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Synergism of mixtures of dicamba and 2,4-dichlorophenoxyacetic acid herbicide formulations on the neotropical fish *Cnesterodon decemmaculatus* (Pisces, Poeciliidae)☆

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ABSTRACT

Dicamba (DIC) and 2,4-dichlorophenoxyacetic acid (2,4-D) are two of the most applied auxinic herbicides worldwide, both individually and as part of a mixture. However, the toxicity and interactions achieved when applied as a mixture have not yet been characterised. The equitoxic and non-equitoxic acute toxicity exerted by binary mixtures of Banvel® (57.71% DIC) and DMA® (58.4% 2,4-D) on the Neotropical fish *Cnesterodon decemmaculatus* were evaluated. Results revealed mean values of 1.02 (range, 0.96–1.08) for the toxic unit (TU) that induced 50% mortality ($TU_{50\ 96\ h}$) to the fish exposed to binary equitoxic mixtures of the commercial formulations Banvel®–DMA®. These results suggest that the mixture is nearly concentration additive. Furthermore, results demonstrated the occurrence of synergistic interaction when non-equitoxic combinations of Banvel®-or DMA®-formulated herbicides were assayed. In this context and regardless of their concentrations, either Banvel®- or DMA®-induced toxicity were synergised by the presence of the counterpart within mixtures. The present study represents the first evidence of the lethality exerted by mixtures of two auxinic herbicides—namely, DIC and 2,4-D—reported to date for fish and other biotic matrices. When *C. decemmaculatus* is used as the target organism, a synergistic pattern is observed following exposure to a mixture of both herbicides.

1. Introduction

Anthropogenic activities, especially agriculture, release large amounts of pesticides daily into the soil, water and air (Ippolito et al., 2015; Liaud et al., 2016). Annually, approximately four million tons of pesticides are applied around the globe (Ippolito et al., 2015) and disseminated by drift, surface runoff, and drainage into the environment (Aparicio et al., 2013; Meffe and de Bustamante, 2014). Accordingly, a mixture of products reaches not only their target species, but also non-target ones, for example, aquatic and terrestrial biota (Liang et al., 2013; Meffe and de Bustamante, 2014).

The application of mixtures of toxicants is a commonly used strategy to control and prevent weed resistance, despite the potential effects that the mixture could exert on non-target organisms (Ge et al., 2014). In addition to the additives that are present in the commercially available pesticide formulations, which are known to modify the toxic effects of the pure compound (Cox and Surgan, 2006; Nikoloff et al., 2014b; Ruiz de Arcaute et al., 2014b), it is known that mixtures of different compounds can interact with each. Those interactions can cause complex changes that are different from the toxic effects exerted by individual components on a biotic matrix (LeBlanc and Wang, 2006; Silva et al., 2015; Svartz et al., 2016; Varona-Uribe et al., 2016). In general terms, a mixture of toxicants can exert complex interactions between its components, including additivity, synergism and antagonism (Calamari and Alabaster, 1980; Calabrese, 1995; Warne, 2003; Lydy et al., 2004; Brodeur et al., 2014). Therefore, it is necessary to evaluate the joint effects of mixtures, especially those with interactions between at least two components (that is, binary mixtures) (Warne, 2003).

Since they were first synthesised after World War II, auxinic herbicides have been frequently used worldwide to eliminate unwanted broadleaf dicotyledons in crops (USEPA, 2006). These

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herbicides include quinolinecarboxylic, benzoic, phenoxyalkanoic and pyridinecarboxilic acids (Garraway and Wain, 1976). They mimic the natural phytohormone auxin, and they cause uncontrolled growth, senescence and death (Grossmann, 2003; USEPA, 2006). Among the benzoic acids, dicamba (DIC), a derivative of chlorinated benzoic acid, is frequently used for controlling weeds in cereal crops, sugar cane, soybeans, wheat, and in grazing and natural fields (USEPA, 2006; CASAFE, 2009). Information regarding the toxicity of DIC is sufficient to evaluate its potential hazards to humans and other aquatic and terrestrial organisms (USEPA, 2006). Taking into account its acute toxic effects, DIC has been ranked by the World Health Organisation (WHO) (2009) as a class II chemical (moderately hazardous) and by the U.S. Environmental Protection Agency (U.S. EPA) (2006) as a type III chemical (moderate toxic). In addition, following U.S. EPA's ecotoxicological classification for aquatic organisms (USEPA, 2008), DIC is considered to be a practically nontoxic pesticide.

2,4-dichlorophenoxyacetic acid (2,4-D)is an auxinic herbicide that belongs to the family of phenoxyalkanoic acids. It has been classified by the U.S. EPA as slightly to moderately toxic (category II–III) (USEPA, 1974), and by WHO as moderately hazardous (class II) (WHO, 2009). Moreover, in 2015, the International Agency for Research on Cancer (IARC) classified 2,4-D as a possibly carcinogenic agent to humans (Group 2B) based on mechanistic studies (IARC, 2015). In addition, and according the ecotoxicological classification proposed for aquatic organisms by the USEPA (2008), 2,4-D is classified as a practically nontoxic pesticide. There are over 172 products containing 2,4-D currently on Argentina (SENASA, 2017) and it is used alone or in combination with other herbicides (for example, in combination with DIC, 4-amino-3,5,6-trichloro-2-pyridinecarboxylic acid [picloran], 3,6-dichloro-2-pyridinecarboxylic acid [clopyralid] and methylchlorophenoxypropionic acid [MCPP]) (NCBI, 2017).

Regarding the toxicity of DIC reported for different fish species, data reviewed in the literature demonstrated $LC_{50\ 96h}$ values ranging from 50 mg/L for the cutthroat trout (*Oncorhynchus clarkii*) to 465 mg/L for the Western mosquitofish (*Gambusia affinis*) (USEPA, 2017). Similarly, $LC_{50\ 96\ h}$ concentrations of 2,4-D that range from 87 mg/L to 707 mg/L have been reported for common carp (*Cyprinus carpio*) and the rainbow trout (*O. mykiss*) (USEPA, 2017). When the acute toxicities of DIC, formulated as Banvel®, and 2,4-D, formulated as DMA®, were assayed, the $LC_{50\ 96\ h}$ concentrations were determined to be 1639 mg/L and 1008 mg/L, respectively, for the ten spotted live-bearer (*Cnesterodon decemmaculatus*) (Ruiz de Arcaute et al., 2014b, 2016). Thus, it could be pinpointed that the latter represents the most resistant fish species reported so far for both DIC and 2,4-D.

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, in surface water at a maximum value of 24 μg/L (Phillips and Bode, 2004; Tagert et al., 2014) and in drinking water reservoirs at values as low as 1.04 μg/L (Donald et al., 2007). Lower environmental concentrations were reported for water in Canada, with values of 0.04 μg/L for DIC and 0.31 μg/L 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 to 0.04 μg/L in Mexico (Félix-Cañedo et al., 2013), and at a concentration of 0.012 μg/L 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 DIC and 2,4-D have extensive and overlapping applications worldwide. Specifically for Argentina, these herbicides are recommended to be applied together in several crops (for example, sugar cane, cereal, corn, sorghum and other cultivars that are of commercial importance) (CASAFE, 2015).

Cnesterodon decemmaculatus (Cyprinodontiformes, Poeciliidae) is an endemic species for Argentina with an extensive distribution in Neotropical America. This is a small ovoviviparous, micro-omnivorous, benthic-pelagic and non-migratory fish (maximum size is approximately 25 mm for males and 45 mm for females), and it is often present in high-density in watercourses. It has an extensive distribution in the La Plata River and other South American basins (for review, see (Soloneski and Larramendy, 2017)). In addition, this species is easy to handle and acclimate to laboratory conditions, and they comply to the conditions requested for toxicity testing, such as a large range of tolerance to many environmental parameters (Di Marzio et al., 2005; de la Torre et al., 2007; Ruiz de Arcaute et al., 2014b, 2016; Vera-Candioti et al., 2015). The species has been employed as a suitable *in vivo* model to analyse pesticide-induced toxicity among aquatic vertebrates. Some of the tested chemicals include the following: chlorpyrifos-based insecticides Lorsban 48E® and CPF Zamba® (Vera-Candioti et al., 2014), the pirimicarb-based insecticides Aficida® (Vera-Candioti et al., 2010) and Patton Flow® (Vera-Candioti et al., 2015), the paraquat-based herbicide Osaquat® (Di Marzio et al., 1998), the glyphosate-based herbicides Credit® and Panzer® (Vera-Candioti et al., 2013), the DIC-based herbicide Banvel® (Ruiz de Arcaute et al., 2014b) and the 2,4-D-formulated product DMA® (Ruiz de Arcaute et al., 2016).

Although detailed testing has been undertaken in terms of *C. decemmaculatus* exposed to individual chemicals (as cited above), no analyses have been completed on the toxic effects exerted when *C. decemmaculatus* is exposed simultaneously to the DIC-based formulation Banvel® and the 2,4-D-formulated product DMA®. This includes possible synergistic, antagonistic or additive patterns. In the present study, we evaluated the acute toxicity and interactions of equitoxic and non-equitoxic binary mixtures of the commercial herbicide formulations Banvel® (57.71% DIC) and DMA® (58.4% 2,4-D) on *C. decemmaculatus* exposed under laboratory conditions using a semi-static acute experimental model.

2. Material and methods

2.1. Chemicals

The DIC-based (3,6-dichloro-2-methoxybenzoic acid; CAS 1918-00-9) formulation Banvel® (57.7% DIC) was obtained from Syngenta Agro S.A. (Argentina). The 2,4-D-based (2,4-dichlorophenoxyacetic acid, CAS 2008-39-1) formulation DMA® (58.4% 2,4-D) was obtained from Dow Agrosciences Argentina S.A., Argentina. $K_2Cr_2O_7$ [Cr(VI); CAS 7778-50-9] was obtained from Merck KGaA (Darmstadt, Germany). Chemicals and solvents of analytical grade were obtained from Sigma Chemical Co. (St. Louis, MO).

2.2. Quality control

DIC and 2,4-D concentrations in test solutions were determined by QV Chem Laboratory (La Plata, Buenos Aires, Argentina) using high-performance liquid chromatography and an ultraviolet detector, following the U.S. Geological Survey Report 01-4134 (Furlong et al., 2011). Active ingredient samples from test solutions (0.25 TU for Banvel® and DMA®, which is equivalent to 410 mg/L of Banvel® and 252 mg/L of DMA®) corresponded to values obtained immediately after preparation (0 h) and after 24 h. The detection limit of the active ingredients was 0.5 mg/L for both herbicide formulations.

2.3. Test organism

C. decemmaculatus specimens were collected from a permanent pond near La Plata city (34°86′S, 58°12′W, Buenos Aires Province, Argentina). After collection, they were acclimatised for 20 days under laboratory conditions (16/8 h light/dark cycle; 20 ± 1 °C with dechlorinated tap water [pH 7.55 ± 0.1; dissolved oxygen, 6.3 ± 0.3 mg/L; ammonium (NH_4+) < 0.2 mg/L; hardness, 143 ± 23.5 mg $CaCO_3$/L]) following the recommendation of the USEPA (1996) and other recommendations specifically reported for the species (Menni, 2004; Somma et al., 2011). Artificial aeration and *ad libitum* fish food (TetraFin®, TetraWerke, Melle, Germany) were provided until the beginning of the experimental protocols. The average body weight and mean body length of the specimens used throughout the experiment was 0.25 ± 0.1 g and 28.5 ± 2.3 mm, respectively. Fish were maintained following the recommendations of the Argentinean National Service for Sanitary and Quality of Agriculture and Food (SENASA) guidelines 617/2002 for biological testing (2013). Specimens were collected with the permission of the Flora and Fauna Direction from the Buenos Aires Province (Buenos Aires, Argentina) (code 22500-22339/13) and the ethical committee from the National University of La Plata (code 11/N754).

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

The acute toxicities of commercial formulations of DIC and 2,4-D were determined in previous experiments assayed in our laboratory (Ruiz de Arcaute et al., 2014b, 2016). The reported $LC_{50\ 96\ h}$ was 1639 mg/L (1,471 mg/L −1808 mg/L) for DIC and 1008 mg/L (929 mg/L −1070 mg/L) for 2,4-D (Ruiz de Arcaute et al., 2014b, 2016).

For both equitoxic and non-equitoxic herbicide mixtures, toxicity was estimated as suggested by 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 (that is, the $LC_{50\ 96\ h}$ value). The toxicity was evaluated for mixtures of DIC-based herbicide formulation Banvel® and 2,4-D-based formulation DMA®. The lethal effect was determined every 24 h in three independent experiments that were run simultaneously for each experimental point.

2.4.1. Acute toxicity of equitoxic binary mixtures of DIC- and 2,4-D-based formulations

For equitoxic mixtures, herbicide formulations were combined in equal concentrations to generate mixtures for which the sum of the TU of both components equalled 0.125, 0.25, 0.5, 1, 1.5, 2, 2.5, 3 and 4 units, as suggested elsewhere (Warne, 2003; Brodeur et al., 2014, 2016). For example, if the test pesticide mixture had a value of 1 TU, it was comprised of 0.5 TUs of DIC-based herbicide formulation Banvel® (50% of the $LC_{50\ 96\ h}$) and 0.5 TUs of 2,4-D-based formulation DMA® (50% of the $LC_{50\ 96\ h}$). Experiments were performed according to standardised methods recommended by the USEPA (1975, 1982, 2002), with minor modifications reported previously for native species (Pérez-Iglesias et al., 2015; Vera-Candioti et al., 2015; Ruiz de Arcaute et al., 2016). For each experimental point, 10 randomly selected specimens were maintained in a 1 L glass container and exposed to the nine herbicide mixtures for 96 h. A negative control (dechlorinated tap water; pH 7.5 ± 0.1; hardness, 143 ± 23.5 mg $CaCO_3$/L) was run simultaneously with the exposed organisms. The test solutions were prepared and replaced every 24 h. Fish were not fed during the experiment. Mortality was registered by visual observation every 24 h. The TU response relationship was derived, and it was used to determine the TU value that caused 50% of mortality (TU_{50}) for each experimental point.

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

For non-equitoxic mixtures, a series of four experimental protocols were conducted following the recommendations of Warne (2003) and Brodeur et al., (2014, 2016). Each series included five fish in the exposed group. In two of the experimental series, the concentration of DIC-based herbicide formulation Banvel® was remained constant at 0.33 TUs or 0.66 TUs, while the concentration of 2,4-D-based formulation DMA® was either 0.01, 0.05, 0.1, 1 or 2 TUs. In the other two series, the concentration of 2,4-D-based formulation DMA® was set at 0.33 or 0.66 TUs, while the concentration of DIC-based herbicide formulation Banvel® was either 0.01, 0.05, 0.1, 1 or 2 TUs. Fish were exposed to these pesticide mixtures for 96 h according to the experimental protocol described above for acute toxicity of equitoxic binary mixtures (See section 2.4.1). A negative control (dechlorinated tap water; pH 7.5 ± 0.1; hardness, 143 ± 23.5 mg $CaCO_3$/L) was run simultaneously with the pesticide-exposed organisms. The TU response relationship was used to determine the TU value that caused 50% of mortality (TU_{50}) for each experimental point.

2.5. Statistical analyses

The concentrations of herbicides and the TU of the mixtures that caused mortality of 50% of specimens were calculated by fitting a four-parameter logistic regression equation to the survival data using the GraphPad Prism software version 6. The level of significance was 0.05 for all tests.

3. Results

3.1. Chemical analysis

No significant changes ($p > .05$) were observed in the concentration of the pure analyte in treatments during the 24 h interval renewals of the testing solutions (concentration range 97% ± 5% recovery). Concentrations assessed throughout the experiment corresponded to the nominal concentrations of the analyte present within the DIC-based formulation Banvel® and 2,4-D-based formulation DMA®.

Table 1
Lethal effects of equitoxic binary mixtures of dicamba (DIC)-based Banvel® and 2,4-dichlorophenoxyacetic acid (2,4-D)-based DMA® commercial formulations on exposed *Cnesterodon decemmaculatus*.

Concentration (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
DIC	2,4-D					
0.0625	0.0625	30	0	0	0	1
0.125	0.125	30	1	1	2	3
0.25	0.25	30	1	1	2	2
0.5	0.5	30	2	4	9	14
0.75	0.75	30	4	23	28	28
1	1	30	2	27	30	30
1.25	1.25	30	12	30	30	30
1.5	1.5	30	30	30	30	30
2	2	30	30	30	30	30

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

3.2. Acute toxicity of equitoxic binary mixtures of DIC- and 2,4-D-based formulations

Data of mortality are presented in Table 1. Survival of the negative control group was 100%. Mortality results allowed for the determination of TUs of the equitoxic mixtures of DIC-based formulation Banvel® and 2,4-D-based formulation DMA® that caused mortality in 50% of the exposed fish. Results revealed a mean value of 1.02 (0.96–1.08) for the $TU_{50\ 96\ h}$ of the binary equitoxic mixtures of the commercial formulations Banvel® and DMA® (Fig. 1).

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

Complete data of mortality are presented in Table 2 for non-equitoxic mixtures of the formulations Banvel® and DMA®. Survival of the negative control group was 100%. Mortality results allowed for the determination of the TUs of the non-equitoxic mixtures that caused mortality in 50% of the exposed fish. The isobologram shown in Fig. 2 summarises data obtained for the series of non-equitoxic mixtures of the commercial formulations Banvel® and DMA®. The diagonal isobole linking the values of 1 TU on both the x- and y-axes correspond to the line effect of a concentration addition. The isobologram clearly demonstrates that combinations of chemicals that cause 50% mortality are below and to the left of the additivity line in the figure. Thus, results demonstrated a synergistic interaction for non-equitoxic combinations of herbicides Banvel® and DMA® (Fig. 2).

Results demonstrate that DIC-based formulation Banvel®-induced toxicity either at a concentration of 0.33 or 0.66 TUs was synergised by the presence of 2,4-D-based formulation DMA® within the mixtures (Fig. 2). Fig. 2 highlights that 0.33 TUs of DIC requires 0.19 (0.10–0.38) TUs of 2,4-D to induce the same toxic effect as when 0.66 TUs was mixed with 0.23 (0.11–0.48) TUs of 2,4-D.

In addition, 2,4-D-based formulation DMA®-induced toxicity was also synergised when mixed with DIC-based formulation Banvel® regardless of whether 0.33 or 0.66 TUs were assayed (Fig. 2). Results demonstrated that 0.33 and 0.66 TUs of 2,4-D required 0.13 (0.04–0.45) and 0.23 (0.13–0.40) TUs of DIC to reach 50% mortality.

Overall, the results also demonstrated that the toxicity exerted by 0.33 TUs of each formulation was slightly higher than the toxicity exerted by 0.66 TUs during testing within mixtures (Fig. 2).

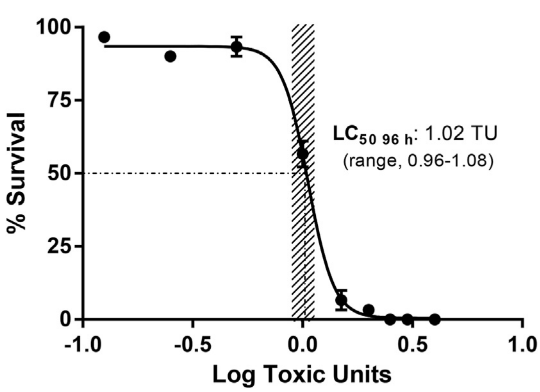

Fig. 1. Survival as a function of the sum of the toxic units (TUs) of equitoxic mixtures of dicamba-based formulation Banvel® and 2,4-dichlorophenoxyacetic acid (2,4-D)-based formulation DMA® in *C. decemmaculatus* after 96 h of exposure. The TU value of the mixture that induces 50% mortality ($LC_{50\ 96\ h}$) is indicated with the 95% confidence in parenthesis. The striped area depicts the "Additivity Zone".

Table 2
Lethal effects of non-equitoxic binary mixtures of dicamba (DIC)-based Banvel® and 2,4-dichlorophenoxyacetic acid (2,4-D)-based DMA® on exposed *Cnesterodon decemmaculatus*.

Concentration (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
DIC	2,4-D	30				
0.33	0.01	30	0	5	6	8
0.33	0.05	30	0	1	4	8
0.33	0.1	30	1	6	7	8
0.33	0.5	30	4	10	12	16
0.33	1	30	6	15	30	30
0.33	2	30	30	30	30	30
0.66	0.01	30	1	3	6	8
0.66	0.05	30	1	4	6	8
0.66	0.1	30	1	3	5	9
0.66	0.5	30	5	9	12	15
0.66	1	30	5	14	22	22
0.66	2	30	30	30	30	30
2,4-D	DIC					
0.33	0.01	30	2	6	8	9
0.33	0.05	30	7	8	10	10
0.33	0.1	30	1	6	8	10
0.33	0.5	30	2	8	9	12
0.33	1	30	7	8	15	24
0.33	2	30	16	27	30	30
0.66	0.01	30	1	2	3	5
0.66	0.05	30	3	4	5	6
0.66	0.1	30	4	6	8	10
0.66	0.5	30	4	6	11	14
0.66	1	30	8	11	23	28
0.66	2	30	27	30	30	30

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

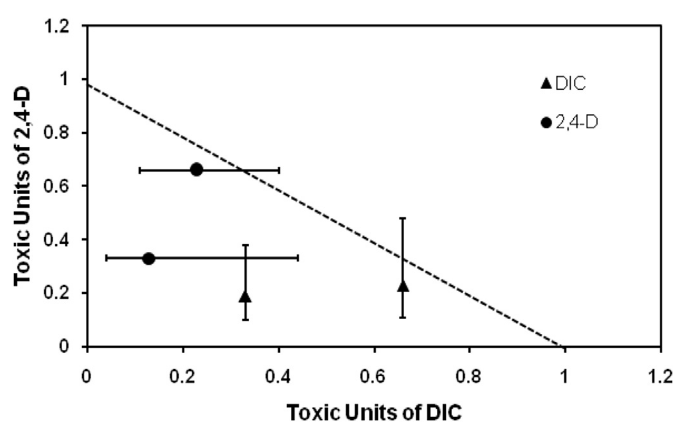

Fig. 2. Isobologram depicting the toxic units (TUs) of non-equitoxic mixtures of dicamba-based formulation Banvel® and 2,4-dichlorophenoxyacetic acid (2,4-D)-based formulation DMA® that cause 50% mortality (TU_{50}) in *C. decemmaculatus* after 96 h of exposure. The error bars from each experimental value represent 95% confidence intervals. The isobole linking the 1-TU values from both axes is the line of concentration addition.

4. Discussion

We analysed the possible toxic interactions—that is, synergy, antagonism or additivity—prevailing in equitoxic and non-equitoxic binary mixtures between two herbicide formulations, the DIC-based Banvel® and the 2,4-D-based DMA®. The target organism, *C. decemmaculatus* (Pisces, Poeciliidae), was exposed under laboratory conditions using a semi-static acute experimental model for toxicity testing (USEPA, 1975, 2002; IRAM, 2008).

Years ago, we used *C. decemmaculatus* as a test species to assess the lethal toxicity of different agrochemical commercial formulations of auxinic herbicides DIC and 2,4-D (Ruiz de Arcaute et al., 2014b, 2016). In these studies,Ruiz de Arcaute (2014b, 2016) analysed the lethality and evaluated the micronuclei frequency and the induction of DNA single-strand breaks by comet assay after an acute exposure to the herbicides.

In the current study, the toxicity of the herbicide mixture was estimated following the protocol described by Warne (2003) in which the TU model was used. According to Warne (2003), a classical toxicity test when analysing equitoxic mixtures should be performed using the method as if a single toxicant is being tested (for example, determination of 50% mortality). However, the toxicity and the concentration of the test solution of the equitoxic mixture—that is, factor S—are expressed in terms of the TU;S is equivalent to the sum of the individual TU values of each toxicant in the mixture (Warne, 2003). In these type of experiments, it has been recommended that at least five different experimental treatments be performed: two treatments possessing S values greater than 1, one treatment having an S value equal to 1, and two treatments with an S value less than 1. Varying upon the results, whether the selected effect S is equal to 1, the equitoxic mixture should be considered as 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).

In current agricultural practice, the simultaneous application of a mixture of agrochemical is common worldwide. Accordingly, mixtures of agrochemicals should be considered as an additional important group of environmental contaminants. In agreement with this concept, there has been an increase in recent years in the number of published reports that evaluate joint toxic and genotoxic effects exerted by mixtures of pesticides. In this regard, a slight synergism has been reported for the toxicity to the water flea *Ceriodaphnia dubia* and the fathead minnow fish *Pimephales promelas* following exposure to glyphosate in combination with imazapir, triclopyr and a modified vegetable oil surfactant (Tatum et al., 2012). Likewise, the effects of a binary mixture of glyphosate and dimethoate were evaluated in the earthworm *Eisenia andrei*, the isopod *Porcellionides pruinosus* and in seeds of the field mustard *Brassica rapa* (Santos et al., 2011). Santos et al. (2011) reported an equivalent synergistic effect at enzymatic levels when invertebrates where exposed to 5- and 10-times the field concentration. When seeds of *B. rapa* were used as the biotic matrix for studying germination capacity, a similar synergistic pattern was observed when realistic binary mix concentration was assayed, but an antagonistic effect at 5- and 10-times the field concentration (Santos et al., 2011). Similarly, a synergistic impact has been reported in the honey bee *Apis mellifera* following exposure to imidacloprid alone and in different binary mixtures with seven other agrochemicals (Zhu et al., 2017), and joint toxicity effects have been reported in the zebrafish *Danio rerio* following exposure to chlorpyrifos and beta-cypermethrin (Zhang et al., 2017). Furthermore, we have recently demonstrated a synergistic pattern for a mixture of glyphosate- and DIC-formulations in introducing primary DNA breaks on circulating blood cells of South American common toad *Rhinella arenarum* tadpoles (Soloneski et al., 2016).

So far, this report constitutes the first study analysing the interactions exerted by a binary mixture of two auxinic herbicides—namely DIC and 2,4-D, which are two of the most used auxinic herbicides worldwide—on the Neotropical fish *C. decemmaculatus*. We observed a mean TU_{50} value of 1.02 (0.96–1.08) for the binary equitoxic mixture of DIC-based formulation Banvel® and 2,4-D-based formulation DMA®. Taking into account that this TU_{50} value is close to 1, it could be suggested that the formulation we analysed exerts an additive interaction for the binary mixtures of DIC and 2,4-D.

Although the *in vivo* treatments in this study covered a wide range of concentrations, the lowest concentration of DIC and 2,4-D assayed was 0.01 TU in non-equitoxic combinations. These values correspond to 17.07 mg/L and 10.08 mg/L for DIC and 2,4-D, respectively. However, these values constitute a relatively high end of the threshold values reported in other countries—that is, 2.1 μg/L of DIC in surface runoff water and 24 μg/L of 2,4-D in surface waters in the United States (Phillips and Bode, 2004; Tagert et al., 2014), and application rates of 1500 cm^3/hL for DIC and 3.5 L/ha for 2,4-D in Argentina (CASAFE, 2015). Thus, the concentrations of both herbicides used in this investigation would be expected to be almost improbable in the environment, perhaps only been observed when specific events occurred (for example, direct application or by accidental discharge). Nevertheless, we cannot rule out that piscine populations, and also occupationally exposed human workers, could be exposed accidentally to these agrochemicals at this range of concentrations.

According to the experimental design used in the present study, the determination of the $TU_{50\ 96\,h}$ is achieved when only one equitoxic mixture is able to induce mortality of 50% in the exposed organism. However, when non-equitoxic mixtures are assayed, and in agreement with the suggestions of Warne (2003), the type of toxicological interaction prevailing in the binary mixture results from at least four independent experimental observations. Thus, it seems evident that conclusions drawn from the latter should be considered more robust and even more representative of the real situation taking place when two agrochemicals are applied together to an exposed population or organism.

When non-equitoxic combinations of the herbicides were analysed to assess the potential toxicity of the binary herbicide mixtures, our observations demonstrated the occurrence of synergistic interactions in all four non-equitoxic combinations of DIC-based formulation Banvel® and 2,4-D-based formulation DMA®. Accordingly, these results suggest that the DIC-based and 2,4-D-based formulation mixtures are more toxic when applied in non-equitoxic combinations compared to the sum of their individual toxicities.

As previously stated, the effect of mixtures of pesticides has been analysed by several studies. However, to the best of our knowledge, only two of those studies have been based on the same experimental design used by our laboratory and described by Warne (2003). Brodeur et al. (2014, 2016) assayed binary mixtures of glyphosate- and cypermethrin-based formulations in *R. arenarum* and in *C. decemmaculatus*. The results clearly demonstrated opposite effects depending upon the species employed. For *R. arenarum*, the mixture of glyphosate and cypermethrin had synergic effects; in contrast, for *C. decemmaculatus*, an antagonistic effect was achieved by inhibition of the cypermethrin toxicity by the former.

In the present study, a DIC-based herbicide containing 57.7% of the active ingredient within the formulation Banvel® and a 2,4-D-based herbicide containing 58.4% of the active ingredient within the formulation DMA® were assayed. Years ago, the USEPA (1982) claimed that the toxicity of an active ingredient can differ significantly from that of the formulation carrying that active ingredient. It is known that the additive compounds present in pesticide commercial formulations have the capability to exert toxicity and cellular damage by themselves rather than that of the active principle ingredient (Mann and Bidwell, 1999; Grisolia et al., 2004; Soloneski et al., 2007; Belden et al., 2010; Soloneski and Larramendy, 2010; Brühl et al., 2013; Nikoloff et al., 2014a; Pérez-

Iglesias et al., 2014; Ruiz de Arcaute et al., 2014a). Unfortunately, the identities of the additive compounds present in the commercial formulations Banvel® and DMA® were not made available to us by the manufacturers. It should be mentioned that according to our Argentinean administration, the excipients present in any agrochemical are not required to be listed on the agrochemical data sheet and can be kept as a "trade secret." Accordingly, caution should be taken with the concept of the binary mixtures of the two commercial formulations we assayed. In the strictest sense, the mixtures we tested throughout the current study represent a much more complex scenario because they involved herbicide formulations, which, as we know, are mixtures of several components by themselves. Furthermore, and extending this concept, we cannot assume that the proportion of each constituent in the mixtures we used follow the same or a similar profile in an environmental aquatic system as the components of the formulations can suffer different transformation or degradation patterns in the environment. Thus, different patterns of occurrence in the water matrix could be produced diverging from that we tested. Further studies are required to reveal whether the pattern of toxicity exerted by the mixtures of Banvel® and DMA® on the *C. decemmaculatus* we observed is attributable to the active ingredients by themselves or results from the presence of xenobiotic(s) within the technical formulation assayed in our study.

Our study represents the first evidence of the lethality exerted by a mixture of two auxinic herbicides (namely, DIC and 2,4-D) reported to date, not only for the fish *C. decemmaculatus*, but also for other biotic matrices. Results of the equitoxic combinations of both herbicides could indicate that under this situation, the mixture could cause an additive interaction. However, results demonstrated the occurrence of a synergistic interaction when non-equitoxic combinations of Banvel®-or DMA®-formulated herbicides were assayed. In this context and regardless of their concentration, either DIC- or 2,4-D-induced toxicity were synergised by the presence of the counterpart within mixtures.

Taking into account the previous studies published by Brodeur et al. (2014, 2016) in which opposite results, namely antagonism or synergism, varied based on the target, further studies are required to corroborate whether the synergic effect we observed in *C. decemmaculatus* is a general effect exerted by the mixture regardless of the target organism.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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