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RESEARCH ARTICLE

Genotoxicity of mixtures of glyphosate with 2,4-dichlorophenoxyacetic acid chemical forms towards *Cnesterodon decemmaculatus* (Pisces, Poeciliidae)

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Abstract

Acute genotoxicity of commercial glyphosate (GLY) (Credit®)-, 2,4-D-acid (2,4-D) (Dedalo Elite)-, 2,4-D-amine (2,4-D DMA) (Weedar Full®)- and 2,4-D-ester (2,4-D BE) (Herbifen Super®)-based herbicide formulations alone and their combinations were analysed in *Cnesterodon decemmaculatus*. Mortality was evaluated as a lethal end-point and the single cell gel electrophoresis (SCGE) bioassay was used as a sublethal end-point. $LC50_{96h}$ values for Dedalo Elite was 0.46 mg/L and Herbifen Super® was 2.67 mg/L based on 2,4-D and 2,4-D BE, respectively. Results reveal a higher toxicity exerted on *C. decemmaculatus* after exposure to 2,4-D- rather than 2,4-D BE-based herbicide formulations. Overall, results demonstrated an enhancement in the genetic damage index committed to an enhancement of damaged erythrocytes of *C. decemmaculatus* when exposed to Credit®, Dedalo Elite, Weedar Full® and Herbifen Super® at 5% and 10% of $LC50_{96h}$ values alone as well as in their combinations. Overall, the combination of GLY plus 2,4-D or GLY plus 2,4-D DMA showed a synergistic pattern whereas the combination of GLY plus 2,4-D BE was antagonic. Furthermore, this research is pioneer in the assessment of lethality and genotoxicity induced by 2,4-D-, 2,4-D DMA- and 2,4-D BE-based formulations when combined with GLY-based formulated herbicides in fish after they are acutely exposed.

Keywords 2,4-D · Glyphosate · Herbicide mixtures · Mortality · SCGE (comet) assay

Introduction

Worldwide, most agrochemicals are manmade compounds employed to reduce the unfavourable consequences of croplands, pastures and domestic pests (Larramendy and Soloneski 2014, 2015). The applications of pesticides have exceptionally increased during the two last decades due to the intensified production model of agriculture, especially in South American countries (Morales and Schaper 2004). This model has stimulated the transition of small cultures to large monocultures as well as to the enhancement of the appearance of resistant weeds to these agrochemicals and to the adopted farming techniques (Dirzo and Raven 2003; Schiesari et al. 2013).

Glyphosate (GLY) (*N*-phosphonomethyl glycine), the most commonly employed active ingredient in commercial formulations worldwide, is a herbicide belonging to the group of organophosphates. GLY is a weak organic acid, formed by one molecule of glycine (carbonyl + amine) and another of phosphonomethyl, which results in different ionic charges as a function of both the pH of the medium and the solubility in water (IPCS 1994). Most organophosphorus compounds are less stable than organochlorine ones and are rapidly broken down by chemical or biochemical reactions. It has been reported that GLY possess very low toxicity by the World Health Organization (WHO-FAO 1997). Additionally, the International Agency for Research on Cancer has considered GLY as a compound "probably carcinogenic in humans" (category 2A) on the bases of

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in vivo and in vitro studies (Guyton et al. 2015; IARC 2018), whereas the United States Environmental Protection Agency (US EPA) claimed that the herbicide is unlikely to be carcinogenic to humans (USEPA 2016).

2,4-D-Dichlorophenoxyacetic acid (2,4-D) is a weak acid herbicide that belongs to the auxinic family with systemic, selective and broad mode of action and presents a broad spectrum low environmental persistence (Liu et al. 2013; Mithila et al. 2011). It is usually applied in pre-planting (or pre-sowing) for desiccation or in post-emergence in several cereal crops (CASAFE 2017). It is part of the chlorophenoxyacetic family. The US EPA has classified it as slightly to moderately toxic chemical (category II–III) (USEPA 1974) or as moderately hazardous agent by WHO (class II) (WHO 2009). After a thorough revision of the available in vivo and in vitro studies, IARC has considered 2,4-D as a compound that is "possibly carcinogenic in humans" (category 2B) (IARC 2018). Formulations of 2,4-D consist of esters, acids and amines which differ in their chemical, environmental fate as well as toxicity properties. These chemical variants can be found in liquid form, soluble powder, granules or pellets (Mithila et al. 2011). In general, it is known that ester formulations are more volatile than amine and salt variants and present higher plant toxicity (WHO-FAO 1997). The amine forms are water soluble and have, then, higher loading properties than salt formulations. They can interact intensely with anions and, to a lesser extent, cations (WHO-FAO 1997).

GLY and 2,4-D are post-emergence herbicides applied in large crop production playing an significant part in the optimisation of global farming production (Mithila et al. 2011; Wright et al. 2010). The Chamber of Agricultural Health and Fertilisers from Argentina suggests to apply GLY and 2,4-D together in several cultures of economic importance (CASAFE 2017) regardless of the potential toxicity that when combined could exert on other than the target species (Ge et al. 2014). Furthermore, the complexity of the chemical interactions between agrochemicals and the extensive use of their combinations result in changes in the physicochemical properties and behaviour and, thus, cause different and negative consequences on environmental and human health (Ge et al. 2014; Silva et al. 2015; Varona-Uribe et al. 2016). It is known that the combination of agrochemicals leads to additive, synergistic or even antagonistic effects (Blouin et al. 2010, 2004; Brodeur et al. 2014, 2016; Deneer 2000; Hernández et al. 2017; Lydy et al. 2004). In general terms, when the response observed is larger than the effects of each constituent applied individually, this is assigned as a synergistic pattern. On the contrary, when the outcome of the interaction is the diminution of the effect caused by any of its constituents, it is referred to as an antagonistic effect. Lastly, an additive consequence is claimed when the effect is similar to the global effects caused by all individual constituents present in the mixture (Blouin et al. 2010; Lydy et al. 2004).

The use of fish as bioindicators of the effects of pollutants in the aquatic environment has shown satisfactory results in bioassays for potentially evaluating the mutagenic, teratogenic and carcinogenic properties of chemicals previously determined to exist in humans since they usually respond to toxic compounds in pathways similar to those found in large vertebrates (Di Giulio and Hinton 2008). In order to evaluate the toxicity of pesticides in fish and other organisms, the single cell gel electrophoresis (comet) (SCGE) assay has been applied on isolated cells due to the sensitivity of the bioassay for detecting genotoxic effects of several xenobiotics, including herbicides (Azqueta and Collins 2013; Collins et al. 2014; Ruiz de Arcaute et al. 2014b, 2016, 2018a).

Several studies have shown that *C. decemmaculatus*, the ten spotted live-bearer, is a valid bioindicator of environmental quality in assessing the lethal and sublethal effects of several pesticides, e.g., pirimicarb (Vera-Candioti et al. 2010, 2013b, 2015), endosulfan (Mugni et al. 2012, 2015), cypermethrin (Carriquiriborde et al. 2007; Mugni et al. 2012), glyphosate (Vera-Candioti et al. 2013a, 2013b), chlorpyrifos (Mugni et al. 2012; Vera-Candioti et al. 2013b, 2014), 2,4-D (Ruiz de Arcaute et al. 2016, 2018a) and dicamba (Ruiz de Arcaute et al. 2014a, 2018a), among others.

We aimed to evaluate the lethality and genotoxicity exerted by the GLY-based herbicide Credit® and the 2,4-D, 2,4-D DMA and 2,4-D BE representative chemical forms included in the commercial formulations namely Dedalo Elite, Weedar Full® and Herbifen Super®, respectively. These commercial herbicides were used alone, as well as in mixtures on *C. decemmaculatus* acutely exposed under semi-static laboratory protocol. Mortality and the SCGE assay were used as biomarkers for lethality and genotoxicity, respectively. Synergistic, antagonistic and additive potential effects resulting from the mixture of these herbicides on the species were also considered.

Material and methods

Chemicals

Herbicides included the 48% isopropylamine salt *N*-phosphonomethyl glycine (GLY; CAS 1071-83-6)-based trade formulation Credit® (Dow AgroSciences Argentina S.A. Buenos Aires, Argentina), the 30% 2,4-dichlorophenoxyacetic acid (2,4-D; CAS 94-75-7)-based commercial formulation Dedalo Elite (Red Surcos, S.A., Buenos Aires, Argentina), the 83.5% 2,4-dimethylamine salt (2,4-D DMA; CAS 2008-39-1)-based commercial formulation Weedar® Full (Nufarm de Argentina, Buenos Aires, Argentina) and 97% butyl ester 2,4-D (2,4-D BE; CAS 94-80-4 or 1713-15-1, or their mixtures)-based commercial formulation Herbifen Super® (Atanor S.C.A., Buenos Aires,

Argentina). Positive control $K_2Cr_2O_7$ (Cr(VI); CAS 7778-50-9) was purchased from Merck KGaA (Darmstadt, Germany). All other chemicals were obtained from Sigma-Aldrich Co. (St. Louis, MO).

Quality control

Analyte determinations of GLY, 2,4-D, 2,4-D DMA and 2,4-D BE herbicides were estimated by HPLC using an ultraviolet detector and fluorenylmethyloxycarbonyl derivatization contained in the commercial formulations Credit®, Dedalo Elite, Weedar® Full and Herbifen Super®, respectively. Analyses were performed by QV Chem Laboratory (La Plata, Buenos Aires, Argentina) according to OSHA Analytical Method PV2067 and US Geological Survey Report 01-4134 (Furlong et al. 2011), respectively. Test solutions correspond to values obtained after preparation (0 h) and 24 h thereafter up to renewal. The detection limit was 0.2 mg/L for GLY and 0.5 mg/L for all 2,4-D variants. The herbicide concentrations employed during the study represent the nominal concentration of the active principle contained in the formulations Credit®, Dedalo Elite, Weedar® Full and Herbifen Super®.

Species

Adult of *C. decemmaculatus* were gathered in a pond, located near La Plata City (Buenos Aires Province, Argentina), away from agricultural areas. Fish were handled and cared according to the guideline of the Argentinean National Service for Sanitary and Quality of Agriculture and Food guidelines 617/2002 for biological testing (SENASA 2013). After collection, specimens were conveyed to the laboratory and acclimatized for 20 days at 16/8 h light/dark cycle, with artificial aeration as reported in detail previously (Ruiz de Arcaute et al. 2018a). Specimens were fed daily with commercial fish food flakes (TetraMin®, TetraWerke, Melle, Germany).

Acute toxicity of herbicide formulations

Lethality analyses were performed according to the standardized recommendations proposed by the US EPA (IRAM 2008; USEPA 1975, 2002), with recommendations informed previously (Ruiz de Arcaute et al. 2016; Vera-Candioti et al. 2013b). Preliminary tests were performed in order to establish the exposure concentrations, as suggested by the USEPA (2002). Each experiment was performed in 1 L glass aquaria containing dechlorinated tap water (see the "Species" section), with 10 individuals selected at random following previous recommendations (Ruiz de Arcaute et al. 2016; Vera-Candioti et al. 2013b). Specimens were treated with 9 concentrations of 2,4-D (0.1, 0.2, 0.25, 0.35, 0.45, 0.55, 0.60, 0.65 and 1 mg/L) or 12 concentrations of 2,4-D BE (1, 2, 2.5, 3, 3.5, 4, 4.5, 5, 6, 7, 12 and 15 mg/L). Negative (dechlorinated tap water; see the "Species" section) and positive (21.4 mg/L $Cr_{(VI)}$-treated fish) controls (Vera-Candioti et al. 2013b) were carried out in parallel with treated specimens. Herbicide solutions were prepared prior to use and fully substituted daily for all experimental points. All experimental points were performed in triplicate and run in parallel. No food was provided to the exposed fish. Dead individuals were determined by visual observation daily before test solution renewal.

Acute toxicity of the GLY-based formulation Credit® and the 2,4-D DMA-based formulation Weedar® Full on *C. decemmaculatus* was determined in our laboratory and complete data reported in detail previously (Ruiz de Arcaute et al. 2018b; Vera-Candioti et al. 2013a).

Single cell gel electrophoresis assay

For each assayed point, 5 fish were placed in a 1-L glass container and treated with two concentrations of the tested herbicides. Fish were treated with 4.58 and 9.17 mg/L GLY, 0.023 and 0.046 mg/L 2,4-D, 33.90 and 67.80 mg/L 2,4-D DMA, 0.023 and 0.13 and 0.26 mg/L 2,4-D BE (see the "Species" section), values equivalent to 5% and 10% of the $LC50_{96h}$ values determined for each formulated product. In addition, fish were exposed to a combination of the single concentrations assayed (i.e. 5% + 5% or 10% + 10% $LC50_{96h}$ values). Negative (see the "Acute toxicity of herbicide formulations" section) and positive controls (10 mg/L CP) were run in parallel with herbicide-exposed individuals. Experimental points were performed in triplicate and run in parallel. No food was provided to the exposed fish. After 96 h of exposure, a blood sample from each specimen was obtained as described in detail previously (Ruiz de Arcaute et al. 2016; Vera-Candioti et al. 2013a) and DNA breaks and alkali-labile sites were analysed following the recommendations proposed by Singh (1996), with minor modifications (Ruiz de Arcaute et al. 2016, 2018a; Vera-Candioti et al. 2013b). Peripheral blood samples were suspended with 1 ml phosphate-buffered saline in Eppendorf microtubes. Then, aliquots of blood cells were combined with 0.5% low-melting-point agarose and sandwiched with 0.5% normal-melting-point agarose on a cleaned slide. After the top layer had solidified (4 °C, 10 min), slides were transferred in an ice-cold freshly prepared lysing solution (1% sodium sarcocinate, 2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, pH 10.0, 1% Triton X-100, 10% DMSO) and then the cells lysed at darkness at 4 °C for at least 1 h. Then, slides were placed into chilled fresh electrophoresis buffer (1 mM Na_2EDTA, 300 mM NaOH) for 25 min at 4 °C to allow the cellular DNA to unwind. Electrophoresis was conducted in the same buffer and temperature for 30 min at 25 V and 250 mA (0.8 V/cm).

Finally, the slides were neutralized three times (5 min each) with a buffer solution comprising 0.4 M Tris-HCl, pH 7.5, and then stained with 4′,6-diamino-2-phenylindole (DAPI; Vectashield mounting medium H1200; Vector Laboratories, Burlingame, CA, USA). Upon staining the slides were immediately examined under an Olympus BX50 fluorescence photomicroscope equipped with an appropriate filter combination. The extent of DNA damage was assessed visually in 100 randomly selected and non-overlapping nucleoids. DNA damage was visually analysed and scores in five classes according to the tail intensity (0–I, undamaged; II, minimum damage; III, medium damage; IV, maximum damage), as suggested previously (Cavaş and Könen 2007). Data are expressed as the percentage of damaged nucleoids (sum of classes II, III and IV). The genetic damage index (GDI) was estimated according Pitarque et al. (1999) using the formula GDI = [1(I) + 2(II) + 3(III) + 4(IV)/N(0–IV)], where 0–IV represents the nucleoid type, and N0–NIV represents the total number of nucleoids scored.

Statistical analysis

Lethality was pondered according to the US EPA Probit statistical software, version 1.5 (USEPA 2002). The relation between variations in LC50 data and exposure time was evaluated with a correlation analysis. Differences in DNA damage among herbicide-exposed and control groups were estimated by one-way ANOVA with Dunnett's test. All tests were performed by the Statistica 7.0 software (StatSoft, OK). For all tests, the significance criterion was 0.05.

Results

Quality control

Results of chemical analyses of the concentration of the pure analyte of GLY, 2,4-D, 2,4-D DMA and 2,4-D BE presented no significant changes ($P > 0.05$) between the testing solutions with 24 h interval renewals (concentration range, 97 ± 5% recovery). Concentrations employed in the present study correspond to the nominal concentrations of pure compound conforming the GLY-based formulation Credit®, 2,4-D-based formulation Dedalo Elite, 2,4-D DMA-based formulation Weedar® Full and 2,4-D BE-based formulation Herbifen Super®, respectively.

Mortality

GLY- and 2,4-D DMA-induced lethality for the species were previously reported by our laboratory previously (Ruiz de Arcaute et al. 2018b; Vera-Candioti et al. 2013a). Data revealed values of 91.73 mg GLY/L (range, 86.80–98.00 mg/L) and 678.04 mg 2,4-D DMA/L (range, 639.35–718.04 mg/L) as $LC50_{96h}$ for Credit® (Vera-Candioti et al. 2013a) and Weedar® Full (Ruiz de Arcaute et al. 2018b), respectively.

Mortality experiments in 2,4-D-based formulation-exposed fish revealed the mean LC50 values of 0.85 mg/L (range, 0.78–0.95), 0.65 mg/L (range, 0.61–0.69), 0.51 mg/L (0.47–0.56) and 0.46 mg/L (range, 0.42–0.51) after 24, 48, 72 and 96 h of exposure, respectively. Correlation analysis demonstrated that 2,4-D-based formulation LC50 values diminished as a significant function of the exposure time ($r = -0.96$; $P < 0.05$).

When fish were exposed to 2,4-D BE-based formulation, results revealed mean LC50 values of 6.12 mg/L (range, 5.06–6.71), 3.67 mg/L (range, 3.44–3.86), 3.04 mg/L (range, 2.82–3.22) and 2.67 mg/L (range, 2.48–2.82) after 24, 48, 72 and 96 h of exposure, respectively. Correlation analysis demonstrated that 2,4-D BE-based formulation LC50 values decreased as a non-significant function of the exposure time ($r = -0.91$; $P > 0.05$).

DNA damage

DNA damage induction in *C. decemmaculatus* specimens, after 96 h of exposure to GLY-, 2,4-D-, 2,4-D DMA- and 2,4-D BE-based formulations alone as well as in their mixtures, is summarized in Table 1. Besides that, the mean frequencies of nucleoids from each damage categories as well as variations in GD are depicted in Figs. 1 and 2, respectively.

CP induced a nearly 6-fold increase in the frequency of damaged nucleoids (Table 1, Fig. 1), as well as in the GDI in relation to negative control values ($P < 0.001$) (Fig. 2). This variation was consequence to the increase in the frequencies of type II, III and IV nucleoids and a concomitant decrease of type 0–I nucleoids ($P < 0.001$) (Fig. 1).

DNA damage in GLY-, 2,4-D-, 2,4-D DMA- and 2,4-D BE-based formulation-exposed fish

In fish exposed to all herbicides, the frequency of damaged cells and GDI values increased when compared to negative control values, regardless of the assayed concentration ($P < 0.001$) (Table 1, Figs. 1 and 2). This effect was due to an increase in the frequencies of type II, III and IV nucleoids ($P < 0.001$), as well as a decrease of type 0–I nucleoids ($P < 0.001$) (Fig. 1). For all tested herbicides, GDI induced by 10% $LC50_{96h}$ 2,4-D-, 2,4-D DMA- and 2,4-D BE-based formulations was higher than that induced by 5% $LC50_{96h}$ of the herbicide ($P < 0.001$). Contrarily, no statistically significant differences were observed between GDI values induced after exposure to 5% and 10% $LC50_{96h}$ GLY-based formulation ($P > 0.05$) (Table 1, Fig. 2).

Table 1 Analysis of DNA damage measured by SCGE assay in peripheral blood erythrocytes of *Cnesterodon decemmaculatus* cells exposed to commercial glyphosate (GLY) (Credit®)-, 2,4-D-acid (2,4-D) (Dedalo Elite)-, 2,4-D-amine (2,4-D DMA) (Weedar Full®)- and 2,4-D-ester (2,4-D BE) (Herbifen Super®)-based herbicides formulations alone and their combinations

Herbicides[a]	Number of animals analysed	Number of cells analysed	% of damaged cells (II + III + IV)	Genetic damage index
Negative control	15	1601	13.35 ± 1.38	1.17 ± 0.01
Positive control[b]	15	1647	78.57 ± 2.99***	2.68 ± 0.06***
GLY				
5%	15	1571	58.18 ± 1.63***	2.10 ± 0.04***
10%	15	1558	69.19 ± 1.51***	2.34 ± 0.04***
2,4-D and mixtures				
5%	15	1502	58.12 ± 1.41***	2.11 ± 0.05***
10%	15	1570	65.29 ± 2.25***	2.45 ± 0.02***
5% GLY + 5% 2,4-D	15	1631	67.57 ± 2.24***	2.42 ± 0.05*** ¤¤¤ ###
10% GLY + 10% 2,4-D	15	1600	69.31 ± 3.50***	2.59 ± 0.10*** ¤ ###
2,4-D DMA and mixtures				
5%	15	1576	68.02 ± 2.05***	2.21 ± 0.02***
10%	15	1590	62.48 ± 2.77***	2.39 ± 0.10***
5% GLY + 5% 2,4-D DMA	13	1365	68.64 ± 1.73***	2.36 ± 0.05*** ¤¤ #
10% GLY + 10% 2,4-D DMA	15	1599	76.11 ± 2.44***	2.72 ± 0.03*** ¤¤¤ ###
2,4-D BE and mixtures				
5%	15	1663	52.38 ± 2.93***	1.91 ± 0.11***
10%	15	1597	66.06 ± 1.43***	2.28 ± 0.06***
5% GLY + 5% 2,4-D BE	15	1681	68.71 ± 2.28***	2.25 ± 0.09*** #
10% GLY + 10% 2,4-D BE	15	1572	72.52 ± 1.18***	2.44 ± 0.03*** ##

[a] Concentrations of herbicide commercial formulations are expressed as percentages of the $LC50_{96h}$ of each active ingredient

[b] Positive control (cyclophosphamide 10 mg/L)

***$P < 0.001$, significant differences with respect to negative control values

¤¤ $P < 0.01$, ¤¤¤ $P < 0.001$, significant differences with respect to GLY-based formulation-induced GDI

# $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$, significant differences with respect to 2,4-D-based formulation-induced GDI

DNA damage in GLY- plus 2,4-D-based formulation-exposed fish

Regardless of the tested concentrations, formulation mixtures induced a significant increase of the GDI compared to the negative control values ($P < 0.001$) as well as those fish treated only with 2,4-D-based ($P < 0.001$) or with GLY-based herbicides ($0.05 > P < 0.001$) (Table 1, Fig. 2). In addition, no significant differences in the GDI were observed between fish exposed to both 5% $LC50_{96h}$ 2,4-D-based plus GLY-based formulations or those exposed to 10% $LC50_{96h}$ 2,4-D-based plus GLY-based formulations ($P > 0.05$) (Table 1, Fig. 2). Overall, exposure to 5% $LC50_{96h}$ 2,4-D-based plus GLY-based formulation concentrations induced an equivalent 1.15-fold increase of the GDI over those induced by a 5% $LC50_{96h}$ 2,4-D-based and 5% $LC50_{96h}$ GLY-based formulation. Nevertheless, fish, when exposed to 10% $LC50_{96h}$ 2,4-D-based plus GLY-based formulations, showed a 1.05- and 1.10-fold increase of the GDI over those induced by a 10% $LC50_{96h}$ 2,4-D-based formulation and 10% $LC50_{96h}$ GLY-based formulation, respectively. Finally, the mixture of 10% $LC50_{96h}$ 2,4-D-based plus GLY-based formulations induced a 1.07-fold enhancement of the GDI over that induced by the mixture of 10% $LC50_{96h}$ of the herbicides (Table 1, Fig. 2).

DNA damage in GLY- plus 2,4-D DMA-based formulation-exposed fish

Regardless of the concentrations tested, formulation mixtures induced a significant increase of the GDI compared to negative control values ($P < 0.001$), as well to those fish exposed only to 2,4-D DMA-based ($P < 0.01$) or to GLY-based herbicides ($P < 0.001$) (Table 1, Fig. 2). Furthermore, no significant differences in the GDI were observed between fish treated with 5% $LC50_{96h}$ 2,4-D DMA-based plus GLY-based formulations and those exposed to 10% $LC50_{96h}$ 2,4-D DMA-based plus GLY-based formulations ($P > 0.05$) (Table 1, Fig. 2). Overall, exposure to 5% $LC50_{96h}$ 2,4-D DMA-based formulation plus GLY-based formulation induced a 1.06- and 1.12-fold increase in GDI over those induced by 5% $LC50_{96h}$ 2,4-D DMA- and 5% $LC50_{96h}$

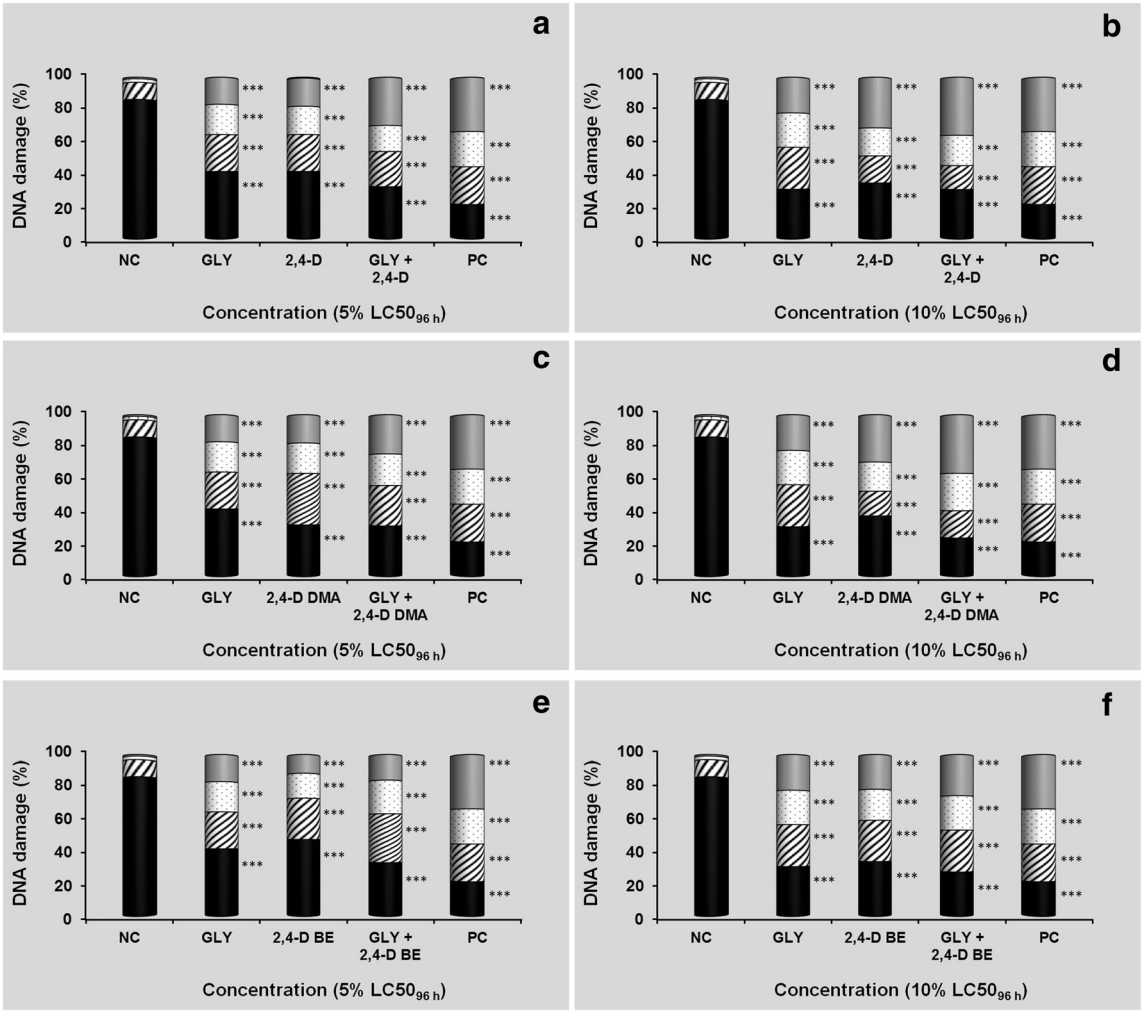

Fig. 1 DNA damage induced by glyphosate (GLY)-based formulation Credit®, the 2,4-D-based formulation Dedalo Elite (**a**–**b**), the 2,4-D DMA-based formulation Weedar® Full (**c**–**d**), the 2,4-D BE-based formulation Herbifen Super® (**e**–**f**) and their mixtures on *Cnesterodon decemmaculatus* exposed for 96 h. The frequencies of undamaged (type 0–I nucleoids; black bar), type II (stripped bar), type III (dotted bar) and type IV (grey bar) were determined in three independent experiments. White bars represent negative (NC, untreated fish) and positive controls (CP, 10 mg cyclophosphamide/L-exposed fish). ***$P < 0.001$: significant differences with respect to negative control values

GLY-based formulation, respectively. However, fish exposed to 10% $LC50_{96h}$ 2,4-D DMA- and GLY-based formulations presented a 1.13- and 1.16-fold increase in GDI in relation to those induced by a 10% $LC50_{96h}$ 2,4-D DMA-based formulation and 10% $LC50_{96h}$ GLY-based formulation, respectively. Finally, mixtures of 5% $LC50_{96h}$ 2,4-D DMA- plus GLY-based herbicides induced a 1.15-fold increase in GDI over that induced by the 10% $LC50_{96h}$ combination (Table 1, Fig. 2).

DNA damage in GLY- plus 2,4-D BE-based formulation-exposed fish

Regardless of the concentrations tested, formulation mixtures induced a significant increase of the GDI compared to negative control values ($P < 0.001$), as well to those fish only exposed to 2,4-D BE-based herbicides ($0.05 > P < 0.01$) (Table 1, Fig. 2). On the other hand, no differences were observed between the GDI value exerted in fish exposed to both herbicide mixtures and that induced in fish when exposed only to a GLY-based product ($P > 0.05$). Furthermore, significant differences in the GDI were observed between fish exposed to 5% $LC50_{96h}$ 2,4-D BE-based plus GLY-based formulations or those exposed to 10% $LC50_{96h}$ 2,4-D BE-based plus GLY-based formulations ($P > 0.05$) the GDI values of the latter being higher than of the former ($P < 0.001$) (Table 1, Fig. 2). Exposure to 5% $LC50_{96h}$ 2,4-D BE-based plus GLY-based formulations induced a 1.17- and 1.07-fold increase in the GDI relative to those induced by the 5% $LC50_{96h}$ 2,4-D BE-based and 5% $LC50_{96h}$ GLY-based products. Fish when exposed to 10% $LC50_{96h}$ 2,4-D BE plus GLY-based formulations showed an equivalent 1.07- and 1.04-fold increase in GDI values compared to those induced by 10% $LC50_{96h}$ 2,4-D BE-based and 10% $LC50_{96h}$ GLY-based formulations, respectively. Overall, the mixture of 5% $LC50_{96h}$ 2,4-D BE

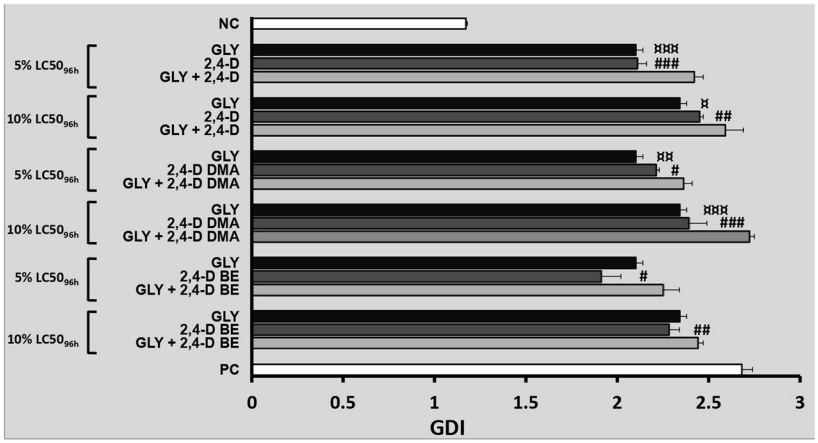

Fig. 2 Analysis of genomic damage index (GDI) measured by comet assay in peripheral blood erythrocytes of *Cnesterodon decemmaculatus* cells exposed to glyphosate (GLY)-based formulation Credit®, the 2,4-D-based formulation Dedalo Elite, the 2,4-D DMA-based formulation Weedar® Full, the 2,4-D BE-based formulation Herbifen Super® and their mixtures. White bars represent negative (NC, untreated fish) and positive controls (CP, 10 mg cyclophosphamide/L-exposed fish). Significant differences with respect to GLY-based herbicide-exposed fish: $^{¤}P < 0.05$, $^{¤¤}P < 0.01$, $^{¤¤¤}P < 0.001$. Significant differences with respect to 2,4-D variant-based herbicides exposed fish: $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$

plus GLY-based formulations induced a 1.08-fold increase in GDI over that induced by the 10% $LC50_{96h}$ mixture of both herbicides (Table 1, Fig. 2).

Discussion

In this study, we determined $LC50_{96h}$ values of 0.46 mg/L (range, 0.42–0.51) and 2.67 mg/L (range, 2.48–2.82) for *C. decemmaculatus* when exposed to 2,4-D-based and 2,4-D BE-based formulations Dedalo Elite and Herbifen Super®, respectively. Thus, considering the toxicological categories for aquatic organisms performed by the United Nations directives (2011), the former commercial herbicide should be classified as a very toxic compound (category 1), whereas the latter as toxic chemical (category 2). Besides, following to the hazard risk categories proposed by the European Union, 2,4-D-based formulation Dedalo Elite can be ranked as a very toxic herbicide (category I), whereas 2,4-D BE-based formulation Herbifen Super® represents a toxic agent for aquatic life (category II) (Mazzatorta et al. 2002). Whether the directives reported by the US EPA for terrestrial and aquatic organisms are considered, 2,4-D-based and 2,4-D BE-based formulations Dedalo Elite and Herbifen Super® should be ascribed as highly and moderately toxic formulations, respectively (USEPA 2001). Nevertheless, the toxicological profile recently obtained for the chemical form 2,4-D DMA-based herbicide Weedar® Full for the species revealed a concentration equivalent to 678.04 mg/L (range, 639.35–718.04) as the $LC50_{96h}$ value (Ruiz de Arcaute et al. 2018b). Thus, this chemical variant has been classified as a harmful compound (category 3) (UN 2011) and as a compound that may cause long-term adverse effects (category IV) (Mazzatorta et al. 2002) or as a product with practically non-toxic effects for aquatic organisms (USEPA 2001). Accordingly, it seems evident that *C. decemmaculatus* results, respectively, nearly 1474 and 254 times more sensitive to 2,4-D-based formulation Dedalo Elite and to 2,4-D BE-based formulation Herbifen Super® than to 2,4-D DMA-based product Weedar® Full. In other words, the results showed that the latter is the least toxic 2,4-D variant among the three tested commercial herbicide formulations on the species. Overall, a progression of toxic effects exerted by 2,4-D and its salt and ester forms is clearly evidenced by the lethal pattern we observed in the species. Results reveal a decreasing toxicity gradient of mortality exerted on *C. decemmaculatus* when exposed to 2,4-D- > 2,4-D BE- > 2-4-D DMA-based herbicide formulations. A comparative analysis of the available data reported for the lethality induced by these auxinic chemical forms would indicate that the acid form of 2,4-D and its salts are less toxic to fish than the 2,4-D BE chemical form (USEPA 2019). A review of the literature indicates $LC50_{96h}$ values ranging from 3.8 up to 1966 mg/L for *Labeo boga* (Cypriniformes, Cyprinidae) (Vardia and Durve 1981) and *Pimephales promelas* (Cypriniformes, Cyprinidae) (Brooke 1989) when exposed to 2,4-D acid, respectively, whereas values ranging from > 100 up to 1530 mg/L were reported for *Lepomis macrochirus* (Perciformes, Centrarchidae)- and *P. promelas*-2,4-D DMA-exposed specimens (Mayer and Ellersieck 1986). Finally, $LC50_{96h}$ values ranging from 0.15 up to 3 mg/L were reported for *Morone saxatilis* (Perciformes, Moronidae) when exposed to 2,4-D BE (Hughes 1973). To the best of our knowledge, in only two fish species, mortality data for the three different chemical forms have been reported so far. When *L. macrochirus* were employed as a biotic matrix, concentration ranges of 180–263, > 100–524 and 0.29–0.56 mg/L were found as the $LC50_{96h}$ values when exposed to 2,4-D, 2,4-D DMA and 2,4-D BE active ingredients, respectively (Alexander et al. 1985;

Mayer 1972; Mayer and Ellersieck 1986; USEPA 1992). Similarly, values of 110–358, > 100–1170 and 0.40–1.44 mg/L were determined in *Oncorhynchus mykiss* (Salmoniformes, Salmonidae) as the $LC50_{96h}$ values when exposed to 2,4-D, 2,4-D DMA and 2,4-D BE active ingredients, respectively (Alexander et al. 1985; Bills et al. 1981; Mayer 1972; Mayer and Ellersieck 1986; USEPA 1992). Thus, a toxicity gradient of mortality, equivalent to 2,4-D BE- > 2,4-D- > 2,4-D DMA active ingredients, exerted on these latter species when exposed to 2,4-D chemical forms result evident. Overall, our current results are in accordance with this observation, indicating that the 2,4-D DMA-based product is the less toxic variant among all three formulated herbicides assayed. On the other hand, we observed that the acid variant of 2,4-D resulted as the most toxic chemical form evaluated, at least in *C. decemmaculatus*-exposed fish. Although not possessing a clear explanation for this finding, a plausible hypothesis to explain this discrepancy could be committed to the fact of using a 2,4-D-based herbicide commercial product instead of the active principle by itself. In agreement, several reports have compared the short-term toxicity exerted by commercial formulations of pesticides vs their active ingredients in aquatic biota (Bach et al. 2018; Pérez-Iglesias et al. 2014; Puglis and Boone 2011; Ruiz de Arcaute et al. 2014a; Schmuck et al. 1994). They all agree in demonstrating that the formulated products produce more toxic results than the respective active ingredients being the excipients included in their formulation responsible for this effect. Our putative explanation is in concordance with this idea.

Our results highlighted the genotoxic effect evaluated by the SCGE assay of GLY-, 2,4-D-, 2,4-D DMA- and 2,4-D BE-based herbicides or their mixtures exerted on *C. decemmaculatus* regardless of the concentrations tested. For fish, an enhancement in the frequency of DNA breaks have been reported after treatment with GLY-based formulated products including Roundup® on *Corydoras paleatus* (Siluriformes, Callichthyidae) (de Castilhos and Cestari 2013), *Carassius auratus* (Cypriniformes, Cyprinidae) (Cavaş and Könen 2007), *Anguilla anguilla* (Anguilliformes, Anguillidae) (Guilherme et al. 2012), *Channa punctatus* (Anabantiformes, Channidae) (Nwani et al. 2013), *Prochilodus lineatus* (Characiformes, Prochilodontidae) (Cavalcante et al. 2008; Cestari Moreno et al. 2014) and Credit® on *C. decemmaculatus* (Vera-Candioti et al. 2013b). Similarly, an increased frequency of DNA breaks have been previously reported after exposure to the auxinic herbicide on *Clarias batrachus* (Siluriformes, Clariidae) (Ateeq et al. 2005) and *O. mykiss* (Martínez-Tabche et al. 2004) treated with the active ingredient 2,4-D in its acid form. Positive induction of DNA breaks have been reported in *C. decemmaculatus* after exposure to the 2,4-D DMA-based formulation DMA® (Ruiz de Arcaute et al. 2014b, 2016, 2018a). Furthermore, positive results have been also found using the fish cell line epithelioma papulosum cyprini (EPC) derived from a skin tumour of carp *Cyprinus carpio* (Cypriniformes, Cyprinidae) after exposure to the 2,4-D acid form (Bokán et al. 2013). At last, it is worth mentioning that to the best of our knowledge no induction of DNA breaks evaluated by the SCGE assay has been reported for fish after exposure to 2,4-D BE so far. Thus, our results could be considered as the first experimental evidence of the genotoxic effect exerted by this chemical form of the auxinic herbicide on fish, at least on *C. decemmaculatus*.

When combining GLY-based Credit® plus 2,4-D-based Dedalo Elite or GLY-based Credit® plus 2,4-D DMA-based herbicide formulation Weedar® Full results demonstrated a synergistic pattern whereas the mixture of GLY-based Credit® plus 2,4-D-based Herbifen Super® followed an antagonic pattern. To the best of our knowledge, no data have been reported on the plausible genotoxicity interactions exerted by these herbicide combinations on fish evaluated by SCGE assay. Thus, our findings represent the first experimental evidence obtained in a fish species, as *C. decemmaculatus* is, as the results of an in vivo exposure to these combinations when the primary DNA damage was employed as genotoxic end-point. However, a similar synergistic pattern has been previously described for the mixture of GLY-based Credit® plus dicamba-based herbicide Banvel® (Soloneski et al. 2016) or by the former when combined with the herbicide imazethapyr-based formulation Pivot® H on *R. arenarum* tadpoles (Carvalho et al. 2019) through the detection of DNA damage by the comet assay. Similarly, years ago, Poletta et al. (2011) verified a synergistic effect on genotoxicity, including the SCGE assay, teratogenesis and metabolic alterations on *Caiman latirostris* (Crocodillia, Alligatoridae) after in ovo exposure to GLY-based Roundup® Full II when mixed with the insecticides cypermethrin-based Cypermethrin Atanor® or endosulfan-based Endosulfan Galgofan®. A synergistic pattern has also been observed for the lethality induced by the combination of GLY-based Credit® plus 2,4-D DMA-based herbicide formulation Weedar® Full (Ruiz de Arcaute et al. 2018b) or by the mixture of 2,4-D DMA-based DMA® plus dicamba-based herbicide Banvel® (Ruiz de Arcaute et al. 2018c) when applied in combination using specimens of the same fish species employed as an experimental matrix in our current study or by the combination. Likewise, when tadpoles of *R. arenarum* were exposed to a mixture of GLY-based Glifosato Atanor® or Glifoglex® plus cypermethrin-based insecticide Xiper® or Glextrin®, a lethal synergistic pattern was also achieved (Brodeur et al. 2014). Finally, when other biotic matrices different than aquatic organisms were employed, a synergistic pattern was also reported for the herbicide mixture of GLY-based Roundup Pro Concentrate® plus a 2,4-D DMA-based (formulation not specified) on the phytotoxicity induced on the poison

ivy *Toxicodendron radicans* (Wehtje and Gilliam 2012). Although not employing the same herbicide mixtures we assayed, an antagonistic pattern was also previously reported for the lethality induction on *C. decemmaculatus* exposed to GLY-based Credit® plus dicamba-based herbicide Kamba® (Ruiz de Arcaute et al. 2018b) or to GLY-based Glifoglex® plus cypermethrin-based insecticide Glextrin® (Brodeur et al. 2016).

According to the Argentinean Administration (CASAFE 2017), the additives contained in any commercialized phytosanitary product, including agrochemicals, are not mandatory to be listed on the product data sheet. Unfortunately, thus, the identities of the excipients present in the trade products Credit®, Dedalo Elite, Weedar® Full and Herbifen Super® were not accessible to us. Additional studies should be performed in order to disclose whether the toxic effects induced by the formulated herbicides assayed are a consequence of the presence of xenobiotics with lethal and genotoxic properties. Furthermore, the aforementioned conundrum could even represent a worst scenario. Since the elements part of the formulations could experience different transformation pathways when moving throughout the environmental compartments, we cannot presume that each member of the combinations will follow a similar toxic pattern in the aquatic environment. Finally, taking into account the lack of information regarding the constituents of tested herbicide formulations, we could neither ensure nor deny whether these excipients take place in the environment under the chemical form we assayed. Besides that, the eventual presence of their metabolites can introduce uncertainties on the conclusions obtained from our current results and hamper the ecological balance cannot be ruled out.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

References

Alexander HC, Gersich FM, Mayes MA (1985) Acute toxicity of four phenoxy herbicides to aquatic organisms. Bull Environ Contam Toxicol 35:314–321

Ateeq B, Farah MA, Ahmad W (2005) Detection of DNA damage by alkaline single cell gel electrophoresis in 2,4-dichlororphenoxyacetic-acid- and butachlor-exposed erythrocytes of *Clarias batrachus*. Ecotoxicol Environ Saf 62:348–354

Azqueta A, Collins AR (2013) The essential comet assay: a comprehensive guide to measuring DNA damage and repair. Arch Toxicol 87: 949–968

Bach NC, Marino DJG, Natale GS, Somoza GM (2018) Effects of glyphosate and its commercial formulation, Roundup® Ultramax, on liver histology of tadpoles of the neotropical frog, *Leptodactylus latrans* (Amphibia: Anura). Chemosphere 202:289–297

Bills TD, Marking LL, Mauck WL (1981) Polychlorinated biphenyl (Aroclor 1254) residues in rainbow trout: effects on sensitivity to nine fishery chemicals. N Am J Fish Manag 1:200–203

Blouin D, Webster E, Bond J (2010) On a method of analysis for synergistic and antagonistic joint-action effects with fenoxaprop mixtures in rice (*Oryza sativa*). Weed Technol 24:583–589

Blouin DC, Webster EP, Wei Z (2004) Analysis of synergistic and antagonistic effects of herbicides using nonlinear mixed-model methodology. Weed Technol 18:464–472

Bokán K, Syberg K, Jensen K, Rank J (2013) Genotoxic potential of two herbicides and their active ingredients assessed with comet assay on a fish cell line, epithelioma papillosum cyprini (EPC). J Toxicol Environ Health 76:1129–1137

Brodeur JC, Melpel S, Anglesio AB, Cristos D, D'Andrea MF, Poliserpi MB (2016) Toxicities of glyphosate- and cypermethrin-based pesticides are antagonic in the tenspotted livebeared fish (*Cnesterodon decemmaculatus*). Chemosphere 155:429–435

Brodeur JC, Poliserpi MB, D'Andrea MF, Sanchez 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

Brooke L (1989) Results of freshwater exposures with the chemicals 2,4-D and diazinon to the larval leopard frog (*Rana pipiens*), juvenile fathead minnows (*Pimephales promelas*), larval midge (*Chironomus riparius*) and adult Oligochaete worms (*Lumbriculus variegatus*). United States Environmental Protection Agency February 15th Memo to RSpehar: 6 pp.

Carriquiriborde P, Díaz J, Mugni H, Bonetto C, Ronco AE (2007) Impact of cypermethrin on stream fish populations under field-use in biotech-soybean production. Chemosphere 68:613–621

Carvalho WF, Ruiz de Arcaute C, Pérez-Iglesias JM, Laborde MRR, Soloneski S, Larramendy ML (2019) DNA damage exerted by mixtures of commercial formulations of glyphosate and imazethapyr herbicides in *Rhinella arenarum* (Anura, Bufonidae) tadpoles. Ecotoxicology 28:367–377

CASAFE (2017) Guía de Productos Fitosanitarios para la República Argentina

Cavalcante DGSM, Martinez CBR, Sofia SH (2008) Genotoxic effects of Roundup® on the fish *Prochilodus lineatus*. Mutat Res 655:41–46

Cavaş T, Könen S (2007) Detection of cytogenetic and DNA damage in peripheral erythrocytes of goldfish (*Carassius auratus*) exposed to a glyphosate formulation using the micronucleus test and the comet assay. Mutagenesis 22:263–268

Cestari Moreno N, Sofia SH, Martinez CBR (2014) Genotoxic effects of the herbicide Roundup Transorb® and its active ingredient glyphosate on the fish *Prochilodus lineatus*. Environ Toxicol Pharmacol 37:448–454

Collins A, Koppen G, Valdiglesias V, Dusinska M, Kruszewski M, Møller P, Rojas E, Dhawan A, Benzie I, Coskun E, Moretti M,

Speit G, Bonassi S, ComNet project (2014) The comet assay as a tool for human biomonitoring studies: the ComNet Project. Mutat Res 759:27–39

de Castilhos GN, Cestari MM (2013) Genotoxic effects of the herbicide Roundup® in the fish *Corydoras paleatus* (Jenyns 1842) after short-term, environmentally low concentration exposure. Environ Monit Assess 185:3201–3207

Deneer JW (2000) Toxicity of mixtures of pesticides in aquatic systems. Pest Manag Sci 56:516–520

Di Giulio RT, Hinton DE (2008) The toxicology of fishes. CRC Press Taylor and Francis Group, Boca Raton

Dirzo R, Raven PH (2003) Global state of biodiversity and loss. Annual Rev Environ Res 28:137–167

Furlong ET, Anderson BD, Werner SL, Soliven PP, Coffey LJ, Burkhardt MR (2011) Methods of analysis by the US Geological Survey National Water Quality Laboratory—determination of pesticides in water by graphitized carbon-based solid-phase extraction and high-performance liquid chromatography/mass spectrometry. US Geological Survey Water Resources Investigations Report 01–4134, 73

Ge H, Lin Z, Yao Z, Gao Z, Cong Y, Yu H (2014) Balance between herbicidal activity and toxicity effect: a case study of the joint effects of triazine and phenylurea herbicides on Selenastrum capricornutum and *Photobacterium phosphoreum*. Aquat Toxicol 150:165–174

Guilherme S, Gaivão I, Santos MA, Pacheco M (2012) DNA damage in fish (*Anguilla anguilla*) exposed to a glyphosate-based herbicide—elucidation of organ-specificity and the role of oxidative stress. Mutat Res 743:1–9

Guyton KZ, Loomis D, Grosse Y, el Ghissassi F, Benbrahim-Tallaa L, Guha N, Scoccianti C, Mattock H, Straif K, International Agency for Research on Cancer Monograph Working Group, IARC, Lyon, France (2015) Carcinogenicity of tetrachlorvinphos, parathion, malathion, diazinon, and glyphosate. Lancet Oncol 16:490–491

Hernández AF, Gil F, Lacasaña M (2017) Toxicological interactions of pesticide mixtures: an update. Arch Toxicol 91:3211–3223

Hughes JS (1973) Acute toxicity of thirty chemicals to striped bass (*Morone saxatilis*). Proceedings of the annual conference of the Western Association of State Game and Fish Commissioners: pp. 15.

IARC (2018) IARC Monographs of the evaluation of carcinogenic risks to humans. DDT, Lindane and 2,4-D vol 113. International Agency for Research on Cancer, Lyon

IPCS (1994) International Programme on Chemical Safety. Environmental health criteria 159. Glyphosate. World Health Organization, Geneva

IRAM (2008) Calidad ambiental-Calidad de agua. Determinación de la toxicidad letal aguda de sustancias en peces de agua dulce. Métodos semiestático. Instituto Argentino de Normalización y Certificación Norma 29112:23

Larramendy ML, Soloneski S (2014) Pesticides—toxic aspects. InTech Publisher, Rijeka

Larramendy ML, Soloneski S (2015) Toxicity and hazard of agrochemicals. InTech Publisher, Rijeka

Liu W, Li H, Tao F, Li S, Tian Z, Xie H (2013) Formation and contamination of PCDD/Fs, PCBs, PeCBz, HzCBz and polychlorophenols in the production of 2,4-D products. Chemosphere 92:304–308

Lydy M, Belden J, Wheelock C, Hammock B, Denton D (2004) Challenges in regulating pesticide mixtures. Ecol Soc 9:1–13

Martínez-Tabche L, Madrigal-Bujaidar E, Negrete T (2004) Genotoxicity and lipoperoxidation produced by paraquat and 2,4-dichlorophenoxyacetic acid in the gills of rainbow trout (*Oncorhynchus mikiss*). Bull Environ Contam Toxicol 73:146–152

Mayer FLJ (1972) Pesticides as pollutants. In: Liptak BG (ed) Environmental engineer's handbook. Chilton Book Co., Radnor, pp 405–418

Mayer FLJ, Ellersieck MR (1986) Manual of acute toxicity: interpretation and data base for 410 chemicals and 66 species of freshwater animals. USDI Fish Wildlife Serv 160:505

Mazzatorta P, Benfenati E, Neagu D, Gini G (2002) The importance of scaling in data mining for toxicity prediction. J Chem Inf Model 42: 1250–1255

Mithila J, Hall CJ, Johnson WG, Kelley KB, Riechers DE (2011) Evolution of resistance to auxinic herbicides: historical perspectives, mechanisms of resistance, and implications for broadleaf weed management in agronomic crops. Weed Sci 59:445–457

Morales C, Schaper M (2004) Las nuevas fronteras tecnológicas: los transgénicos y sus impactos en América Latina y el Caribe. In: Bárcena C, Katz J, Morales C, Schaper M (eds) Los transgénicos en América Latina y el Caribe: un debate Abierto, vol 1. CEPAL, Santiago de Chile, pp 191–267

Mugni H, Paracampo A, Demetrio P, Scalise A, Solís M, Fanelli S, Bonetto C (2015) Acute toxicity of endosulfan to the non-target organisms *Hyalella curvispina* and *Cnesterodon decemmaculatus*. Bull Environ Contam Toxicol 95:363–367

Mugni HA, Paracampo A, Marrochi N, Bonetto C (2012) Cypermethrin, chlorpyrifos and endosulfan toxicity to two non-target freshwater organisms. Fresenius Environ Bull 21:2085–2089

Nwani CD, Nagpure NS, Kumar R, Kushwaha B, Lakra WS (2013) DNA damage and oxidative stress modulatory effects of glyphosate-based herbicide in freshwater fish, *Channa punctatus*. Environ Toxicol Pharmacol 36:539–547

Pérez-Iglesias JM, Ruiz de Arcaute C, Nikoloff N, Dury L, Soloneski S, Natale GS, Larramendy M (2014) The genotoxic effects of the imidacloprid-based insecticide formulation Glacoxan Imida on Montevideo tree frog *Hypsiboas pulchellus* tadpoles (Anura, Hylidae). Ecotoxicol Environ Saf 104:120–126

Pitarque M, Creus A, Marcos R, Hughes JA, Anderson D (1999) Examination of various biomarkers measuring genotoxic endpoints from Barcelona airport personnel. Mutat Res Genet Toxicol Environ Mutagen 440 (2):195–204

Poletta GL, Kleinsorge E, Paonessa A, Mudry MD, Larriera A, Siroski PA (2011) Genetic, enzymatic and developmental alterations observed in *Caiman latirostris* exposed *in ovo* to pesticide formulations and mixtures in an experiment simulating environmental exposure. Ecotoxicol Environ Saf 74:852–859

Puglis HJ, Boone MD (2011) Effects of technical-grade active ingredient vs. commercial formulation of seven pesticides in the presence or absence of UV radiation on survival of green frog tadpoles. Arch Environ Contam Toxicol 60:145–155

Ruiz de Arcaute C, Larramendy ML, Soloneski S (2018a) Genotoxicity by long-term exposure to the auxinic herbicides 2,4-dichlorophenoxyacetic acid and dicamba on *Cnesterodon decemmaculatus* (Pisces: Poeciliidae). Environ Pollut 243:670–678

Ruiz de Arcaute C, Pérez-Iglesias JM, Nikoloff N, Natale GS, Soloneski S, Larramendy ML (2014a) Genotoxicity evaluation of the insecticide imidacloprid on circulating blood cells of Montevideo tree frog *Hypsiboas pulchellus* tadpoles (Anura, Hylidae) by comet and micronucleus bioassays. Ecol Indic 45:632–639

Ruiz de Arcaute C, Soloneski S, Larramendy M (2014b) Evaluation of the genotoxicity of a herbicide formulation containing 3,6-dichloro-2-metoxybenzoic acid (dicamba) in circulating blood cells of the tropical fish *Cnesterodon decemmaculatus*. Mutat Res 773:1–8

Ruiz de Arcaute C, Soloneski S, Larramendy M (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

Ruiz de Arcaute C, Soloneski S, Larramendy ML (2018b) Opposite effects of mixtures of commercial formulations of glyphosate with auxinic herbicides on the ten spotted live-bearer fish *Cnesterodon decemmaculatus* (Pisces, Poeciliidae). Environ Pollut 240:858–866

Ruiz de Arcaute C, Soloneski S, Larramendy ML (2018c) Synergism of binary mixtures of dicamba and 2,4-dichlorophenoxyacetic acid herbicide formulations on the ten spotted live-bearer fish *Cnesterodon decemmaculatus* (Pisces, Poeciliidae). Environ Pollut 236:33–39

Schiesari L, Waichman A, Brock T, Adams C, Grillitsch B (2013) Pesticide use and biodiversity conservation in the Amazonian agricultural frontier. Philos TransR Soc London Series B 368:1–9

Schmuck R, Pflüger W, Grau R, Hollihn U, Fischer R (1994) Comparison of short-term aquatic toxicity: formulation vs active ingredients of pesticides. Arch Environ Contam Toxicol 26:240–250

SENASA (2013) Servicio Nacional de Sanidad y Calidad Agroalimentaria. http://www.senasa.gov.ar/contenido.php?to=n&in=1506&io=18122. Accessed June 2019

Silva AR, Cardoso DN, Cruz A, Lourenco J, Mendo S, Soares AM, Loureiro S (2015) Ecotoxicity and genotoxicity of a binary combination of triclosan and carbendazim to *Daphnia magna*. Ecotoxicol Environ Saf 115:279–290

Singh NP (1996) Microgel electrophoresis of DNA from individual cells. In: Pfeifer GP (ed) Technologies for detection of DNA damage and mutations. Springer, Boston. https://doi.org/10.1007/978-1-4899-0301-3_1

Soloneski S, Ruiz de Arcaute C, 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

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

USEPA (1974) United States Environmental Protection Agency. Compendium of Registered Pesticides US Government Printing Office, Washington

USEPA (1975) Methods for acute toxicity tests with fish, macroinvertebrates, and amphibians. US Government Printing Office, Washington 660/3-75-009:62

USEPA (1992) United States Environmental Protection Agency. Pesticide Ecotoxicity Database (formerly: Environmental Effects Database (EEDB)). Environmental Fate and Effects Division, Washington

USEPA (2001) United States Environmental Protection Agency Report. Environmental Hazard Assess Ecol Risk Assess Methodology. Appendix H

USEPA (2002) United States Environmental Protection Agency. Methods for measuring the acute toxicity of effluents and receiving waters to freshwater and marine organisms, 5th edn. US Government Printing Office, Washington 821-R-02-012

USEPA (2016) United States Environmental Protection Agency. Glyphosate issue paper: evaluation of carcinogenic potential. US Government Printing Office, Washington, p 227

USEPA (2019) United States Environmental Protection Agency. ECOTOX knowledgebase. https://cfpub.epa.gov/ecotox/. Last accessed May, 2019. US Government Printing Office, Washington

Vardia HK, Durve VS (1981) Bioassay study on some freshwater fishes exposed to 2,4-dichlorophenoxyacetic acid. Acta Hydrochim Hydrobiol 92:219–223

Varona-Uribe ME et al (2016) Exposure to pesticide mixtures and DNA damage among rice field workers. Arch Environ Occupat Health 71:3–9

Vera-Candioti J, Soloneski S, Larramendy M (2010) Genotoxic and cytotoxic effects of the formulated insecticide Aficida® on *Cnesterodon decemmaculatus* (Jenyns, 1842) (Pisces: Poeciliidae). Mutat Res 703:180–186

Vera-Candioti J, Soloneski S, Larramendy M (2015) Pirimicarb-based formulation induced genotoxicity and cytotoxicity on the fresh water fish *Cnesterodon decemmaculatus* (Jenyns, 1842) (Pisces, Poeciliidae). Toxicol Ind Health 31:1051–1060

Vera-Candioti J, Soloneski S, Larramendy ML (2013a) Evaluation of the genotoxic and cytotoxic effects of glyphosate-based herbicides in the ten spotted live-bearer fish *Cnesterodon decemmaculatus* (Jenyns, 1842). Ecotoxicol Environ Saf 89:166–173

Vera-Candioti J, Soloneski S, Larramendy ML (2013b) Single-cell gel electrophoresis assay in the ten spotted live-bearer fish, *Cnesterodon decemmaculatus* (Jenyns, 1842), as bioassay for agrochemical-induced genotoxicity. Ecotoxicol Environ Saf 98:368–373

Vera-Candioti J, Soloneski S, Larramendy ML (2014) Chlorpyrifos-based insecticides induced genotoxic and cytotoxic effects in the ten spotted live-bearer fish, *Cnesterodon decemmaculatus* (Jenyns, 1842). Environ Toxicol 29:1390–1398

Wehtje G, Gilliam CH (2012) Cost-effectiveness of glyphosate, 2,4-D, and triclopyr, alone and in select mixtures for poison ivy control. Weed Technol 26:469–473

WHO-FAO (1997) Pesticides residues in food vol 145. World Health Organization and Food and Agriculture Organization of the United Nations, Rome

WHO (2009) The WHO recommended classification of pesticides by hazard and guidelines to classification: 2009. World Health Organization, Geneva, p 81

Wright TR et al (2010) Robust crop resistance to broadleaf and grass herbicides provided by aryloxyalkanoate dioxygenase transgenes. Proc Natl Acad Sci U S A 107:20240–202405