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Opposite effects of mixtures of commercial formulations of glyphosate with auxinic herbicides on the ten spotted live-bearer fish *Cnesterodon decemmaculatus* (Pisces, Poeciliidae)☆

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ABSTRACT

We analyzed the acute toxicity of the 48% glyphosate (GLY)-based Credit®, the 57.71% dicamba (DIC)-based Kamba®, and the 83.5% 2,4-dichlorophenoxyacetic acid (2,4-D)-based Weedar® Full, alone and as mixtures on the fish *Cnesterodon decemmaculatus*. Mortality revealed the $LC_{50\ 96h}$ values of 91.73 mg L^{-1} (range: 86.80–98.00 mg L^{-1}), 1401.57 mg L^{-1} (range: 1243.78–1527.35) and 678.04 mg L^{-1} (range: 639.35–718.04 mg L^{-1}) for GLY, DIC and 2,4-D, respectively. Mean values for the toxic unit (TU) that induced 50% mortality ($TU_{50\ 96h}$) of fish exposed to equitoxic mixtures were 1.67 (range: 1.65–1.69) for Credit® and Kamba® and 1.28 (range: 1.20–1.36) for Credit® and Weedar® Full suggesting that both mixtures are antagonic. Non-equitoxic combinations demonstrated an antagonistic interaction of herbicides Credit® and Kamba®, whereas a synergistic effect was observed for Credit® and Weedar® Full formulations. GLY and DIC as a mixture demonstrated lower toxicity on non-target species compared to GLY and 2,4-D in combination, at least for *C. decemmaculatus*, leading to the conclusion that the former combination could be strongly recommended in further agricultural practices.

1. Introduction

Glyphosate (GLY) [*N*-(phosphonomethyl) glycine], generally used as a GLY isopropylamine salt, is traditionally employed in pre- and post-emergent herbicide formulations with extensive agricultural usage but also employed in urban and forest areas (Tsui and Chu, 2008). GLY acts by inhibition of plant development by blocking the biosynthesis of essential aromatic amino acids inhibiting the enzyme 5-enolpyruvil shikimate-3-phosphate pathway present in plants, fungi and bacteria (Sharps, 1984). It possesses high water solubility and thus the herbicide contaminates surface and ground waters (WHO-FAO, 1997). The World Health Organization (WHO) considers the toxicity exerted by GLY as very low, and significantly dependent upon other compounds (i.e., excipients present within commercial products) to increase its absorption and efficacy (Mann et al., 2009; Wagner et al., 2013). According to epidemiologic available data, the WHO International Agency for Research on Cancer (IARC) classified GLY as a “probably carcinogenic in humans” (category 2A) compound (IARC, 2017). Available literature demonstrates that the analytical standard is less toxic than GLY-commercial formulated products to aquatic organisms (Cedergreen and Streibig, 2005; Peixoto, 2005; Pereira et al., 2009; Sobrero et al., 2007). In general, products containing GLY present as a trimethylsulfonium salt are more hazardous to aquatic biota than those containing GLY as an isopropylamine salt (Pettersson and Ekelund, 2006). In addition, included within the latter group is Roundup Original®, the first commercial and most commonly used GLY-based herbicide. Its toxicity is mainly committed to the POEA, the surfactant polyethoxylated tallow amine, which renders the formulated product more toxic than the pure compound and other POEA-free commercial herbicides (Bolognesi et al., 1997; Brodeur et al., 2014; Moore et al., 2012; Tsui and Chu, 2003; Tsui and Chu, 2008). However, Guilherme et al. (2012) reported a similar toxicity pattern induced not only by GLY but also by Roundup® and POEA when the European eel *Anguila anguila* was employed as test organism.

A commonly used strategy of modern agricultural practices is the application of herbicides, such as GLY, in combination with

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other pesticides to control and prevent weed resistance, despite of the potential effect that the mixture could exert on non-target organisms (Ge et al., 2014). Moreover, mixtures of compounds can produce complex interactions that cause additive, synergistic or even antagonistic effects (Brodeur et al., 2014; Lydy et al., 2004). Auxinic herbicides are frequently used worldwide to control unwanted broadleaf dicotyledons (USEPA, 2006). They mimic the natural phytohormone auxin, which causes uncontrolled growth, senescence and plant death (Grossmann, 2003; USEPA, 2006).

Dicamba (DIC) is a chlorinated benzoic acid-derivative that is a member of the auxinic group of herbicides. It is frequently used in cereal crops, sugar cane, soybeans and wheat, as well as in grazing and natural fields (CASAFE, 2009; USEPA, 2006). DIC has been reported as a contaminant in cultivated and urban areas (Woudneh et al., 2007), in surface drinking-water reservoirs (Donald et al., 2007) and in estuarine waters (Bushek et al., 2007). Its risks on aquatic and terrestrial organisms are well known (USEPA, 2006). Toxicological information on DIC is available to assess its potential negative effect to living species (USEPA, 2006). DIC has been classified as Class II (moderately hazardous) by WHO (www.who.int/ipcs/publications/pesticides_hazard/en/) and as Class III (slightly toxic) by the U.S. Environmental Protection Agency (U.S. EPA) (2006).

2,4-D is also an auxinic herbicide, and it belongs to the phenoxyalkanoic acids family. The U.S. EPA classified 2,4-D as slightly to moderately toxic (category II-III) (USEPA, 1974) whereas WHO (2009) as Class II (moderately hazardous). Additionally, in 2015, IARC arrived to the conclusion that 2,4-D is possibly carcinogenic to humans (Group 2B) based on mechanistic studies (IARC, 2015). Concentrations of 2,4-D have been detected in surface, ground and in potable water supplies (Félix-Cañedo et al., 2013; Glozier et al., 2012; Loos et al., 2010; Tagert et al., 2014).

When pesticides are release into the environment, they reach their target species and also other non-target aquatic biota, including fish (Liang et al., 2013). Fish are sensitive to low concentrations of environmental pollutants, which make them a suitable organism to be used as an environmental bioindicator. The species *Cnesterodon decemmaculatus* is a member of the Poeciliidae family. This is a small zooplanctophagous non-migratory organism that is often present in high densities in watercourses. It has an extensive distribution in South American basins. This species is easy to manipulate and adapted to laboratory; it has characteristics that are optimal for toxicity testing, such as large range of tolerance to many environmental parameters (de la Torre et al., 2007; Di Marzio et al., 2005; Ruiz de Arcaute et al., 2014b, 2016; Vera-Candioti et al., 2015). Moreover, it has been used as an *in vivo* model to analyse the toxicity exerted by pesticides in aquatic vertebrates, *e.g.*, the insecticide chlorpyrifos present in the Lorsban 48E® and CPF Zamba® formulations (Vera-Candioti et al., 2014), the insecticide pirimicarb contained in Aficida® (Vera-Candioti et al., 2010) and Patton Flow® formulated products (Vera-Candioti et al., 2015), the paraquat as Osaquat® commercial formulation (Di Marzio et al., 1998), the GLY-based herbicides Credit® and Panzer® (Vera-Candioti et al., 2013), the herbicides DIC and 2,4-D present in the formulations Banvel® (Ruiz de Arcaute et al., 2014b) and DMA® (Ruiz de Arcaute et al., 2016), respectively.

In the current report, we analysed the acute toxicity and interactions of equitoxic and non-equitoxic mixtures using the herbicide formulated products Credit® (48% GLY), Kamba® (57.7% DIC) and Weedar® Full (83.5% 2,4-D). These three agrochemicals are commonly used worldwide and they have extensive and overlapping applications. Particularly, in Argentina mixing of these herbicides are employed to control the growth of different undesired broadleaf weeds in soybean, wheat, sorghum, among other economical crops (CASAFE, 2017). The acute toxic effects of these three chemicals were tested using specimens of *C. decemmaculatus* (Jenyns, 1842) that were exposed under semi-static experimental laboratory conditions. We aimed to determine the toxic interactions — specifically synergy, antagonism or additivity — that prevails when these herbicides are applied simultaneously as mixtures.

2. Material and methods

2.1. Chemicals

The following herbicide commercial formulations employed were 48% GLY [*N*-(phosphonomethyl) glycine; CAS 1071-83-6] Credit®, 57.7% DIC (3,6-dichloro-2-methoxybenzoic acid; CAS 1918-00-9) Kamba®, and 83.5% 2,4-D (2,4-dichlorophenoxyacetic acid, CAS 2008-39-1) Weedar® Full. All herbicides were kindly provided by Nufarm Ltd. Argentina (Buenos Aires, Argentina). All pesticide formulations included proprietary excipients of undisclosed molecular nature not provided by the manufacturers. $K_2Cr_2O_7$ [$Cr_{(VI)}$] (CAS 7778-50-9) was purchased from Merck KGaA (Darmstadt, Germany). All other reagents of analytical grade were obtained from Sigma Chemical Company (St. Louis, MO).

2.2. Quality control

GLY, DIC and 2,4-D concentrations were determined in test solutions by QV Chem Laboratory (La Plata, Buenos Aires, Argentina) following the criteria of the U.S. Geological Survey Report 01-4134 (Furlong et al., 2011) and OSHA Analytical Method PV2067. Analyte levels were determined by high-performance liquid chromatography (HPLC) using an ultraviolet detector and derivatization with fluorenylmethyloxycarbonyl. Pure substance samples from test solutions (50, 500 and 500 mg L^{-1} for Credit®, Kamba® and Weedar® Full, respectively) correspond to values obtained immediately after preparation (0 h) and at 24 h. The detection limit was 0.2 mg L^{-1} for GLY and 0.5 mg L^{-1} for DIC and 2,4-D.

2.3. Test organism

Specimens were collected from a pond away from agricultural zones near La Plata city (Buenos Aires Province, Argentina). The specimens were carried to the laboratory and kept for 20 days in dechlorinated tap water with 16/8 h light/dark cycles in aquaria. Physicochemical parameters of the water (mean ± SE) were: temperature, 20 ± 1 °C; pH, 7.55 ± 0.1; dissolved oxygen, 6.3 ± 0.3 mg L^{-1}; ammonium ($NH4^{+}$),< 0.2 mg L^{-1}; hardness, 143 ± 23.5 mg L^{-1} $CaCO_3$. The specimens were provided with aeration and an *ad libitum* supply of fish food (TetraFin®, Tetra-Werke, Melle, Germany). Organisms were cared following the recommendations of Argentinean National Service for Sanitary and Quality of Agriculture and Food guidelines 617/2002 for biological testing (SENASA, 2013).

2.4. Acute toxicity of GLY-, DIC-, and 2,4-D-based formulations

Experiments were performed according to standardized methods for piscine testing recommended by the U.S. EPA (IRAM, 2008; USEPA, 1975, 2002), with minor modifications as reported elsewhere (Ruiz de Arcaute et al., 2014b, 2016; Vera-Candioti et al., 2013). The concentrations employed in the toxicity tests were determine using preliminary assays, based on suggestions proposed by the U.S. EPA (2002). For each end-point, 10 fish were selected at random and were transferred to an aquaria containing 1 L of dechlorinated tap water according to previous recommendations (Mugni et al., 2015; Ruiz de Arcaute et al., 2014b, 2016;

Vera-Candioti et al., 2010, 2013, 2014). Then, the organisms were exposed to 12 concentrations of DIC (300, 500, 1000, 1200, 1500, 1550, 1650, 1700, 1750, 2000, 3000 and 900 mg L^{-1}) or 13 concentrations of 2,4-D (400, 500, 600, 650, 700, 750, 800, 1100, 1200, 1300, 1400, 1600 and 2200 mg L^{-1}) for 96 h. Negative (dechlorinated tap water; see Section 2.3) and positive (21.4 mg L^{-1} $Cr_{(VI)}$-treated fish) controls (Vera-Candioti et al., 2013) were performed in parallel with fish exposed to DIC or 2,4-D. All solutions were prepared before use and renovated completely every 24 h. Specimens were not fed throughout the experiment. Lethal effects were determined every 24 h. Experiments were carried out in triplicate and performed in simultaneous for each experimental point.

LC_{50} values for the GLY contained in the formulation product Credit® were determined previously in our laboratory by Vera-Candioti et al. (2013).

2.5. Acute toxicity of mixtures of GLY-, DIC-, and 2,4-D-based formulations

For treatments using both equitoxic and non-equitoxic pesticide mixtures, toxicity was estimated as suggested elsewhere (Brodeur et al., 2014, 2016; Warne, 2003). Accordingly, a toxic unit (TU) was arbitrarily assigned to the concentration of the pesticide that was able to induce 50% mortality in *C. decemmaculatus* after 96 h of exposure ($LC_{50\ 96h}$ value). The toxicity of mixtures was evaluated for the GLY contained in the formulation Credit® and the DIC contained in the Kamba®, and for the GLY contained in the formulation Credit® and the 2,4-D contained in the formulation Weedar® Full.

A negative control (dechlorinated tap water; see Section 2.3) was performed simultaneously with exposed organisms. Test solutions were prepared before of the experimental treatment and replaced every 24 h. Lethal effect was assessed visually every 24 h. Fish were not fed throughout the experiment. Experiments were performed in triplicate and run simultaneously for each experimental point. The TU–response relationship was obtained and was used to determine the TU value that caused 50% mortality (TU_{50}) for each experimental point.

2.5.1. Acute toxicity of equitoxic mixtures of GLY-, DIC-, and 2,4-d-based formulations

For equitoxic mixtures, herbicide formulations were mixed in equal concentrations to achieve different combinations in which the sum of the TUs of both components equalled 0.125, 0.25, 0.5, 0.75, 1, 1.5, 1.7, 1.9, 2, 2.2, 2.3, 2.5, 3, and 4, as suggested elsewhere (Brodeur et al., 2014, 2016; Warne, 2003). For example, if the test pesticide mixture for GLY/DIC had a value of 1 TU, that means the mixture contained 0.5 TUs of the GLY-based formulation Credit® (50% of the $LC_{50\ 96h}$) and 0.5 TUs of the DIC-based formulation Kamba® (50% of the $LC_{50\ 96h}$). Similarly, if the test pesticide mixture GLY/2,4-D had a value of 0.5 TUs, that means the mixture contained 0.25 TUs of the GLY-based formulation Credit® (25% of the $LC_{50\ 96h}$) and 0.25 TUs of the 2,4-D-based formulation Weedar® Full (25% of the $LC_{50\ 96h}$). Experiments were performed following suggestions proposed by the U.S. EPA (1975, 1982, 2002) standardised methods with minor modifications reported previously for native species (Pérez-Iglesias et al., 2015; Ruiz de Arcaute et al., 2016; Vera-Candioti et al., 2015). For each experimental point, 10 specimens were selected at random. The specimens were maintained in a 1-L glass container and exposed to the 14 pesticide mixtures for 96 h.

2.5.2. Acute toxicity of non-equitoxic mixtures of GLY-, DIC-, and 2,4-d-based formulations

For non-equitoxic mixtures, eight series of experimental protocols that included nine fish exposed groups were conducted following the recommendations of Warne (2003) and Brodeur et al. (Brodeur et al., 2014, 2016). In a series of four experiments, the concentration of the GLY-based formulation Credit® was fixed at 0.33 or 0.66 TUs, while the concentration of the DIC-based formulation Kamba® or the 2,4-D-based formulation Weedar® Full were either 0.01, 0.05, 0.10, 0.50, 0.75, 1.00, 1.50, 2.00 or 2.25 TUs. Following the same criteria, a series of four other experiments were performed in which the concentration of either the DIC-based formulation Kamba® or the 2,4-D-based formulation Weedar® Full was fixed at 0.33 or 0.66 TUs, while the concentration of the GLY-based formulation Credit® was 0.01, 0.05, 0.10, 0.50, 0.75, 1.00, 1.50, 2.00 or 2.25 TUs.

Fish were exposed to the nine pesticide mixtures (0.01, 0.05, 0.10, 0.50, 0.75, 1.00, 1.50, 2.00 or 2.25 TUs) for 96 h according to the experimental protocol described above for acute toxicity of equitoxic mixtures (See section 2.5.1).

2.6. Statistical analysis

Data of acute lethality tests for the individual formulations on *C. decemmaculatus* were analyzed using the U.S. EPA Probit Analysis, Version 1.5 statistical software (http://www.epa.gov/nerleerd/stat2.htm#tsk) and based on Finney's method (Finney, 1971). When analyzing the mixtures, a four-parameter logistic regression equation was performed to calculate the concentrations of pesticides with their respective 95% confidence intervals and the TUs of that resulted in the mortality of 50% of specimens. For these analyses, the GraphPad Prism software version 6 was employed as suggested elsewhere (Brodeur et al., 2014, 2016). All results, unless otherwise indicated, were considered significant at $P < 0.05$.

3. Results

3.1. Chemical analysis

No significant differences ($P > 0.05$) were observed in the concentration of the active ingredient in treatments during the 24 h interval renewals of the testing solutions (concentration range 97 ± 5% recovery). Concentrations assessed throughout the study represent the nominal concentrations of active ingredients that are present within the formulations Kamba® and Weedar® Full.

3.2. Acute toxicity of GLY-, DIC-, and 2,4-D-based formulations

Acute toxic effects of the Credit® formulation on *C. decemmaculatus* was reported previously by Vera-Candioti et al. (2013). Mortality experiments revealed the LC_{50} values for the GLY contained in the Credit® formulation of 98.50 mg L^{-1} (range, 93.60–105.80 mg L^{-1}), 93.73 mg L^{-1} (range, 89.10–100.20 mg L^{-1}), 91.73 mg L^{-1} (range, 86.80–98.00 mg L^{-1}), and 91.73 mg L^{-1} (range, 86.80–98.00 mg L^{-1}) after 24, 48, 72, and 96 h of exposure, respectively. No significant time-dependent increases in the mortality rate were identified when the exposure time increased from 24 to 96 h ($r = -0.83$, $P > 0.05$) (Vera-Candioti et al., 2013).

A Probit statistical analysis of the lethality data allowed the determination of the LC_{50} values for the DIC contained in the Kamba® formulation after 24, 48, 72, and 96 h of exposure. Results revealed the following mean LC_{50} values: 24h = 2918.16 mg L^{-1} (range, 2572.05–3522.48 mg L^{-1}), $LC_{50\ 48h}$ = 2264.98 mg L^{-1} (range, 2264.98–2613.82 mg L^{-1}), $LC_{50\ 72h}$ = 1690.50 mg L^{-1} (1502.66–1880.06 mg L^{-1}), and $LC_{50\ 96h}$ = 1401.57 mg L^{-1} (range, 1243.78–1527.35 mg L^{-1}). Complete mortality data are presented in Table 1. Overall, a regression analysis demonstrated a concentration-dependent increase in mortality rate ($r = -0.98$, $P < 0.01$).

Mortality experiments revealed the LC_{50} values of the 2,4-D

Table 1
Lethal effects of the dicamba-based herbicide Kamba® on *Cnesterodon decemmaculatus* exposed fish.

Number of individuals	Concentration (mg L^{-1})	Mortality (number of dead individuals)			
		24 h	48 h	72 h	96 h
30	Negative control	0	0	0	1
30	300	1	1	1	2
30	500	1	1	1	2
30	1000	0	1	1	6
30	1200	3	7	7	12
30	1500	3	7	7	13
30	1550	2	4	4	16
30	1650	6	17	17	23
30	1700	6	20	20	24
30	1750	6	18	18	24
30	2000	9	18	18	25
30	3000	12	19	23	26
30	9000	30	30	30	30
30	Positive control[a]	5	8	12	16

[a] Cr(VI) (21.4 mg L-1) was used as positive control.

present within the based formulation Weedar® Full of 1203.29 mg L^{-1} (range, 1145.79–1254.03 mg L^{-1}), 919.81 mg L^{-1} (range, 771.11–1371.89 mg L^{-1}), 756.82 mg L^{-1} (range, 716.73–802.62 mg L^{-1}), and 678.04 mg L^{-1} (range, 639.35–718.04 mg L^{-1}) after 24, 48, 72, and 96 h of exposure, respectively. Complete mortality data are presented in Table 2. Regression analysis reveals a trend toward a concentration-dependent increase in the mortality rate, although the increase did not reach statistical significance ($r = -0.94$, $P < 0.05$).

3.3. Acute toxicity of equitoxic mixtures of GLY-, DIC- and 2,4-D-based formulations

Complete mortality data for the equitoxic mixtures of GLY-, DIC- and 2,4-D-based formulations are presented in Table 3. Survival of the negative control group was 100%. Analysis of lethal effects allowed a determination of the TUs for the equitoxic mixtures of GLY/DIC and GLY/2,4-D that caused a mortality of 50% of the exposed fish. Results of the $TU_{50\ 96h}$ values for the equitoxic mixtures revealed means of 1.67 (range, 1.65–1.69) for the mixture of Credit® and Kamba® (Fig. 1A) and 1.28 (range, 1.20–1.36) for the mixture of Credit® and Weedar® Full (Fig. 1B).

Table 2
Lethal effects of the 2,4-D-based herbicide Weedar Full® on *Cnesterodon decemmaculatus* exposed fish.

Number of individuals	Concentration (mg L^{-1})	Mortality (number of dead individuals)			
		24 h	48 h	72 h	96 h
30	Negative control	0	0	0	0
30	400	1	1	2	2
30	500	0	2	4	9
30	600	0	3	6	8
30	650	1	4	6	11
30	700	0	6	11	17
30	750	1	9	12	16
30	800	1	13	18	20
30	1100	6	19	27	29
30	1200	18	30	30	30
30	1300	21	30	30	25
30	1400	28	30	30	30
30	1600	28	30	30	30
30	2200	30	30	30	30
30	Positive control[a]	5	8	12	16

[a] Cr(VI) (21.4 mg L-1) was used as positive control.

Table 3
Lethal effects of equitoxic mixtures of the glyphosate (GLY)-based Credit®, dicamba (DIC)-based herbicide Kamba® and 2,4-D-based herbicide Weedar Full® comercial formulations on *Cnesterodon decemmaculatus* exposed fish.

Concentrations (Tus)[a]		Number of individuals	Mortality (number of dead individuals)			
			24 h	48 h	72 h	96 h
Negative control		30	0	0	0	0
GLY	DIC					
0.0625	0.0625	30	0	1	1	1
0.125	0.125	30	0	1	1	1
0.25	0.25	30	0	0	0	0
0.5	0.5	30	0	0	0	1
0.75	0.75	30	0	3	4	4
0.85	0.85	30	13	14	16	17
0.95	0.95	30	28	29	29	29
1	1	30	29	29	29	29
1.1	1.1	30	30	30	30	30
1.15	1.15	30	30	30	30	30
1.25	1.25	30	30	30	30	30
1.5	1.5	30	30	30	30	30
2	2	30	30	30	30	30
GLY	2,4-D					
0.0625	0.0625	30	1	1	1	1
0.125	0.125	30	0	0	0	0
0.25	0.25	30	1	1	3	4
0.5	0.5	30	0	0	2	6
0.75	0.75	30	12	14	16	19
0.85	0.85	30	30	30	30	30
0.95	0.95	30	30	30	30	30
1	1	30	30	30	30	30
1.1	1.1	30	30	30	30	30
1.15	1.15	30	30	30	30	30
1.25	1.25	30	30	30	30	30
1.5	1.5	30	30	30	30	30
2	2	30	30	30	30	30

[a] Toxic unit (TU), concentration value of individual pesticide that causes 50% of mortality.

3.4. Acute toxicity of non-equitoxic mixtures of GLY-, DIC- and 2,4-D-based formulations

Survival of the negative control group was 100%. Mortality results allowed the determination of the TUs of the non-equitoxic mixtures that caused 50% mortality in the exposed fish; the mixtures were the GLY-based formulation Credit® with the DIC-based formulation Kamba® and with the 2,4-D-based formulation Weedar® Full. The isobolograms shown in Fig. 2 summarise the results obtained for the series of non-equitoxic mixtures using the commercial formulations Credit® and Kamba® (Fig. 2A) and Credit® and Weedar® Full (Fig. 2B). The diagonal isobole that links the values of 1 TU of both the *X*- and *Y*-axes corresponds to the line effect of a concentration addition. The isobologram depicted in Fig. 2A clearly demonstrated that combinations that cause 50% mortality were located above and to the right of the additivity line. Thus, these results demonstrated the occurrence of antagonistic interactions of non-equitoxic combinations of herbicides that contain GLY- and DIC-formulated products (Fig. 2A). In contrast, the isobologram depicted in Fig. 2B clearly demonstrated that combinations that cause 50% mortality were located below and to the left of the additivity line. Thus, these results demonstrated the occurrence of synergistic interactions of non-equitoxic combinations of herbicides that contain GLY- and 2,4-D-formulated products (Fig. 2B).

Results demonstrated that when mixtures of the commercial formulations of GLY and DIC were assayed, the toxicity induced by the GLY-based formulation Credit® either at a concentration of 0.33 or 0.66 TUs was antagonised by the presence of the DIC-based formulation Kamba® within mixtures (Fig. 2A). Fig. 2A highlights

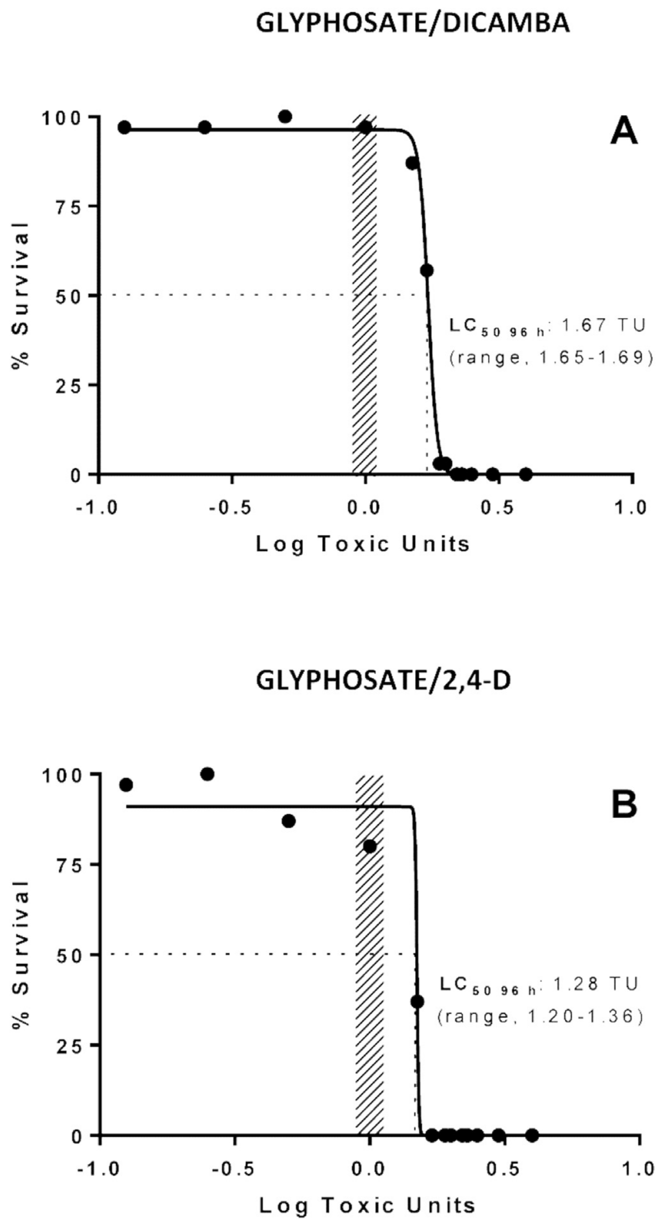

Fig. 1. Dose-response survival curve as a function of the sum of toxic units (TUs) of equitoxic mixtures for the DIC-based formulation Kamba® and the 2,4-D-based formulation Weedar® Full in *C. decemmaculatus* after 96 h of exposure. The sum of the TUs of the mixture that cause 50% mortality ($LC_{50\ 96h}$) is indicated with the 95% confidence interval in parenthesis. The striped area depicts the "Additivity Zone", which is the interval of TUs within which the $LC_{50\ 96h}$ value would be found if the mixture was additive.

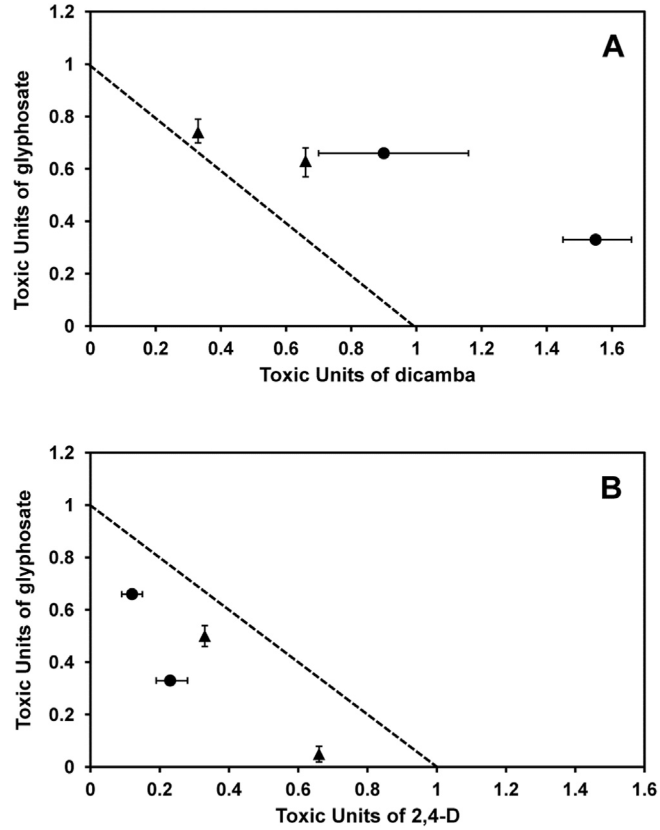

Fig. 2. Isobologram illustrating the composition in toxic units (TUs) of non-equitoxic mixtures that cause 50% mortality (TU_{50}) in *C. decemmaculatus* following 96 h of exposure to the dicamba-based formulation Kamba® and the 2,4-D-based formulation Weedar® Full. The error bars for each experimental value represents 95% confidence intervals. The diagonal isobole that links the values on the ordinate and abscissa axes with values of 1 TU is the line of concentration addition.

that 0.33 TUs of GLY required 0.74 TUs of DIC (range, 0.70–0.79) to induce the same toxic effect that was observed when 0.66 TUs was combined with 0.63 TUs of DIC (range, 0.57–0.68). Additionally, when DIC was fixed at 0.33 or 0.66 TUs, it was necessary to add 1.55 (range, 1.45–1.66) and 0.90 (range, 0.70–1.16) TUs of GLY, respectively, to reach 50% mortality.

On the other hand, results of the mixtures of the commercial formulations of GLY and 2,4-D revealed a synergism regardless of whether 0.33 or 0.66 TUs were assayed for both GLY and 2,4-D (Fig. 2B). Results demonstrated that 0.33 and 0.66 TUs of GLY required 0.23 (range, 0.19–0.28) and 0.12 (range, 0.09–0.15) TUs of 2,4-D, respectively, to induce 50% mortality. In addition, 0.33 and 0.66 TUs of 2,4-D required 0.50 (range, 0.46–0.54) and 0.05 (range, 0.03–0.08) TUs of GLY, respectively, to reach the same level of mortality.

4. Discussion

We used the Neotropical zooplanctophagous fish *C. decemmaculatus* (Pisces, Poeciliidae) as a test organism to study the acute toxicity and the possible toxic interactions (i.e., synergy, antagonism or additivity) that result after exposure to equitoxic and non-equitoxic mixtures of two herbicide commercial formulations — the DIC contained in the formulation Kamba® and the 2,4-D contained in the formulation Weedar® Full — when applied together with GLY contained in the formulation Credit®.

Previously, we evaluated two other formulations based on the same active ingredients, the DIC-based formulation Banvel® (57.71% DIC) and the 2,4-D-based formulation DMA® (58.4% 2,4-D) on the same experimental species (Ruiz de Arcaute et al., 2014b, 2016). We analysed the pesticide-induced lethal effects as an end point for mortality, and the frequency of micronuclei and DNA single-strand breaks evaluated by the comet assay as end points for genotoxicity (Ruiz de Arcaute et al., 2014b, 2016). In these studies, $LC_{50\ 96h}$ values for the tested species were 1639 mg L^{-1} (range, 1471–1808 mg L^{-1}) and 1008 mg L^{-1} (range, 929–1070 mg L^{-1}) for DIC and 2,4-D, respectively (Ruiz de Arcaute et al., 2014b, 2016). In our current study, the $LC_{50\ 96h}$ values were 1401.57 mg L^{-1} (range, 1243.78–1527.35 mg L^{-1}) for DIC in the formulated product Kamba® and 678.04 mg L^{-1} (range, 639.35–718.04 mg L^{-1}) for 2,4-

D in the formulated product Weedar® Full. Therefore, comparing both data sets, it can be concluded that DIC is able to exert the same mortality in *C. decemmaculatus* regardless of the formulation employed, at least for Banvel® and Kamba®. On the other hand, it is evident that *C. decemmaculatus* is 1.49 times more resistant to DMA® than to Weedar® Full, although the active ingredient is present in both formulated products as 2,4-D isopropylamine salt. Thus, the differences that we observed in lethality between these two 2,4-D formulations were most likely due to the excipients present within the formulations rather than the active ingredient. It is well known that in a pesticide formulated product, the pure compound is mixed with several additives to achieve an optimal penetration and performance (WHO, 1990). These additives are not usually included in the discussion of the effects on living non-target organisms, and their toxic effects can go beyond those of the pure compound. Several authors have proved and agree in demonstrating that the excipients present in pesticide commercial products, including herbicides, are able to induce both toxicity and cellular damage by themselves rather than the pure compound either *in vitro* or *in vivo* (Mann and Bidwell, 1999; Mansano et al., 2016a, 2016b; Molinari et al., 2013; Nikoloff et al., 2014; Pérez-Iglesias et al., 2014; Ruiz de Arcaute et al., 2014a; Vera-Candioti et al., 2010). Regrettably, the identities as well as the quantities of the additive compounds present in the formulations DMA® and Weedar® Full were not revealed to us by the manufacturers. According to the Argentinean directives, additive compounds present in any pesticide are not obligatory for listing on agrochemical data sheets and can be kept as a "trade secret." It is worth mentioned that the U.S. EPA (1982) highlighted that the toxic effects of formulated products can diverge considerably from that of the end-use formulation that contains that active ingredient. Our results are in concordance with this notion and pinpoint the necessity of further studies, at least on *C. decemmaculatus*, using pure 2,4-D as the test compound. The aim would be to reveal whether the jeopardising effects we observed were specific to 2,4-D or if the increased toxicity of the commercial product resulted from synergistic effects of the other ingredients present in the commercial formulations DMA® and Weedar® Full.

Finally, based on the acute toxicity found in our study, according to the scoring used by the Office of Pollution Prevention and Toxics of the U.S. EPA (2001), GLY could be ranked as a practically nontoxic agrochemical, and DIC and 2,4-D as compounds having with low ecotoxicity concern for the ten spotted live-bearer fish *C. decemmaculatus*. Additionally, according to the hazard risk assessment categories of the European Union directives (category IV) (Mazzatorta et al., 2002), GLY can be considered to be a herbicide that is harmful to aquatic organisms, whereas DIC and 2,4-D as compounds that could cause long-term adverse effects on *C. decemmaculatus* in the aquatic environment. Moreover, GLY, DIC, and 2,4-D can be also classified as harmful compounds for aquatic organisms (category III) according the classification proposed by the United Nations (UN, 2011).

The application of mixtures of pesticides is a common and extensive agricultural practice that represents an important group of environmental pollutants. In an attempt to address some of the concern about the effects of these pollutants, several reports have been published about the complexity of the toxic effects exerted by mixtures of pesticides. The toxicity of GLY in combination with imazapyr and triclopyr and modified vegetable oil surfactant was evaluated by Tatum et al. (2012) in the water flea *Ceriodaphnia dubia* and in the fathead minnow fish *Pimephales promelas*. The authors demonstrated that the mixture of these herbicides had a slight synergistic effect on the LC_{50} values for the models that were used (Tatum et al., 2012). Santos et al. (2011) observed a synergistic patter in enzymatic biomarkers in the earthworm *Eisenia andrei* and in the isopod *Porcellionides pruinosus* following exposure to a mixture of GLY and dimethoate. However, for the same mixture, both synergistic and antagonistic effects were reported when germination success was studied in seeds of the field mustard *Brassica rapa* (Santos et al., 2011). Recently, a synergistic toxicity of imidacloprid alone and in mixtures with seven other pesticides in the honey bee *Apis mellifera* (Zhu et al., 2017) or the joint toxicity effects of chlorpyrifos and beta-cypermethrin in the zebrafish *Danio rerio* (Zhang et al., 2017) were reported. Finally, we have recently demonstrated a synergistic effect of a mixture of GLY- and DIC-based-commercial herbicide formulations on the induction of DNA damage on circulating blood cells of tadpoles of *Rhinella arenarum* (Soloneski et al., 2016).

In our current study, the toxicity of herbicide mixtures was analysed following the model originally described by Sprague (1970) and the protocol detailed by Warne (2003). In this sense, a value of 1 TU was arbitrarily assigned to a concentration of a toxicant that was able to inflict a particular response (Warne, 2003). In our study, we defined the particular response as equivalent to the concentration of the mixture of the two pesticides that was able to induce 50% mortality in the species after 96 h of exposure, (i.e., $LC_{50\ 96h}$).

According to Warne (2003), the toxicity and the concentration of the test solution of an equitoxic mixture, so-called factor S, is expressed in terms of the TU rather than mg/L, and it is equivalent to the sum of the individual TUs of each toxicant that is part of the pesticide mixture. If the selected toxic effect S is equal to 1, then the equitoxic mixture should be considered to be concentration additive. However, if S is less than or greater than 1, the mixture should be considered to be synergistic or antagonistic, respectively (Warne, 2003). Previously, this model had only been employed for analysing the toxicity of mixtures of the GLY and cypermethrin commercial formulations in *R. arenarum* and in *C. decemmaculatus* (Brodeur et al., 2014, 2016). Brodeur et al. (2014, 2016) demonstrated opposite effects depending upon the species investigated. For *R. arenarum*, the mixture of GLY and cypermethrin had synergic effects, whereas an antagonistic pattern was observed when the experiment investigated the effects on *C. decemmaculatus*.

This report represents the first study that analyses the interactions between the mixtures of GLY with two auxinic herbicides, namely DIC and 2,4-D on *C. decemmaculatus*. The results obtained for the equitoxic mixtures revealed mean TU_{50} values of 1.67 (range, 1.65–1.69) for the GLY contained in the formulation Credit® combined with DIC contained in the formulation Kamba® and 1.28 (range, 1.20–1.36) for the GLY contained in the formulation Credit® combined with the 2,4-D contained in the formulation Weedar® Full. Thus, we suggest that the auxinic formulations that were analysed exerted an antagonistic interaction when combined in equitoxic proportions with GLY.

Previous reports indicate that GLY was detected in water bodies near agricultural crops at concentrations up to 3.7 mg L^{-1} (Giesy et al., 2000). However, specifically for Argentina, Peruzzo et al. (2008) found GLY values between 0.10 and 0.70 mg L^{-1} in Pampasic water streams. In addition, Castro Berman et al. (2018) reported maximum concentrations of GLY of 4.52 μg L^{-1} for surface water, 0.13 μg L^{-1} for suspended particulate matter and 20.34 μg kg^{-1} for sediment samples, respectively. DIC and 2,4-D have been found in urban, agricultural and agricultural–urban sites and in surface drinking-water reservoirs (Loos et al., 2010; Glozier et al., 2012; Félix-Cañedo et al., 2013; Tagert et al., 2014). They contaminate both surface and groundwater because they are highly mobile and adsorb poorly onto soil particles (Li et al., 2009). In the United States, DIC and 2,4-D have been detected in surface water runoff at a maximum value of 2.1 μg L^{-1}, in surface water at a maximum value of 24 μg L^{-1} and in drinking water reservoirs at values as low as 1.04 μg L^{-1} (Donald et al., 2007; Phillips and Bode, 2004; Tagert et al., 2014). Lower environmental concentrations were reported for water in Canada, with values of 0.04 μg L^{-1} for DIC and 0.31 μg L^{-1} for 2,4-D (Woudneh et al., 2007; Tierney et al., 2011; Glozier et al., 2012). Concentrations of 2,4-D were found in surface and ground water in the range of 0.005 μg L^{-1} to 0.04 μg L^{-1} in Mexico (Félix-Cañedo et al., 2013), and at a concentration of 0.012 μg L^{-1} in European ground water (Loos et al., 2010). However, to the best of our knowledge, no data have been published on the environmental concentrations of DIC and 2,4-D in Argentina. It is worth mentioning that the TU50 value obtained for the equitoxic mixture containing GLY present in the formulation Credit® when co-applied with DIC present in the formulation Kamba® had a mean value of 1.67, value corresponding to 76.59 mg L^{-1} GLY plus 1170.31 mg L^{-1} DIC. Similarly, the TU50 value obtained for the equitoxic mixture containing GLY present in the formulation Credit® combined with 2,4-D contained in the formulation Weedar® Full had a mean value of 1.28 and comprised 58.71 mg L^{-1} GLY plus 433.94 mg L^{-1} 2.4-D. Thus, GLY, DIC and 2,4-D concentrations present in equitoxic mixtures in this study represent a relatively high end of the values found in the environment, perhaps only registered when specific events would occur, e.g., a direct application adjacent to surface waters in creeks, ponds and drainage ditches by accidental discharge, among others. Even though, we cannot exclude that fish populations and other living species could be exposed accidentally to these herbicides to concentration-range.

It is worth mentioning that when non-equitoxic mixtures were assayed and in agreement with the suggestions of Warne (2003), the type of toxicological interaction prevailing in the mixture results from at least four independent experimental observations. Thus, conclusions drawn from non-equitoxic mixtures should be considered more robust and even more representative of the real situation that takes place when an organism is exposed to two agrochemicals applied together. In agreement, it has been observed that fish species in the Pampasic Region of Argentina could be more suitable to be exposed to non-equitoxic mixtures than to equitoxic combinations. Commercial products available in the market containing GLY and 2,4-D as active ingredients indicate that they include 1.80–2.35 times more GLY than 2,4-D (SENASA, 2017). Besides, when using this model, our observations demonstrated the occurrence of antagonistic interactions in all four non-equitoxic combinations of the GLY-based formulation Credit® and the DIC-based formulation Kamba®. Contrarily, in all four non-equitoxic combinations of the GLY-based formulation Credit® and the 2,4-D-based formulation Weedar® Full, a synergistic interaction was observed. This result suggests that the concomitant presences of the GLY- and 2,4-D-based formulations in non-equitoxic mixtures are more toxic than the sum of their individual effects. Furthermore, it is worth mentioning that herbicide mix employed in our study represent a much more complex scenario since they involve herbicide formulations, which, as we know, are mixtures of several components by themselves. Additional studies are required to reveal whether the pattern of toxicity exerted by the mixtures of GLY-based formulation Credit® with the DIC-based formulation Kamba® or the 2,4-D-based formulation Weedar® Full, on the *C. decemmaculatus* we observed is referable to the active ingredients by themselves or results from the presence of xenobiotic(s) within the commercial formulations assayed in our study. In addition, caution should be taken as we cannot assume whether the proportion of each component of the mixtures employed follow a similar profile in aquatic environment as the different constituents of the formulations can suffer different transformation or degradation patterns in the environment. Thus, different pattern of occurrence in the water matrix could be produced diverging from that we tested. Extending this concept, considering the unknown nature of the excipients contained in the herbicide formulations we assayed, we cannot ensure or deny whether the constituents of the addressed commercial herbicides occur in aquatic environments in the chemical forms tested or if the eventual predominance of their metabolites can introduced uncertainties on the conclusions of our observations.

Finally, according to our results, the use of the combination of GLY and DIC as a mixture demonstrated lower toxic effects on non-target species compared to GLY and 2,4-D in combination when, *C. decemmaculatus* is employed as the biotic matrix. Thus, this observation could lead to the conclusion that the former combination of herbicides, i.e., GLY and DIC, could be recommended in further agricultural practices rather than the mixture GLY and 2,4-D. However, whether this scenario is committed to *C. decemmaculatus* or it could be also valid for other species is still an open question.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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