticides and fish on yellow-legged frogs. Ecol Appl 17:587–597

De Gerónimo E, Aparicio VC, Bárbaro S, Portocarrero R, Jaime S, Costa JL (2014) Presence of pesticides in surface water from four sub-basins in Argentina. Chemosphere 107:423–431

Del Conte E, Sirlin L (1951) The first stages of *Bufo arenarum* development. Acta Zool Lilloana 12:495–499

Deneer JW (2000) Toxicity of mixtures of pesticides in aquatic systems. Pest Management Science: formerly Pesticide Science 56:516–520

Edginton AN, Sheridan PM, Stephenson GR, Thompson DG, Boermans HJ (2004) Comparative effects of pH and Vision (R) herbicide on two life stages of four anuran amphibian species. Environ Toxicol Chem 23:815–822

EPA (1988) Users guide for a computer program for PROBIT analysis of data from acute and short-term chronic toxicity test with aquatic organisms. Biological Methods. Environmental monitoring and Support Lab

Etchegoyen MA, Ronco AE, Almada P, Abelando M, Marino DJ (2017) Occurrence and fate of pesticides in the Argentine stretch of the Paraguay-Paraná basin. Environ Monit Assess 189:63

Freitas JS, Girotto L, Goulart BV, Alho LOG, Gebara RC, Montagner CC, Schiesari L, Espíndola ELG (2019) Effects of 2, 4-D-based herbicide (DMA® 806) on sensitivity, respiration rates, energy reserves and behavior of tadpoles. Ecotoxicol Environ Saf 182: 109446

Fuentes L, Moore LJ, Rodgers JH Jr, Bowerman WW, Yarrow GK, Chao W (2011) Comparative toxicity of two glyphosate formulation (original formulation of Roundup and Roundup Weathermax) to six North American larval anurans. Environ Toxicol Chem 30:2756–2761

Green DM, Lannoo MJ, Lesbarrères D, Muths E (2020) Amphibian population declines: 30 years of progress in confronting a complex problem. Herpetologica 76:97–100

Health Canada Pest Management Regulatory Agency (2016)Re-evaluation note REV2016-08, special review of 2,4-D: proposed decision for consultation, https://www.canada.ca/content/dam/hc-sc/migration/hc-sc/cps-spc/alt_formats/pdf/pest/part/consultations/rev2016-08/REV2016-08-eng.pdf

Howe CM, Berrill M, Pauli BD, Helbing CC, Werry K, Veldhoen N (2004) Toxicity of glyphosate-based pesticides to four north American frog species. Environ Toxicol Chem 23:1928–1938

International Agency for Research on Cancer I (2015) Some organophosphate insecticides and herbicides: diazinon, glyphosate, malathion, parathion, and tetrachlorvinphos. IARC monographs on the evaluation of carcinogenic risks to humans. 112

Islam F, Wang J, Farooq MA, Khan MS, Xu L, Zhu J, Zhao M, Muños S, Li QX, Zhou W (2018) Potential impact of the herbicide 2, 4-dichlorophenoxyacetic acid on human and ecosystems. Environ Int 111:332–351

Kwet A, Reichle S, Silvano D, Úbeda C, Baldo D, Di Tada I (2004): *Rhinella arenarum*. The IUCN Red List of Threatened Species 2004: e.T54576A11169255. https://doi.org/10.2305/IUCN.UK.2004.RLTS.T54576A11169255.en. .

LaChapelle AM, Ruygrok ML, Toomer M, Oost JJ, Monnie ML, Swenson JA, Compton AA, Stebbins-Boaz B (2007) The hormonal herbicide, 2,4-dichlorophenoxyacetic acid, inhibits *Xenopus oocyte* maturation by targeting translational and posttranslational mechanisms. Reprod Toxicol 23:20–31

Lajmanovich RC, Sandoval MT, Pelzer PM (2003) Induction of mortality andmalformation in Scinaxnasicus tadpoles exposed to glyphosate formulations. Bull Environ Contam Toxicol 70:612–618

Lajmanovich RC, Attademo AM, Peltzer PM, Junges CM, Cabagna MC (2011) Toxicity of four herbicide formulations with glyphosate on *Rhinella arenarum* (Anura: Bufonidae) tadpoles: B-esterases and glutathione S-transferase inhibitors. Arch Environ Contam Toxicol 60:681–689

Lajmanovich RC, Attademo AM, Simoniello MF, Poletta GL, Junges CM, Peltzer PM, Cabagna-Zenklusen MC (2015) Harmful effects of the dermal intake of commercial formulations containing chlorpyrifos, 2, 4-D, and glyphosate on the common toad *Rhinella arenarum* (Anura: Bufonidae). Water Air Soil Pollut 226:427

Lajmanovich RC, Peltzer PM, Attademo AM, Martinuzzi CS, Simoniello MF, Colussi CL, Cuzzio Boccioni AP, Sigrist M (2019) First evaluation of novel potential synergistic effects of glyphosate and arsenic mixture on *Rhinella arenarum* (Anura: Bufonidae) tadpoles. Heliyon 5:e02601

Leiva APD, Picapietra G (2012): Compatibilidad para mezclas de tanque de tres herbicidas utilizados en barbecho químico. Pergamino, Grupo Protección Vegetal–INTA. Grupo Protección Vegetal-INTA, Estación Experimental Agropecuaria Pergamino. Disponible en https://www.aapresid.org.ar/wp-content/uploads/sites/3/2013/02/ensayo_mezclas_tanque_agosto2012.pdf

Lenhardt PP, Brühl CA, Berger G (2015) Temporal coincidence of amphibian migration and pesticide applications on arable fields in spring. Basic Appl Ecol 16:54–63

Lima IB, Machado JR, Machado JFF, Rivaroli L (2020) Effect of exposure to glyphosate based herbicide-roundup original®-and nutritional therapy with folic acid and selenium on cardiac histogenesis of bullfrog (*Lithobates catesbeianus*, Shaw-1802). Revista Acta Ambiental Catarinense 17:73–85

Lozano VL, Vinocur A, García CS, Allende L, Cristos DS, Rojas D, Wolansky M, Pizarro H (2018) Effects of glyphosate and 2, 4-D mixture on freshwater phytoplankton and periphyton communities: a microcosms approach. Ecotoxicol Environ Saf 148:1010–1019

Lupi L, Miglioranza KS, Aparicio VC, Marino D, Bedmar F, Wunderlin DA (2015) Occurrence of glyphosate and AMPA in an agricultural watershed from the southeastern region of Argentina. Sci Total Environ 536:687–694

Mann RM, Bidwell JR (1999) The toxicity of glyphosate and several glyphosateformulations to four species of southwestern Australian frogs. Arch Contam Toxicol 36:193–199

Mann RM, Bidwell JR (2000) Application of the FETAX protocol to assess the developmental toxicity of nonylphenol ethoxylate to *Xenopus laevis* and two Australian frogs. Aquat Toxicol 51:19–29

Mann RM, Hyne RV, Choung CB, P. WS (2009) Amphibians and agricultural chemicals: Review of the risks in a complex environment. Environ Pollut 157:2903–2927

Merini LJ, Cuadrado V, Giulietti AM (2008) Spiking solvent, humidity and their impact on 2, 4-D and 2, 4-DCP extractability from high humic matter content soils. Chemosphere 71:2168–2172

Morgan MK, Scheuerman PR, Bishop CS, Pyles RA (1996) Teratogenic potential of antrazine and 2,4-D using FETAX. J Toxicol Environ Health 48:151–168

Pavan FA, Samojeden CG, Rutkoski CF, Folador A, Da Fré SP, Müller C, Hartmann PA, Hartmann MT (2021) Morphological, behavioral and genotoxic effects of glyphosate and 2, 4-D mixture in tadpoles of two native species of South American amphibians. Environ Toxicol Pharmacol 85:103637

Peltzer PM, Lajmanovich RC, Sanchez LC, Attademo AM, Junges CM, Bionda CL, Martino AL, Basso A (2011) Morphological abnormalities in amphibian populations. Herpetol Conserv Biol 6:432–442

Peluso J, Aronzon CM, Acquaroni M, Coll CSP (2020a) Biomarkers of genotoxicity and health status of *Rhinella fernandezae* populations from the lower Paraná River Basin, Argentina. Ecol Indic 117: 106588

Peluso J, Aronzon CM, de Molina MdCR, Rojas DE, Cristos D, Pérez Coll CS (2020b) Integrated analysis of the quality of water bodies from the lower Paraná River basin with different productive uses by physicochemical and biological indicators. Environ Pollut:114434

Pengue WA (2004) Transgenic crops in Argentina and its hidden costs, Proceedings of IV Biennial International Workshop “Advances in Energy Studies”. Unicamp, Campinas, SP, Brazil, pp. 91-101

Pérez Coll CS, Aronzon CM, Svartz GV (2017) Chapter 17 Developmental Stages of *Rhinella arenarum* (Anura, Bufonidae) in Toxicity Studies: AMPHITOX, a Customized Laboratory Assay, Ecotoxicology and Genotoxicology: Non-traditional Aquatic Models. The Royal Society of Chemistry, pp. 407-424

Relyea RA, Jones DK (2009) The toxicity of Roundup Original Max to 13 speciesof larval amphibians. Environ Toxicol Chem 28:2004–2008

Romero DM, Ríos de Molina MC, Juárez AB (2011) Oxidative stress induced by a commercial glyphosate formulation in a tolerant strain of *Chlorella kessleri*. Ecotoxicol Environ Saf 74:741–747

Ruiz de Arcaute C, Soloneski S, Larramendy ML (2016) Toxic and genotoxic effects of the 2, 4-dichlorophenoxyacetic acid (2, 4-D)-based herbicide on the Neotropical fish *Cnesterodon decemmaculatus*. Ecotoxicol Environ Saf 128:222–229

Sasal MC, Wilson MG, Sione SM, Beghetto SM, Gabioud EA, Oszust JD, Paravani EV, Demonte L, Repetti MR, Bedendo DJ, Medero SL (2017) Monitoreo de glifosato en agua superficial en Entre Ríos. La investigación acción participativa como metodología de abordaje. RIA. Revista de investigaciones agropecuarias 43, 195-205

SENASA (2017): Importación de Fitoterapicos 2013, 2014, 2015, data on website.

Sing Yadav S, Giri S, Singha U, Boro F, Giri A (2013) Toxic and genotoxic effects of Roundup on tadpoles of the Indian skittering frog (Euflictiscyanophlyctis) in the presence and absence of predator stress. Aquat Toxicol 1-8:1–8

Soloneski S, de Arcaute CR, Larramendy ML (2016) Genotoxic effect of a binary mixture of dicamba-and glyphosate-based commercial herbicide formulations on *Rhinella arenarum* (Hensel, 1867) (Anura, Bufonidae) late-stage larvae. Environ Sci Pollut Res 23:17811–17821

Solomon DK, Thompson (2011) (2003) Ecological Risk Assessment for Aquatic Organisms from Over-Water Uses of Glyphosate. J Toxicol Environ Health Part B 6(3):289–324. https://doi.org/10.1080/10937400306468

Sprague JB (1970) Measurement of pollutant toxicity to fish. II. Utilizing and applying bioassay results. Water Res 4:3–32

Stebbins-Boaz B, Fortner K, Frazier J, Piluso S, Pullen S, Rasar M, Reid W, Sinclair K, Winger E (2004) Oocyte maturation in *Xenopus laevis* is blocked by the hormonal herbicide, 2,4-dichlorophenoxy acetic acid. Mol Reprod Dev 67:233–242

UN (2011) Peligros para el medio ambiente. Naciones Unidas Parte 4: 229–258

WHO WHO (1990) Public health impact of pesticides used in agriculture

Yu Y, Li X, Yang G, Wang Y, Wang X, Cai L, Liu X (2019) Joint toxic effects of cadmium and four pesticides on the earthworm (*Eisenia fetida*). Chemosphere 227:489–495

Zimdahl RL (2018) Fundamentals of weed science. Academic press

Terms and Conditions

Springer Nature journal content, brought to you courtesy of Springer Nature Customer Service Center GmbH (“Springer Nature”).

Springer Nature supports a reasonable amount of sharing of research papers by authors, subscribers and authorised users (“Users”), for small-scale personal, non-commercial use provided that all copyright, trade and service marks and other proprietary notices are maintained. By accessing, sharing, receiving or otherwise using the Springer Nature journal content you agree to these terms of use (“Terms”). For these purposes, Springer Nature considers academic use (by researchers and students) to be non-commercial.

These Terms are supplementary and will apply in addition to any applicable website terms and conditions, a relevant site licence or a personal subscription. These Terms will prevail over any conflict or ambiguity with regards to the relevant terms, a site licence or a personal subscription (to the extent of the conflict or ambiguity only). For Creative Commons-licensed articles, the terms of the Creative Commons license used will apply.

We collect and use personal data to provide access to the Springer Nature journal content. We may also use these personal data internally within ResearchGate and Springer Nature and as agreed share it, in an anonymised way, for purposes of tracking, analysis and reporting. We will not otherwise disclose your personal data outside the ResearchGate or the Springer Nature group of companies unless we have your permission as detailed in the Privacy Policy.

While Users may use the Springer Nature journal content for small scale, personal non-commercial use, it is important to note that Users may not:

1. use such content for the purpose of providing other users with access on a regular or large scale basis or as a means to circumvent access control;
2. use such content where to do so would be considered a criminal or statutory offence in any jurisdiction, or gives rise to civil liability, or is otherwise unlawful;
3. falsely or misleadingly imply or suggest endorsement, approval , sponsorship, or association unless explicitly agreed to by Springer Nature in writing;
4. use bots or other automated methods to access the content or redirect messages
5. override any security feature or exclusionary protocol; or
6. share the content in order to create substitute for Springer Nature products or services or a systematic database of Springer Nature journal content.

In line with the restriction against commercial use, Springer Nature does not permit the creation of a product or service that creates revenue, royalties, rent or income from our content or its inclusion as part of a paid for service or for other commercial gain. Springer Nature journal content cannot be used for inter-library loans and librarians may not upload Springer Nature journal content on a large scale into their, or any other, institutional repository.

These terms of use are reviewed regularly and may be amended at any time. Springer Nature is not obligated to publish any information or content on this website and may remove it or features or functionality at our sole discretion, at any time with or without notice. Springer Nature may revoke this licence to you at any time and remove access to any copies of the Springer Nature journal content which have been saved.

To the fullest extent permitted by law, Springer Nature makes no warranties, representations or guarantees to Users, either express or implied with respect to the Springer nature journal content and all parties disclaim and waive any implied warranties or warranties imposed by law, including merchantability or fitness for any particular purpose.

Please note that these rights do not automatically extend to content, data or other material published by Springer Nature that may be licensed from third parties.

If you would like to use or distribute our Springer Nature journal content to a wider audience or on a regular basis or in any other manner not expressly permitted by these Terms, please contact Springer Nature at

onlineservice@springernature.com