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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*

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ABSTRACT

Acute toxicity and genotoxicity of the dicamba-based commercial herbicide formulation Banvel® were evaluated on *Cnesterodon decemmaculatus* (Pisces, Poeciliidae) exposed under laboratory conditions. A lethal effect was used as the end point for mortality, whereas frequency of micronuclei (MNs) and DNA single-strand breaks evaluated by the single cell gel electrophoresis assay were employed as end points for genotoxicity. Mortality studies revealed an $LC_{50\,96\,h}$ value of 1639 mg/L (range, 1471–1808) of dicamba. Furthermore, behavioral changes, *e.g.*, gathering at the bottom of the aquarium, slowness in motion, abnormal swimming, and slow reaction, were observed. Whereas increased frequency of MNs was observed when 1229 mg/L dicamba was assayed for 48 h, no induction of MNs was observed in fish exposed to the herbicide for 96 h, regardless of the concentration of dicamba. Furthermore, other nuclear abnormalities, *i.e.*, binucleated cells and lobed and notched nuclei, were induced in fish exposed for 48 h but not 96 h. Increase in the genetic damage index was observed in those treatments (lasting for both 48 and 96 h) within the 410–1229 mg/L dicamba concentration-range. This study represents the first evidence of acute lethal and sublethal effects exerted by dicamba on a piscine species native to Argentina. The results could indicate that dicamba-based formulation Banvel® is the less toxic emerging pollutant reported so far for *C. decemmaculatus*. Finally, our findings highlight the properties of this herbicide that jeopardize nontarget living species exposed to this agrochemical.

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1. Introduction

Nowadays, it is almost impossible for many countries to decrease their use of pesticides without reducing crop yields [1]. These compounds can also be hazardous if not used appropriately, and many of them (if not all) may represent potential hazards to the environment due to the contamination of food, water, and air [2]. Furthermore, increasing their jeopardizing effects, anthropogenic activities are continuously introducing extensive amounts of these compounds into the environment regardless of their persistence, bioaccumulation, and toxicity (www.epa.gov/pesticides/). However, it is well known that pesticides not only affect target organisms, but concomitantly also exert side effects on nontarget organisms [3,4].

Auxinic herbicides are among the most used pesticides worldwide. They are employed as agrochemicals for their selective control on broadleaf weeds. They comprise several compounds belonging to four different chemical families, *i.e.*, phenoxyalkanoic acids, pyridinecarboxilic acids, quinolinecarboxylic acids, and benzoic acids [5].

Dicamba is a chlorinated benzoic acid-derivate compound that was first registered in 1967 in the United States as a postemergent herbicide [6]. It is currently used in agriculture and gardening to control the growth of different unwanted vegetable species, mainly in cereal grain crops, but also on sugar cane and soybeans, among others [7]. The herbicide is available in numerous commercial formulations as a single component or coformulated with a wide variety of other herbicides, such as 2,4-D, sulfonylureas or

Abbreviations: AS, abnormal swimming; BL, blebbed nuclei; BN, binucleated erythrocyte; CP, cyclophosphamide; GBA, gathering at the bottom of the aquarium; GDI, genetic damage index; LC_{50}, lethal concentration 50; LB, lobed nuclei; LOEC, lowest observed effect concentration; MN, micronucleus; NOEC, no observed effect concentration; NT, notched nuclei; SCGE, single cell gel electrophoresis; SM, slowness in motion; SR, slow reaction.

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triazines, to provide a broader spectrum of weed control. These formulations are registered and sold in many countries all over the world [8]. As dicamba shares characteristics with other acidic herbicides, *e.g.*, high water solubility, low volatility, and heavy agricultural use, that facilitate their incorporation into the aqueous environment, a growing number of studies have been performed stressing its environmental effects. Dicamba has been detected as a pollutant in agricultural, urban, and mixed agricultural/urban sites [9], in surface drinking-water reservoirs [10], and even in estuarine waters [11]. Furthermore, much is known about its effects on aquatic plants and risks to terrestrial organisms [7]. Toxicological data on this chlorinated acid herbicide are available and considered adequate to assess its potential hazards not only to humans, but also to other living species [7].

So far, dicamba's potential genotoxic hazard has been revealed by diverse *in vivo* [12–14] and *in vitro* assays [12,15,16]. Some recent studies demonstrated that dicamba should be considered a DNA-damaging agent. Induction of sister chromatid exchanges (SCEs) and alteration in both cell-cycle progression and mitotic indices in human lymphocytes *in vitro* and Chinese hamster ovary (CHO-K1) cells have been reported [17,18]. Additionally, the herbicide was reported to induce DNA-strand breaks revealed by the single cell gel electrophoresis (SCGE) assay [18] as well as micronuclei (MNs) in CHO-K1 cells [19]. Finally, positive results were also reported when applying the *Tradescantia*-micronucleus assay [14].

The dicamba DNA damage mechanism is not yet totally known. It is known that dicamba induces tissue damage and cell death in cleavers (*Gallium aparine* L.) by lipid peroxidation [20]. Furthermore, it was suggested recently that dicamba-induced lesions on DNA could be attributable to reactive oxygen species delivered *in vitro* [17,20,21].

Fish are suitable aquatic vertebrates to be employed as reliable environmental lethality, cytotoxicity, and genotoxicity bioindicator organisms due to either their role in the aquatic trophic chain and their sensitivity to low concentrations of emerging pollutants, characteristic of polluted aquatic environments. Furthermore, the capability of fish to efficiently metabolize and accumulate chemical pollutants is well documented [22,23]. The MN as well as the SCGE tests, due to their sensitivity, reliability, and the simplicity of use, have become widely used in piscine erythrocytes to assess the genotoxicity of many xenobiotics [22–32]. Genotoxicity in fish associated with pesticide exposure analyzed using both the MN and the SCGE bioassays in fish erythrocytes is well documented [23–31].

The aim of the present study is to characterize the acute toxicity of the Argentinean dicamba-containing auxinic herbicide commercial formulation Banvel® (57.71% dicamba) on *Cnesterodon decemmaculatus*, an endemic species with an extensive distribution in Neotropical America, exposed under laboratory conditions using a static acute experimental method. Whereas a lethal effect was used as the end point for mortality, the frequency of MNs and DNA single-strand breaks evaluated by the SCGE assay were employed as end points for genotoxicity on circulating blood cells.

2. Materials and methods

2.1. Chemicals

Dicamba (3,6-dichloro-2-methoxybenzoic acid, CAS 1918-00-9) commercial-grade trade formulation Banvel® (57.71% dicamba) was kindly provided by Syngenta Agro S.A. (Buenos Aires, Argentina). Cyclophosphamide (CP; CAS 6055-19-2) and dimethyl sulfoxide (CAS 67-68-5) were purchased from Sigma Chemical Co. (St. Louis, MO), whereas $K_2Cr_2O_7$ [$Cr_{(VI)}$] (CAS 7778-50-9) was obtained from Merck KGaA (Darmstadt, Germany). All other chemicals and solvents of analytical grade were purchased from Sigma Chemical Co.

2.2. Quality control

Determination of the concentration levels of dicamba in the test solutions was performed by QV Chem Laboratory (La Plata, Buenos Aires, Argentina) according to U.S. Geological Survey Report 01-4134 [33]. Dicamba levels were analyzed by high performance liquid chromatography using an ultraviolet detector. Active ingredient samples from test solutions (820 mg/L) correspond to values obtained immediately after preparation (0 h) and 24 h thereafter. The detection limit for dicamba was 0.5 mg/L. Concentrations assessed throughout the study represent the nominal concentrations of active ingredient present within the dicamba-based formulation Banvel®.

2.3. Test organisms

C. decemmaculatus was selected as the target organism. This species is an endemic fish member of the family Poeciliidae with an extensive distribution in Neotropical America and which attains high densities in a large variety of water bodies within the whole La Plata River and other South American basins. This is a small ovoviviparous, microomnivorous, benthic-pelagic and nonmigratory fish (maximum size, ≈25 mm and 45 mm for ♂♂ and ♀♀, respectively), and it is often the most abundant and sometimes the only species present in small watercourses. This species is easy to handle and acclimate to laboratory conditions. Ranges of tolerance of *C. decemmaculatus* to many environmental parameters, *e.g.*, temperature, salinity, and pH, are comparatively large, requested conditions for toxicity testing [34]. Furthermore, several reports found this species suitable as a test organism in acute and chronic toxicity bioassays [25–30,35,36]. Specimens were collected from a permanent pond away from agricultural areas, in the vicinity of La Plata city (Buenos Aires Province, Argentina). Adults were transported to the laboratory and then acclimatized for at least 20 days to 16/8 h light/dark cycle in aquaria at 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) with artificial aeration and an *ad libitum* daily supply of commercially available fish food (TetraMin®, TetraWerke, Melle, Germany) until the beginning of the experimental procedures.

2.4. Determination of LC_{50}

Experiments were carried out for toxicity assessment following recommendations proposed by the U.S. EPA standardized methods for acute piscine toxicity testing [37,38] with minor modifications reported previously for the species [25,27–30]. The average body weight of the specimens used throughout the experiment was 0.26 ± 0.1 g, and the mean body length was 29.5 ± 2.7 mm. Briefly, for each experimental point, 10 specimens (5 ♂♂ and 5 nongravid ♀♀) were maintained in a 1 L glass container and exposed to nine different concentrations of dicamba (816, 1152, 1296, 1440, 1584, 1728, 2208, 2688, and 2880 mg/L) for 96 h. Negative (dechlorinated tap water, pH 7.55 ± 0.1; dissolved oxygen, 6.3 ± 0.3 mg/L; NH_4^+ <0.2 mg/L; hardness, 143 ± 23.5 mg $CaCO_3$/L) and positive controls (21.4 mg/L $Cr_{(VI)}$-treated fish) [26] were conducted and run simultaneously with Banvel®-exposed fish. All test solutions were prepared immediately before use and replaced every 24 h. Fish were not fed throughout the experiment. A lethal (mortality) effect was determined every 24 h in three independent experiments run simultaneously for each experimental point.

2.5. Sublethal end points

2.5.1. Behavioral changes

The behavioral changes of the fish subjected to different concentrations of dicamba (816, 1152, 1296, 1440, 1584, 1728, 2208, 2688, and 2880 mg/L) and those of healthy fish were monitored and determined every 24 h before the application of Banvel® test solutions in those fish employed for determining LC_{50} values. Optomotor responses were classified following the criteria reported elsewhere [39].

2.5.2. Micronuclei and other erythrocytic nuclear abnormalities

MN assay was performed on peripheral circulating blood erythrocytes according to the procedure described previously [25,27–30]. Experiments were performed following the same experimental design described in Section 2.4, with specimens exposed to three different concentrations of Banvel® equivalent to 25%, 50%, and 75% of the corresponding $LC_{50\,96\,h}$ value. To achieve these concentrations, specimens were exposed to 410, 820, and 1229 mg/L of dicamba, respectively (see Section 2.4). Negative (dechlorinated tap water, see Section 2.4) and positive (10 mg/L CP) controls were conducted and run simultaneously with Banvel®-exposed fish. All test solutions were prepared immediately before each experiment. The frequency of MNs was determined in peripheral mature erythrocytes at 48 and 96 h after the initial treatment. Experiments were performed in triplicate and run simultaneously for each experimental point. Fish were killed according to American Society of Ichthyologists and Herpetologists [40] criteria. At the end of each experiment, anesthesia was induced by immersion in ice water and blood samples were obtained by sectioning behind the operculum. Peripheral blood smears were performed for each animal onto clean slides, air dried, fixed with 100% (v/v) cold methanol (4 °C) for 20 min, and then stained with 5% Giemsa solution for 12 min. Slides were coded and blind-scored by one researcher at 1000 × magnification. Data are expressed as the

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

Number of individuals	Concentration (mg/L)	Mortality (number of dead individuals)			
		24 h	48 h	72 h	96 h
30	Control	1	1	1	1
30	816	3	3	3	3
30	1152	2	2	2	7
30	1296	2	3	9	12
30	1440	2	6	10	12
30	1584	5	6	10	13
30	1728	0	13	16	17
30	2208	3	9	16	18
30	2688	3	13	21	27
30	2880	15	22	24	30
30	Positive control[a]	4	8	11	14

[a] $Cr_{(VI)}$ (21.4 mg/L) was used as positive control.

total number of MNs per 1000 cells, as suggested previously [25]. MN frequency was determined following the examination criteria reported previously [25]. Briefly, the criteria employed in identifying MNs were as follows: a diameter smaller than 1/3 of that of the main nuclei, nonrefractibility, the same staining intensity as or staining intensity lighter than that of the main nuclei, no connection or link with the main nuclei, no overlapping with the main nuclei, MN boundary distinguishable from the main nuclei boundary, and no more than four MNs associated with the nuclei.

Other erythrocytic nuclear abnormalities were blind-scored from 1000 erythrocytes per experimental point from each experiment at 1000× magnification. Examination criteria followed those established previously [41,42]. Briefly, cells with two nuclei were considered binucleated cells (BNs), whereas cells with one nucleus presenting a relatively small evagination of the nuclear membrane containing euchromatin were classified as blebbed nuclei (BLs). Nuclei with evaginations of the nuclear membrane larger than that of a BL, which could have several lobes, were considered lobed nuclei (LBs). Finally, nuclei with vacuoles and appreciable depth into a nucleus without containing nuclear material were recorded as notched nuclei (NTs).

2.5.3. Single cell gel electrophoresis assay

Specimens exposed for MN assay (see Section 2.5.1) were also employed for SCGE assay. Negative (dechlorinated tap water, see Section 2.4) and positive (10 mg/L CP) controls were conducted and run simultaneously with dicamba-based formulation Banvel®-exposed specimens. The SCGE assay was performed following the alkaline procedure described by Singh et al. [43] with minor modifications. Blood samples were diluted with 1 ml phosphate-buffered saline. An aliquot of 30 μl the diluted samples was mixed with 70 μl 0.5% low-melting-point agarose and was then layered on a slide precoated with 100 μl 0.5% normal-melting-point agarose. The slide was covered with a coverslip and placed at 4 °C for 10 min. After solidification, the coverslip was removed, and the slide was covered with a third layer of 50 μl 0.5% low-melting-point agarose. After solidification, the coverslip was removed, and slides were immersed in ice-cold freshly prepared lysis 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 lysed at darkness for 1 h period (4 °C). Then, slides were placed in an electrophoresis buffer (1 mm Na_2EDTA, 300 mm NaOH) for 25 min at 4 °C to allow the cellular DNA to unwind, followed by electrophoresis in the same buffer and temperature for 30 min at 25 V and 250 mA (0.8 V/cm). Finally, the slides were neutralized with a solution comprising 0.4 m Tris–HCl, pH 7.5, stained with 4′,6-diamino-2-phenylindole (DAPI; Vectashield mounting medium H1200; Vector Laboratories, Burlingame, CA, USA). Slides were examined under an Olympus BX50 fluorescence photomicroscope equipped with an appropriate filter combination. The extent of DNA damage was quantified by the length of DNA migration, which was visually determined in 100 randomly selected and nonoverlapping cells. DNA damage was classified in four classes (0–I, undamaged; II, minimum damage; III, medium damage; IV, maximum damage), as suggested previously [44]. Data are expressed as the mean number of damaged cells (sum of Classes II, III, and IV) and the mean comet score for each treatment group. The genetic damage index (GDI) was calculated for each test compound following Pitarque et al. [45] using the formula GDI = [I (I) + 2 (II) + 3 (III) + 4 (IV)/*N* (0–IV)], where 0–IV represents the nucleoid type, and N_0–N_{IV} represents the total number of nucleoid scored.

2.6. Statistical analysis

A *t*-test was performed for comparisons in chemical analyses. Mortality data were analyzed using the U.S. EPA Probit Analysis statistical software, version 1.5 (http://www.epa.gov/nerleerd/stat2.htm.), based on Finney's method [46]. The proportion of individuals affected per test chamber ($n = 5$) was calculated for lethal and sublethal end points (mortality, behavior, MNs, BNs, BLs, LBs, NTs, damaged cells, and GDI). Each proportion was angular transformed and a one-way ANOVA with Dunnett's test was performed, whereas a one-way ANOVA with Tukey's test was performed for comparison between negative and solvent control data. ANOVA assumptions were corroborated with Barlett's test for homogeneity of variances and a χ^2 test for normality. In cases which did not perform the assumptions of normality was made a Kruskal–Wallis test. No observed effect concentration (NOEC) and lowest observed effect concentration (LOEC) values were estimated for behavioral end points. The relationships between concentration and GDI and MN data were evaluated by simple linear regression and correlation analyses. Concentration–response (C–R) curves at 96 h were estimated with their 95% confidence limits. Regression and correlation coefficients were calculated for each C–R curve. Tests of the significance of the regressions and correlation coefficients were performed. The level of significance chosen was 0.05 unless indicated otherwise.

3. Results

Results obtained from the *t*-test between chemical analyses showed no significant changes ($P > 0.05$) in the concentration of the pure analyte in treatments during the 24 h interval renewals of the testing solutions (concentration range, 97 ± 5% recovery).

3.1. Mortality

Probit analysis of the mortality data allowed determination of the LC_{50} values of dicamba present within the formulation Banvel® after 24, 48, 72, and 96 h of exposure. Results revealed mean values of $LC_{50\ 24\,h}$ = 2358 mg/L (range, 714–7812), $LC_{50\ 48\,h}$ = 2473 mg/L (range, 2144–3087), $LC_{50\ 72\,h}$ = 1941 mg/L (range, 1725–2216), and $LC_{50\ 96\,h}$ = 1639 mg/L (range, 1471–1808). As revealed by regression analysis, LC_{50} values were not affected by the exposure time ($r = -0.90$, $P > 0.05$). Complete data of mortality are presented in Table 1.

3.2. Behavioral changes

The behavioral changes observed in those *C. decemmaculatus* exposed to different concentrations of dicamba present within the formulation Banvel® were gathering at the bottom of the aquarium (GBA), slowness in motion (SM), abnormal swimming (AS), and slow reaction (SR). NOEC and LOEC values for all observed changes are summarized in Table 2.

3.3. MN and other erythrocytic nuclear abnormality frequencies

Table 3 shows the results of the analysis of MNs in peripheral blood erythrocytes of *C. decemmaculatus* induced by the dicamba-based herbicide formulation Banvel®. An increased frequency of MNs was observed in fish exposed to CP (positive control) for 48 h ($P < 0.05$), but not in fish exposed for 96 h ($P > 0.05$). The results reveal that while increased frequency of MNs was observed in those fish exposed to the highest concentration (1229 mg/L) of dicamba present within the formulation Banvel® for 48 h ($P < 0.001$), no induction of MNs was observed in fish exposed to the herbicide for 96 h ($P > 0.05$), regardless of the concentration

Table 2
Behavioral effects of *Cnesterodon decemmaculatus* exposed to the dicamba-based herbicide Banvel®.[a]

Exposure time (h)	Effect	NOEC[b]	LOEC[b]
24	SM[c]	1296	1440***
	GBA[c]	1296	1440***
	SR[c]	1296	1440**
	As[c]	1584	1728***
48	SM	1152	1296**
	GBA	1584	1728*
	SR	1728	2208***
	AS	1440	1584*
72	SM	1152	1296***
	GBA	0	1152***
	SR	0	1152***
	AS	0	1152***
96	SM	1296	1440***
	GBA	1152	1296*
	SR	1296	1440*
	AS	0	1152*

[a] Fish were exposed to 816, 1152, 1296, 1440, 1584, 1728, 2208, 2688, and 2880 mg/L of dicamba.
[b] Results are expressed in mg/L.
[c] SM, slowness in motion; GBA, gathering at the bottom of the aquarium; SR, slow reaction; AS, abnormal swimming.
* $P<0.05$.
** $P<0.01$.
*** $P<0.001$; significant differences with respect to control values.

of herbicide assayed. When other nuclear abnormalities rather than MNs were analyzed, significant increased frequencies were observed only for treatments lasting 48 h. An increase in the frequency of NTs was observed in fish exposed to 410, 820, and 1229 mg/L dicamba present within the formulation Banvel® ($P<0.001$, $P<0.05$, and $P<0.001$, respectively). In addition, only the treatment with 1229 mg/L of the herbicide showed an increased frequency of BNs ($P<0.01$) and LBs ($P<0.01$). On the other hand, herbicide treatments, regardless of concentration, did not modify the frequencies of BLs in regard to control values ($P>0.05$). Finally, results revealed that, regardless of the concentration of the herbicide assayed, the frequency of other nuclear abnormalities rather than MNs in fish exposed to the herbicide for 96 h did not differ from that of negative controls ($P>0.05$). However, a trend to an increased frequency of LBs was observed in fish exposed to 1229 mg/L of the herbicide, although not reaching statistical significance ($P>0.05$) (Table 3).

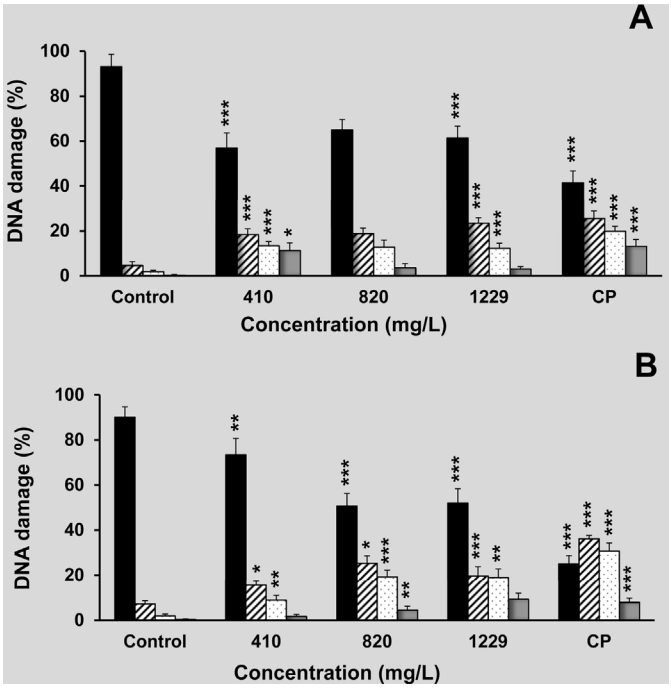

Fig. 1. The dicamba-based herbicide formulation Banvel®-induced DNA damage measured by single cell gel electrophoresis assay in circulating blood cells from *Cnesterodon decemmaculatus* (Pisces, Poeciliidae) exposed for 48 h (A) and 96 h (B). The frequencies of undamaged (type 0–I nucleoids; black bar), type II (stripped bar), type III (dotted bar), and type IV (gray bar) were determined by analyzing 100 nucleoids from each specimen. Results are presented as percentages of pooled data from three independent experiments. Negative (untreated fish) and positive controls (CP, 10 mg cyclophosphamide/L-treated fish) were conducted and run simultaneously with Banvel®-exposed fish. *$P<0.05$; **$P<0.01$; ***$P<0.001$; significant differences with respect to control values.

3.4. DNA damage

The results of the SCGE assay obtained for peripheral blood erythrocytes of *C. decemmaculatus* exposed for 48 and 96 h to the dicamba-based herbicide formulation Banvel® are presented in Table 4, and the mean frequencies of cells from each damage grade are depicted in Fig. 1. CP treatment (positive control) induced an enhancement of the GDI as well as in the frequency of damaged cells compared to negative controls in specimens exposed for

Table 3
Frequencies (‰) of MNs and other nuclear abnormalities in peripheral blood erythrocytes of *Cnesterodon decemmaculatus* exposed to the dicamba-based herbicide Banvel®.[a]

Exposure time (h)	Concentration (mg/L)	No. of individuals analyzed	No. of cells analyzed	MNs[b]	Other nuclear abnormalities[b]			
					NTs	LBs	BNs	BLs
48	Control	15	22,599	0.09 ± 0.04	1.86 ± 0.34	0.04 ± 0.04	0.00 ± 0.00	1.77 ± 0.33
	Positive control[c]	15	22,632	0.44 ± 0.15*	4.52 ± 0.81*	0.09 ± 0.06	0.00 ± 0.00	4.07 ± 0.59
	410	15	23,032	0.09 ± 0.09	6.13 ± 0.98***	0.18 ± 0.12	0.00 ± 0.00	3.10 ± 0.88
	820	15	23,132	0.22 ± 0.10	3.94 ± 0.92*	0.17 ± 0.07	0.04 ± 0.04	3.54 ± 0.84
	1229	15	22,837	0.44 ± 0.14*	8.43 ± 1.55***	0.70 ± 0.28**	0.35 ± 0.15**	4.63 ± 1.30
96	Control	15	22,583	0.13 ± 0.07	2.96 ± 0.71	0.04 ± 0.04	0.00 ± 0.00	1.77 ± 0.44
	Positive control[c]	15	23,035	0.47 ± 0.16	8.24 ± 2.18	0.44 ± 0.16	0.13 ± 0.09	4.10 ± 1.09
	410	15	22,771	0.48 ± 0.22	7.00 ± 2.42	0.22 ± 0.18	0.04 ± 0.04	4.12 ± 1.21
	820	15	22,664	0.22 ± 0.08	4.35 ± 0.93	0.53 ± 0.23	0.00 ± 0.00	2.78 ± 0.77
	1229	14	22,731	0.13 ± 0.07	7.15 ± 1.87	0.81 ± 0.34	0.04 ± 0.04	3.56 ± 0.77

[a] Results are expressed as mean number of abnormalities/1000 cells ± SE.
[b] MNs, micronuclei; NTs, notched nuclei; LBs, lobed nuclei; BNs, binucleated nuclei; BLs, blebbed nuclei.
[c] Ciclophosphamide (10 mg/L) was used as positive control.
* $P<0.05$.
** $P<0.01$.
*** $P<0.001$; significant differences with respect to control values.

Table 4
Analysis of DNA damage measured by comet assay in *Cnesterodon decemmaculatus* cells exposed to the dicamba-based herbicide Banvel®.

Chemicals	Concentration (mg/L)	Exposure time (h)	No. of individuals analyzed	No. of cells analyzed	% of damaged cells (II + III + IV)	GDI ± SE[a]
Negative control		48	13	1126	6.93	0.73 ± 0.08
		96	15	1607	9.89	0.67 ± 0.06
Banvel®	410	48	15	1531	43.04***	1.65 ± 0.15***
		96	15	1442	26.63**	1.23 ± 0.09***
	820	48	15	1631	35.07***	1.21 ± 0.14***
		96	15	1596	49.37***	1.60 ± 0.11***
	1229	48	15	1647	38.68***	1.28 ± 0.09***
		96	13	1345	48.03***	1.70 ± 0.14***
Positive control[b]	10	48	15	1528	58.57***	1.90 ± 0.12***
		96	15	1567	74.98***	2.14 ± 0.09***

[a] GDI: genetic damage index.
[b] Cyclophosphamide (10 mg/L) was used as positive control.
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$; significant differences with respect to negative control values.

both 48 and 96 h ($P < 0.001$) (Table 4, Fig. 1). In herbicide-exposed organisms, a significant increase of the GDI was observed in all treatments (410, 820, and 1229 mg/L) lasting for both 48 and 96 h ($P < 0.001$) (Table 4). In specimens exposed for 48 h, such alteration was due to an enhanced frequency of type II and III nucleoids ($P < 0.001$) and a concomitant decrease of type 0–I nucleoids ($P < 0.001$) (Fig. 1A). In addition, an increased frequency of type IV nucleoids in fish treated with 410 mg/L for 48 h was observed ($P < 0.05$) (Fig. 1A). The results demonstrate that the alteration in the GDI found in specimens treated for 96 h was due to an increase in the frequency of type II and III nucleoids within the 410–1229 mg/L concentration range ($0.05 > P < 0.001$) and a concomitant decrease of type 0–I nucleoids ($0.01 > P < 0.001$) (Fig. 1B). In addition, enhanced frequencies of type IV nucleoids ($P < 0.01$) were also observed in organisms treated with 810 and 1229 mg/L dicamba for 96 h (Fig. 1B). Overall, a regression analysis demonstrated that whereas the GDI varied as a function of the herbicide concentration assayed in fish treated for 96 h, since a significant dose-dependent increase in the GDI was observed ($r = -0.4018$, $P < 0.01$), no association was observed in specimens exposed for 48 h ($r = 0.1215$, $P > 0.05$).

4. Discussion

C. decemmaculatus (Jenyns, 1842) (Pisces, Poeciliidae), the ten spotted live-bearer fish, attains high densities in a large variety of water bodies within the whole La Plata River and other South American basins [34]. Several reports found this species suitable as a test organism in acute and chronic toxicity bioassays [26,35,36]. Recently we used *C. decemmaculatus* as a laboratory-exposed target species to evaluate the lethal and sublethal toxicity of several agrochemical commercial formulations used most widely for cereal and leguminous crop production as well as in garden control, not only in Argentina but also worldwide. Among them, two 48% chlorpyrifos-based insecticides (Lorsban 48E® and CPF Zamba®) [28], two 50% pirimicarb-based insecticides (Aficida® and Patton Flow®) [25,27], and two 48% glyphosate-based herbicides (Panzer® and Credit®) [29] were included. In all cases, whereas LC_{50} estimation was employed as a biomarker for toxicity, induction of MNs and alterations in the erythrocyte/erythroblast frequencies were employed as biomarkers of genotoxicity and cytotoxicity, respectively. Furthermore, the induction of DNA single-strand breaks in peripheral blood cells of *C. decemmaculatus* exposed under laboratory conditions was also evaluated bythe SCGE assay to further characterize the genotoxic effects of the aforementioned pesticide technical formulations [30].

Regarding the acute lethal effects, the studied pesticide can be considered an herbicide that may cause long-term adverse effects on *C. decemmaculatus* in the aquatic environment (category IV, following the European Union directives) [47]. Furthermore, the acute lethality data of LC_{50} values reported here for herbicide contribute new information for this type of effect for *C. decemmaculatus*. The herbicide increased the frequency of MNs in peripheral erythrocytes of fish when exposed for 48 h to the highest dose assayed (1229 mg/L). On the other hand, regardless of the concentration of the herbicide assayed, no alteration in the frequency of MNs was observed in fish exposed for 96 h. Furthermore, the herbicide was able to induce other nuclear abnormalities, *i.e.*, BN cells and NT and LB nuclei, in fish only when exposed for 48 h. Besides, we observed that acute exposure to all dicamba concentrations within the 410–1229 mg/L range increased the frequencies of primary DNA lesions estimated by alkaline SCGE in those fish exposed both for 48 h and 96 h. Additionally, our data revealed that the SCGE assay was more sensitive than the MN test in detecting early DNA damage when the same herbicide concentrations were employed for fish exposure.

The variability in pesticide-induced toxicity to different fish species is a phenomenon known worldwide (for review, see [48] and references therein). If we compare the $LC_{50\,96\,h}$ values reported here for dicamba with those from the literature for several fish species, *C. decemmaculatus* can be considered the least sensitive fish reported so far. Previous dicamba-induced fish lethality studies revealed $LC_{50\,96\,h}$ values of 465 mg/L for the western mosquitofish (*Gambusia affinis*) [49], 180 mg/L for the sheepshead minnow (*Ciprinodon variegatus*) [50], 153 mg/L for the rainbow trout (*Oncorhynchus mykiss*) [50], and as low as 50 mg/L for the cutthrout trout (*Oncorhynchus clarkii*) [51]. Our results revealed a concentration of 1639 mg/L dicamba (range, 1471–1808) as the $LC_{50\,96\,h}$ value for *C. decemmaculatus*. Previously reported maximum $LC_{50\,96\,h}$ values for adult exposed specimens include 87.80 mg/L for $ZnCl_2$ [52], 21.40 mg/L for Cr(VI) [26], and 0.157 mg/L for $CuSO_4$ [53]. Similarly, pesticide-induced lethality analyses performed on *C. decemmaculatus* revealed $LC_{50\,96\,h}$ values of 91.73 mg/L and 15.68 mg/L for the glyphosate-based herbicides Credit® and Panzer®, respectively [29]; 67.40 mg/L for the paraquat-based herbicide Osaquat® [54]; 0.03 mg/L and 0.21 mg/L for the chlorpyrifos-based insecticides Lorsban 48E® and CPF Zamba® [28], respectively; and, finally, values of 225.0 mg/L and 88.00 mg/L for the pirimicarb-based insecticides Aficida® [25] and Patton Flow® [27], respectively. Accordingly, it seems evident that

the herbicide dicamba is the least toxic emerging pollutant reported so far for *C. decemmaculatus*. Furthermore, it could be valid to suggest that the species seems to be more sensitive to pesticides belonging to the phosphonoglycine, bipyridylium, organophosphate, and carbamate chemical groups than to chlorinated benzoic auxinic pesticides.

In our study, a dicamba-based herbicide containing only 57.71% of the active ingredient within the formulation, Banvel®, was assayed, an aspect that should be considered further. It is well known that in agriculture, pesticides are usually applied in their formulated forms, where the active ingredient is combined with organic solvents and emulsifying and wetting agents, which affect the pesticide penetration and performance [55]. The additives may synergize or antagonize the toxicity of the active ingredient. Although additive compounds frequently comprise part of a commercial pesticide formulation, they are not usually included in any discussion of the effects on living organisms, and their adverse effects may exceed those of the active ingredient. Although pesticides are developed through very strict regulation processes to function with reasonable certainty and minimal impact on human health and the environment, serious concerns have been raised about health risks resulting from occupational exposure and from residues in food and drinking water [55]. Several investigations have demonstrated that the additive compounds present in pesticide commercial formulations have the ability to induce toxicity and cellular damage by themselves, separate from the active ingredient [56–59]. Hence, risk assessment must also consider additional toxic effects caused by the excipient(s). Unfortunately, the identities of the additive compounds present in the commercial herbicide formulation Banvel® were not made available to us by the manufacturers. It should be mentioned that according to the 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." Years ago, the U.S. EPA [60] claimed that the acute toxicity of a technical active ingredient can differ significantly from that of the end-use formulation containing that active ingredient. Our results are in total agreement with this concept and pinpoint the necessity of further studies on *C. decemmaculatus* employing pure dicamba as test compound to reveal whether the observed jeopardizing effects we determined are specific to dicamba or result from the presence of xenobiotic(s) within the formulated technical formulation Banvel®. Previous reports on CHO-K1 cells *in vitro* employing, comparatively, the active ingredient dicamba as well as Banvel® demonstrated that although the dicamba-based commercial formulation mimics the genotoxic effect inflicted by dicamba, it contains within other xenobiotic agent(s) able to induce cytotoxicity and DNA damage by different mechanism(s) [21].

Genotoxicity in fish associated with pesticide exposure using the MN test in fish erythrocytes is well documented [23–25,27–29,31]. Furthermore, employing *C. decemmaculatus* as an experimental model, Vera-Candioti et al. [25,27–29] demonstrated the induction of MNs after exposure to glyphosate-based herbicides and chlorpyrifos- and pirimicarb-based insecticides. Accordingly, our current results represent the first evidence of the acute genotoxic effects exerted by the dicamba-based commercial herbicide formulation Banvel® on *C. decemmaculatus* under laboratory conditions. The results reveal that only increased frequency of MNs was observed in those fish exposed to the highest concentration (1229 mg/L) of dicamba for 48 h but not in fish exposed to the herbicide for 96 h, regardless of the concentration of herbicide assayed. So far, we do not have any exact explanation for this finding. It could be suggested that concentrations lower than 1229 mg/L dicamba are not sufficient enough to exert genotoxic/cytotoxic damage leading to MN formation. Furthermore, the possibility of induction of a selective cell loss by herbicide-induced cell death of the most damaged cells after treatment, leaving only a reduced proportion of undamaged cells capable of reaching the M1 status nearly after 96 h of exposure, cannot be ruled out. Whether the latter is true or not, the present results could confirm the importance of studying complete agrochemical formulations in toxicity screenings because the excipient(s) may have toxicological properties completely different from those of the active ingredients alone, and their impacts may be quite different, as demonstrated previously in different biotic matrices [57,58,61]. Finally, regardless of the aforementioned speculations, the present results highlight the genotoxic capability of the test compound under study and verify previous studies on vertebrate and plant cells when exposed to dicamba [14,19]. Finally, nuclear abnormalities other than MNs, *i.e.*, BNs and LB as well as BL nuclei, were also induced after the herbicide exposure for 48 h in those fish exposed within the 410–1229 mg/L concentration range. Previous reports demonstrated that these morphological nuclear abnormalities can be considered genetic damage indicators [22,62]. Our results agree well with these observations.

The SCGE test has become extensively valuable as a biomarker in fish to monitor contaminated areas (*in situ* assay) [63,64] as well as for screening xenobiotics after direct or indirect exposure (*in vivo* assay) [23,24,30]. We observed that, regardless of the length of treatment, dicamba acute exposure within the 410–1229 mg/L concentration range increased the frequency of primary DNA lesions estimated by alkaline SCGE, a result opposite that of the MN test. One possible explanation for this observation could be related to the different cellular status of the target cells that are included for analysis for each end point. Whereas DNA damage is estimated by SCGE in a heterogeneous circulating cell population, MNs are determined on proliferating cells with lesions that have lasted for at least one mitotic cell cycle and that probably retain their repair properties, as suggested elsewhere [42]. Furthermore, our current results represent the first *in vivo* evidence of the induction of primary DNA damage exerted by dicamba using the SCGE assay on piscine erythrocytes. Finally, our findings support the view that the SCGE assay is a highly sensitive method for the detection of DNA damage induced by environmental pollutants.

Although the *in vivo* dicamba treatments in this study covered a wide and high range of concentrations, the concentration range assayed represents a relatively high end of the threshold values of up to 13 μg/L and even 1×10^{-5} μg/L dicamba found in stream and ground waters, respectively (http://www.chem-online.org/agrochemical/dicamba.htm), even considering that the recommended application rates to foliage or soil range from approximately 50 to 320 g dicamba/ha [65], or as high as 1500 cm^3/hl reported for Argentina [66]. Thus, the concentrations of dicamba employed in this investigation would be expected to be almost improbable in the environment, perhaps only observed when specific events or circumstances occurred (*e.g.*, direct application, in drainage ditches, or by accidental discharge). Although, we cannot rule out that piscine populations, and also occupationally exposed human workers, could be exposed accidentally to these agrochemicals at this range of concentration.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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