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Toxic and genotoxic effects of the 2,4-dichlorophenoxyacetic acid (2,4-D)-based herbicide on the Neotropical fish *Cnesterodon decemmaculatus*

C. Ruiz de Arcaute [a,b], S. Soloneski [a,b], M.L. Larramendy [a,b,*]

[a] Cátedra de Citología, Facultad de Ciencias Naturales y Museo, Universidad Nacional de La Plata, Calle 64 Nº 3, B1904AMA, La Plata, Argentina
[b] Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina

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ABSTRACT

Acute toxicity and genotoxicity of the 54.8% 2,4-D-based commercial herbicide DMA® were assayed on *Cnesterodon decemmaculatus* (Pisces, Poeciliidae). Whereas lethal effect was used as the end point for mortality, frequency of micronuclei (MNs), other nuclear abnormalities and primary DNA damage evaluated by the single cell gel electrophoresis (SCGE) assay were employed as end points for genotoxicity. Mortality studies demonstrated an $LC_{50\ 96\ h}$ value of 1008 mg/L (range, 929–1070) of 2,4-D. Behavioral changes, e.g., gathering at the bottom of the aquarium, slowness in motion, slow reaction and abnormal swimming were observed. Exposure to 2,4-D within the 252–756 mg/L range increased the frequency of MNs in fish exposed for both 48 and 96 h. Whereas blebbed nuclei were induced in treatments lasting for 48 and 96 h, notched nuclei were only induced in fish exposed for 96 h. Regardless of both concentration and exposure time, 2,4-D did not induce lobed nuclei and binucleated erythrocytes. In addition, we found that exposure to 2,4-D within the 252–756 mg/L range increased the genetic damage index in treatments lasting for either 48 and 96 h. The results represent the first experimental evidence of the lethal and several sublethal effects, including behavioral alterations and two genotoxic properties namely the induction of MNs and primary DNA strand breaks, exerted by 2,4-D on an endemic organism as *C. decemmaculatus*.

1. Introduction

It has been estimated that less than 0.1 percent of pesticides applied worldwide into crops reaches their specific targets, leaving large amount of toxic residues free to move into different environmental compartments (Pimentel et al., 1993; WHO, 1990, 2009). Pesticides are able to contaminate soil and air, as well as surface and ground water, affecting also non-target organisms such as aquatic biota, plants, mammals and soil microorganisms, among others (Liang et al., 2013; Meffe and de Bustamante, 2014). Living species are inevitably exposed to pesticides, including agrochemicals, and they represent both a significant ecological and public health concern. Furthermore, pesticides are hazardous to the environment due to their persistence, bioaccumulation and toxicity. Unfortunately, it is difficult to decrease the use of these agrochemicals without reducing crop yields (McLaughlin and Kinzelbach, 2015). In this sense, according to FAO (2006), the global agricultural production is growing and it must increase more than 70% by 2050.

2,4-Dichlorophenoxyacetic acid, also commonly known as 2,4-D, is a widely employed pre- and post-emergence systemic herbicide belonging to the phenoxyalkanoic acid (or phenoxyacetic) family of herbicides. It was the first synthetic herbicide to be commercially developed and has commonly been employed worldwide for almost 70 years for the selective control of broad-leaf weeds and plants in agriculture, forestry, right-of-way (*e.g.*, roadside, rail track, and power line), lawn/turf, and aquatic weed control (www.pan-uk.org/pestnews/Actives/24d_pn65.htm). Whereas at low concentrations the main function of 2,4-D is to mimic natural auxins to promote cell division and elongation, at high concentrations functions as a herbicide controlling broad-leaf growth. It selectively kills dicots without affecting monocots mimicking natural auxin at the molecular level (Grossmann, 2003). Physiological responses of dicots sensitive to auxinic

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

* Corresponding author at: Cátedra de Citología, Facultad de Ciencias Naturales y Museo, Universidad Nacional de La Plata, Calle 64 Nro. 3 (esq. 120), B1904AMA, La Plata, Argentina.
E-mail address: marcelo.larramendy@gmail.com (M.L. Larramendy).

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herbicides include abnormal growth, senescence, and plant death (Grossmann, 2003). The identification of auxin receptors, auxin transport carriers, transcription factors response to auxin, and cross-talk among phytohormones provided new insights on the molecular action mode of 2,4-D as a herbicide (Song, 2014). However, the underlying molecular mechanism of how auxinic herbicides selectively kill dicots and spare monocots is not understood yet (Song, 2014). 2,4-D has been classified, on the basis of its acute toxicity, as a class II member (moderately hazardous) by WHO (http://www.who.int/ipcs/publications/pesticideshazard/en/) and slightly to moderately toxic (category II–III) by U.S. EPA (1974). Furthermore, based on mechanistic studies, the International Agency for Research on Cancer (IARC) classified 2,4-D as a possibly carcinogenic agent to humans (Group 2B) (IARC, 2015).

Fish, an integral part of aquatic biota are organisms sensitive to any change in the environment. Several studies have demonstrated that these vertebrates can be used as valid indicator species for environmental monitoring (Annabi et al., 2015; Benitez et al., 2014; Greene et al., 2015; Schlenk et al., 2012). The capability of fish to efficiently metabolize and accumulate chemical pollutants is well documented (Cavalcante et al., 2008; Lazartigues et al., 2013; Zhao et al., 2014). In recent decades, fish populations have been reported to suffer a significant decline worldwide, a phenomenon in most cases committed to pollution of both natural and agricultural areas with the use of pesticides. Although environmental pollution might interfere with normal fish growth, development and susceptibility to disease, the induction of genetic damage into DNA after acute and chronic exposure to agrochemicals is ended the most relevant jeopardizing effect (Benitez et al., 2014; King et al., 2013; Ogada, 2014; Schlenk et al., 2012; Tierney et al., 2010). Furthermore, a positive correlation between the decline of fish population and the use of agrochemicals has been reported (Ogada, 2014; Tierney et al., 2010).

Fish represent important aquatic vertebrates as reliable environmental lethality, cytotoxicity, and genotoxicity bioindicator organisms due to both their role in the trophic chain and their sensitivity to low concentrations of emerging pollutants (McKenzie et al., 2007). The micronucleus (MN) as well as the single cell gel electrophoresis (SCGE) end points, due to their sensitivity, reliability, and the simplicity of use, has become widely used in piscine erythrocytes to assess the genotoxicity of many xenobiotics, including pesticides. Furthermore, genotoxicity in fish associated with pesticide exposure analyzed using both the MN and the SCGE bioassays in peripheral circulating erythrocytes is well documented (Ali et al., 2009; Cavalcante et al., 2008; Cavaş, 2011; Cavaş and Könen, 2008; Ruiz de Arcaute et al., 2014; Vera-Candioti et al., 2010, 2011, 2013a, 2013b, 2013c, 2015).

Cnesterodon decemmaculatus (Jenyns, 1842) (Pisces, Poeciliidae) is an endemic member of the fish family Poeciliidae with an extensive distribution in Neotropical America attaining high densities in a large variety of water bodies within the whole La Plata River and other South American basins. The 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 (Menni et al., 1996).

Previous studies have highlighted that *C. decemmaculatus* can be considered suitable test aquatic vertebrate model in the risk assessment of lethal and sublethal effects exerted by several agrochemicals. Among them, the insecticides pirimicarb (Vera-Candioti et al., 2010, 2013c, 2015), endosulfan (Mugni et al., 2012b), cypermethrin (Carriquiriborde et al., 2007; Mugni et al., 2012b), chlorpyrifos (Mugni et al., 2012a, 2012b; Vera-Candioti et al., 2013a, 2013c) and paraquat (Di Marzio et al., 1998; Di Marzio and Tortorelli, 1994) as well as the herbicides glyphosate (Carriquiriborde et al., 2007; Menendez-Helman et al., 2012; Vera-Candioti et al., 2013b, 2013c), and dicamba (Ruiz de Arcaute et al., 2014) are included. To the best of our knowledge, no further reports have been published analyzing the relationship between other pesticide exposure and risk assessment in the species, particularly 2,4-D exposition.

The aim of the present study is to characterize the acute toxicity of the 2,4-D-based herbicide formulation DMA® (54.8% 2,4-D) on the native molly *C. decemmaculatus* exposed under laboratory conditions using a static acute experimental method. A lethal effect was used as the end point for mortality, whereas frequency of MNs and other nuclear abnormalities as well as DNA single-strand breaks evaluated by the SCGE were employed as end points for genotoxicity. Our current results represent the first experimental evidence of the lethal and several sublethal effects, including behavioral alterations and two genotoxic properties namely the induction of MNs and primary DNA strand breaks, exerted by the auxinic herbicide 2,4-D on an endemic organism as *C. decemmaculatus*.

2. Materials and methods

2.1. Chemicals

2,4-D (2,4-dichlorophenoxyacetic acid, CAS 94-75-7) commercial-grade trade formulation DMA® (58.4% 2,4-D) was kindly provided by Dow AgroSciences Argentina S.A. (Buenos Aires, Argentina). $K_2Cr_2O_7$ [$Cr_{(VI)}$] (CAS 7778-50-9) was obtained from Merck KGaA (Darmstadt, Germany) whereas cyclophosphamide (CP, CAS 6055-19-2). All other chemicals and solvents of analytical grade were purchased from Sigma-Aldrich Co.

2.2. Quality control

Determination of the concentration levels of 2,4-D in the test solutions was performed by QV Chem Laboratory (La Plata, Buenos Aires, Argentina) according to U.S. Geological Survey Report 01-4134 (Furlong et al., 2011). 2,4-D levels were analyzed by high performance liquid chromatography using an ultraviolet detector. Active ingredient samples from test solution (100, 250 and 400 mg/L) correspond to values obtained immediately after preparation (0 h) and 24 h thereafter. The detection limit for 2,4-D was 0.5 mg/L. Concentrations assessed throughout the study represent the nominal concentrations of active ingredient present within the 2,4-D-based formulation DMA®.

2.3. Test organisms

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. Individuals were cared according to SENASA (Argentinean National Service for Sanitary and Quality of Agriculture and Food) guidelines 617/2002 for biological testing (SENASA, 2013).

2.4. Determination of LC_{50}

Bioassays were carried out for toxicity assessment following recommendations proposed by the U.S. EPA standardized methods

for acute piscine toxicity testing (IRAM, 2008; USEPA, 1975, 2002) with minor modifications reported previously (Ruiz de Arcaute et al., 2014; Vera-Candioti et al., 2013c). The average body weight of the specimens used throughout the experiments was 0.20 ± 0.1 g, and the mean body length was 25.5 ± 2.2 mm. For each experimental point, 10 randomly selected specimens were maintained in a 1 L glass container according recommendations reported elsewhere (Mugni et al., 2016; Ruiz de Arcaute et al., 2014; Vera-Candioti et al., 2010, 2013a, 2013b, 2013c, 2015). To determine the concentrations used in the acute toxicity tests, preliminary assays were performed according the recommendations proposed by the U.S. USEPA (2002). Then, test organisms were exposed to ten different concentrations of 2,4-D (100, 250, 400, 800, 1100, 1200, 1300, 1400, 1600 and 2200 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) (Vera-Candioti et al., 2013c) were conducted and run simultaneously with DMA®-exposed fish. All test solutions were prepared immediately before use and replaced completely every 24 h. Fish were not fed throughout the experiment. Experiments were performed in triplicate and run simultaneously for each experimental point. 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

Behavioral changes of the fish subjected to various concentrations of DMA® and those of non-exposed fish were monitored and registered every 24 h before the application of DMA® test solutions in those fish employed for determining LC_{50} values (Section 2.4). Changes were registered after gently swirling the water five times with a glass rod and observing for 1 min the swimming activity of each organism. Optomotor responses were classified following the criteria reported elsewhere (Sarikaya and Yilmaz, 2003; Yilmaz et al., 2004). Briefly, responses were classified as slow motion (SM), when the motility of fish become extremely slow; gathering at the bottom of the aquarium (GBA), when individuals congregate in the lower part of the water column; slow reaction (SR), when the response to stimulus decay; and abnormal swimming (AS), when the swimming ability of the individuals was decreased or erratic. The prevalence of a type of abnormality was calculated by dividing the number of individuals with the particular abnormality by the number of individuals examined.

2.5.2. Micronuclei and other erythrocytic nuclear abnormalities

MN assay was performed on peripheral circulating blood erythrocytes according to the procedure described previously (Ruiz de Arcaute et al., 2014; Vera-Candioti et al., 2013c). Experiments were performed following the same experimental conditions described in Section 2.4. For each experimental point, 5 randomly selected specimens were maintained in a 1 L glass container and exposed to three different concentrations of DMA® equivalent to 25%, 50%, and 75% of the corresponding LC50 96 h value. To achieve these concentrations, specimens were exposed to 252, 504, and 756 mg/L of 2,4-D, respectively. Negative (dechlorinated tap water, see Section 2.4) and positive (10 mg/L CP) controls were conducted and run simultaneously with DMA®-exposed fish. Experiments were performed in triplicate and run simultaneously for each experimental point. 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 following recommendations reported elsewhere for piscine micronuclei bioassay (Cavaş and Ergene-Gözükara, 2005; de Lemos et al., 2008; Ruiz de Arcaute et al., 2014; Vera-Candioti et al., 2010, 2013a, 2013b). At the end of each experiment, hypothermia, a non-chemical anesthetic method was performed by immersion of fish in ice water (Ackerman et al., 2005; Summerfelt and Smith, 1990), and then, blood samples were obtained by sectioning behind the operculum according recommendations reported elsewhere (Ruiz de Arcaute et al., 2014; Vera-Candioti et al., 2010, 2013a, 2013b, 2013c, 2015). 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 total number of MNs per 1,000 cells, as suggested previously (Vera-Candioti et al., 2010). MN frequency was determined following the examination criteria reported previously (Fenech, 2007; Vera-Candioti et al., 2010). 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 (Cavaş and Ergene-Gözükara, 2003; Nikoloff et al., 2014; Strunjak-Perovic et al., 2009). 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 2.5.2) were also employed for SCGE assay. The SCGE assay was performed following the alkaline procedure described by Singh (1996) with minor modifications reported elsewhere (Nikoloff et al., 2014; Ruiz de Arcaute et al., 2014). 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 nucleoids. DNA damage was

classified in five classes (0–I, undamaged; II, minimum damage; III, medium damage; IV, maximum damage), as suggested previously (Cavaş and Könen, 2007). Data are expressed as the mean number of damaged nuceoids (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. (1999) using the formula GDI = [1(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 (1971) method. The results were analyzed using the Statistica 7.0. software. The proportion of individuals affected per test chamber (n=10) was calculated for lethal and sublethal end points (mortality, behavior, MNs, BNs, BLs, LBs, NTs, damaged nucleoids 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

3.1. Chemical analysis

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).

3.2. Mortality

Probit analysis of the mortality data allowed determination of the LC_{50} values of 2,4-D present within the formulation DMA® after 24, 48, 72, and 96 h of exposure. Results revealed mean values of $LC_{50\ 24\ h}$=1647 mg/L (range, 1556–1777), $LC_{50\ 48\ h}$ = 1247 mg/L (range, 1192–1295), $LC_{50\ 72\ h}$=1081 mg/L (range, 1007–1142), and $LC_{50\ 96\ h}$=1008 mg/L (range, 929–1070). As revealed by regression analysis, a trend to LC_{50} values to be dependent of the exposure time was observed although not reaching statistical significance ($r=-0.94$, $P>0.05$).

3.3. Behavioral changes

The behavioral changes observed in those *C. decemmaculatus* exposed to different concentrations of 2,4-D present within the formulation DMA® were GBA, SM, AS, and SR ($P<0.01$–$P<0.001$). NOEC and LOEC values for all observed changes are summarized in Table 1. GBA was observed only in fish after 24 h of exposure ($P<0.01$, F=5.92) with NOEC and LOEC values of 400 and 800 mg/L 2,4-D, respectively. With similar NOEC and LOEC values of 400 and 800 mg/L 2,4-D, SM was detected in fish after 24 ($P<0.01$, F=4.52) and 48 h ($P<0.001$, F=11.19) with of exposure, respectively. SR was observed in specimens exposed either during 24 ($P<0.001$, F=11.84), 48 ($P<0.01$, F=5.00) as well 72 h ($P<0.001$, F=9.04) to the herbicide. Whereas NOEC and LOEC values were 400 and 800 mg/L 2,4-D for 24 and 48 h treatments, respectively, 800 and 1100 mg/L 2,4-D were determined as the a NOEC and LOEC values for 72 h-exposed fish, respectively. Finally, the only abnormality observed throughout the experiment was AS ($P<0.01$–$P<0.001$; F=5.63, F=15.84, F=11.88, and F=9.29, for 24, 48, 72, and 96 h, respectively) with NOEC and LOEC values of 400 and 800 mg/L 2,4-D for all experimental points (Table 1).

Table 1
Behavioral effects of *Cnesterodon decemmaculatus* exposed to the 2,4-D-based formulation DMA®[a].

Exposure time (h)	Effect	NOEC		LOEC	
		No. of animals analyzed	Value	No. of animals analyzed	Value
24	GBA	30	400 mg/L	27	800 mg/L**
	SM	30	400 mg/L	27	800 mg/L**
	SR	30	400 mg/L	27	800 mg/L***
	AS	30	400 mg/L	27	800 mg/L**
48	GBA		–		–
	SM	30	400 mg/L	26	800 mg/L***
	SR	30	400 mg/L	26	800 mg/L**
	AS	30	400 mg/L	26	800 mg/L***
72	GBA	–	–	–	–
	SM	–	–	–	–
	SR	25	800 mg/L	18	1100 mg/L***
	AS	30	400 mg/L	25	800 mg/L***
96	GBA	–	–	–	–
	SM	–	–	–	–
	SR	–	–	–	–
	AS	30	400 mg/L	23	800 mg/L**

GBA, gathering at the bottom of the aquarium; SM, slow motion; SR, slow reaction; AS, abnormal swimming.

** $P<0.01$; significant differences with respect to control values.

*** $P<0.001$; significant differences with respect to control values.

[a] Results are expressed in mg/L.

3.4. MN and other erythrocytic nuclear abnormality frequencies

Table 2 and Fig. 1 show the results of the analysis of MNs in peripheral blood erythrocytes of *C. decemmaculatus* induced by the 2,4-D-based herbicide formulation DMA®. Results revealed an increase in the frequency of MNs in fish exposed to CP (positive control) at 48 h ($P<0.05$, $H=15.69$), but not in fish exposed for 96 h ($P>0.05$). After 48 h, a significant increase in the frequency of MNs was found in fish exposed to 252, 504 and 756 mg/L 2,4-D-treated individuals with respect to negative control values ($P<0.05$, $P<0.01$ and $P<0.001$, respectively). Additionally, a significant increase in the frequency of MNs was observed at 96 h of treatment for all tested concentrations of 2,4-D compared to negative controls ($P<0.001$, F=11.5) (Table 2). Overall, a regression analysis revealed that the increase in MN frequency was not affected by 2,4-D concentrations neither at 48 h ($r=-0.24$, $P>0.05$) nor at 96 h of exposure ($r=-0.05$, $P>0.05$).

When other nuclear abnormalities rather than MNs were analyzed, an increase in the frequency of NTs was observed only in individuals exposed to 504 mg/L for 96 h ($P<0.01$). In addition, an increase in the frequency of BLs was observed after 48 h only in fish exposed to 756 mg/L 2,4-D ($P<0.05$, F=3.3) and after 96 h in fish exposed to 504 mg/L 2,4-D ($P<0.05$, F=3.3). 2,4-D

Table 2
Frequencies (‰) of MNs and other nuclear abnormalities in peripheral blood erythrocytes of *Cnesterodon decemmaculatus* exposed to 2,4-D-based formulation DMA®[a].

Exposure time (h)	Concentration (mg/L)	No. of cells analyzed	MNs	Other Nuclear Abnormalities			
				NTs	LBs	BNs	BLs
48	Negative control	22814	0.09 ± 0.06	3.78 ± 0.95	0.00 ± 0.00	0.00 ± 0.00	1.01 ± 0.24
	Positive control[b]	22595	0.53 ± 0.11*	4.74 ± 0.78	0.09 ± 0.06	0.00 ± 0.00	3.94 ± 0.60**
	252	22571	0.58 ± 0.15*	3.15 ± 0.71	0.09 ± 0.06	0.04 ± 0.04	0.93 ± 0.25
	504	22189	0.94 ± 0.26**	4.55 ± 1.15	0.09 ± 0.09	0.00 ± 0.00	1.27 ± 0.38
	756	22568	1.06 ± 0.21***	3.06 ± 0.85	0.00 ± 0.00	0.09 ± 0.08	3.77 ± 1.19*
96	Negative control	22601	0.13 ± 0.07	2.12 ± 0.65	0.04 ± 0.04	0.00 ± 0.00	1.33 ± 0.42
	Positive control[b]	22921	0.43 ± 0.16	8.88 ± 2.13	0.44 ± 0.16	0.09 ± 0.08	4.29 ± 1.09**
	252	22586	1.06 ± 0.23***	3.54 ± 0.73	0.00 ± 0.00	0.00 ± 0.00	2.08 ± 0.49
	504	22621	1.46 ± 0.25***	5.43 ± 0.82**	0.66 ± 0.47	0.09 ± 0.08	3.48 ± 0.82*
	756	21130	1.37 ± 0.29***	2.94 ± 0.47	0.14 ± 0.10	0.05 ± 0.04	1.14 ± 0.34

MNs, micronuclei; NTs, notched nuclei; LBs, lobed nuclei; BNs, binucleated nuclei; BLs, blebbed nuclei.
The number of animals analyzed was 15 for all concentrations in both 48 and 96 h exposure time, but 14 in 756 mg/L 96 h concentration.

[a] Results are expressed as mean number of abnormalities/1000 cells ± SE.
[b] Cyclophosphamide (10 mg/L) was used as positive control.
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$; significant differences with respect to control values.

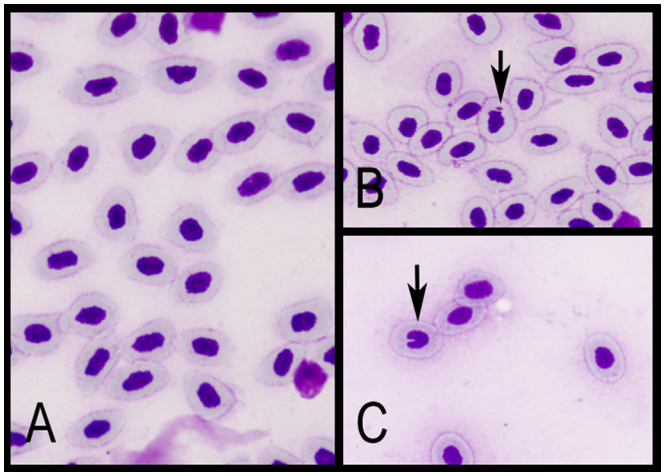

Fig. 1. Photomicrographs from blood smears of *C. decemmaculatus* showing erythrocytes with normal nucleus (A), an erythrocyte with a micronucleus (B, arrow) and an erythrocyte depicting a notched nucleus (C, arrow). Cells were stained with 5% Giemsa and viewed at 1000 times magnification. Cells are approximately 10 μm along the long axis.

treatments, regardless of both concentration and exposure time, did not modify the frequencies of LBs and BNs in regard to negative control values ($P > 0.05$) (Table 2).

3.5. DNA damage

Table 3 and Fig. 2 show the results of the SCGE assay obtained for *C. decemmaculatus* after 2,4-D DMA® exposure. CP treatment (positive control) induced an enhancement of the GDI and in the frequency of damaged nucleoids compared to 48 and 96 h negative controls ($P < 0.001$; F=45.35 for 48 and F=73.74 for 96 h, respectively) (Table 3, Fig. 2). In herbicide-exposed fish, a significant increase of the GDI was observed in all treatments (252, 504, and 756 mg/L 2,4-D) lasting for both 48 and 96 h ($P < 0.001$; F=55.56 for 48 and F=91.56 for 96 h, respectively) (Table 3). In specimens exposed for 48 h, such alteration was due to an enhanced frequency of type II and III nucleoids ($P < 0.001$; F=44.42 for nucleoids type II and F=30.20 for nucleoids type III, respectively). In addition, an increased frequency of type IV nucleoids in fish treated with 504 mg/L for 48 h was observed ($P < 0.01$; F=5.45) (Fig. 2A). When the analysis was performed in fish exposed for 96 h, the alteration in the GDI was due to an increase in the frequency of type II, III and IV nucleoids for all treatments performed ($P < 0.001$; F=76.35, F=60.83 and H=24.13 for nucleoids type II, III and IV, respectively) (Fig. 2B). Overall, a regression analysis demonstrated that the GDI varied as a dependent function of 2,4-D concentration in fish treated for 48 h ($r = -0.42$, $P < 0.01$), but not in fish exposed for 96 h ($r = 0.14$, $P > 0.05$).

Table 3
Analysis of DNA damage measured by comet assay in *Cnesterodon decemmaculatus* cells exposed to the 2,4-D-based formulation DMA®.

Chemicals	Concentration (mg/L)	Exposure time (h)	No. of nucleoids analyzed	% of damaged nucleoids (II+III+IV)	GDI ± SE[a]
Negative control		48	1547	11.25	0.69 ± 0.09
		96	1601	6.75	0.60 ± 0.05
DMA®	256	48	1639	58.08***	1.57 ± 0.08***
		96	1575	84.70***	2.26 ± 0.12***
	504	48	1414	74.40***	2.29 ± 0.09***
		96	1704	82.22***	2.00 ± 0.10***
	756	48	1728	78.59***	1.85 ± 0.10***
		96	1584	80.30***	2.13 ± 0.07***
Positive control[b]	10	48	1633	58.67***	1.83 ± 0.09***
		96	1463	75.60***	2.26 ± 0.08***

Number of animals analyzed: 15 for all concentrations in both 48 and 96 h exposure time.

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

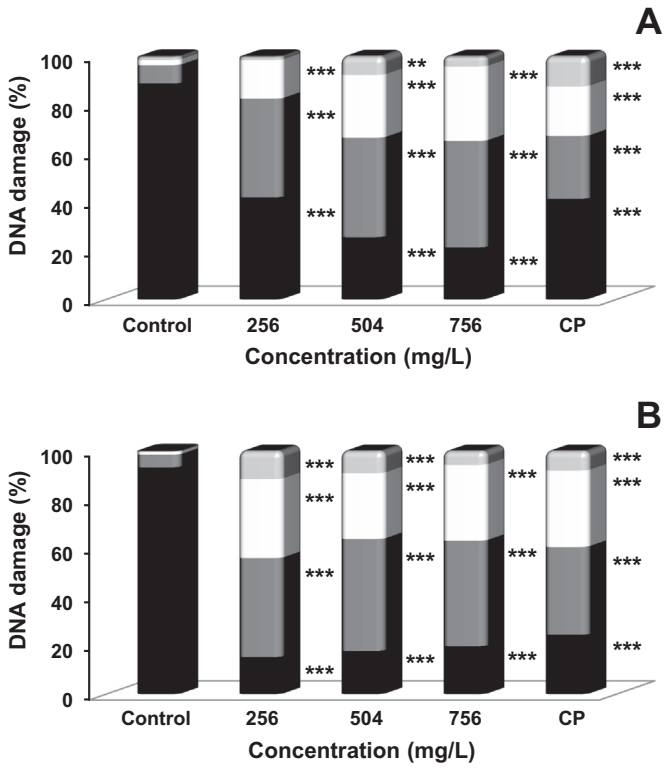

Fig. 2. The 2,4-D based herbicide formulation DMA®-induced DNA damage measured by single cell gel electrophoresis assay in circulating blood cells from *Cnesterodon decemmaculatus* (Pisces, Poeciliidae) exposed for 48 (A) and 96 h (B). The frequencies of undamaged (type 0-I nucleoids; black bar), type II (dark gray bar), type III (white bar) and type IV (light 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 DMA®-exposed fish. **, $P<0.01$; ***, $P<0.001$; significant differences with respect to control values.

4. Discussion

Taking into account the acute lethal effect of the pesticide on the species, the 2,4-D tested herbicide could be ranked, according the scoring used by the Office of Pollution Prevention and Toxics of the U.S. EPA (2001), as a compound with low ecotoxicity concern for the ten spotted live-bearer fish *C. decemmaculatus*. Besides, 2,4-D can be classified as harmful compounds for aquatic organisms (category III) following the classification criteria proposed by the United Nations directives (2011). Finally, 2,4-D can be considered as compound that may cause long lasting harmful effects to aquatic life, according to the hazard risk assessment categories of the European Union directives (Mazzatorta et al., 2002).

Our results reveal a concentration of 1008 mg/L 2,4-D (confidence limits, 95%, 929–1070) as the $LC_{50\ 96\ h}$ value for *C. decemmaculatus*. Among pesticides, previously maximum $LC_{50\ 96\ h}$ values reported for the species comprises 1639 mg/L for the dicamba-based herbicide formulation Banvel® (Ruiz de Arcaute et al., 2014), 225.5 mg/L for the pirimicarb-based insecticide formulation Aficida® (Vera-Candioti et al., 2010), 100 mg/L for the herbicide glyphosate (a.i.) (Carriquiriborde et al., 2007), 91.73 mg/L for the glyphosate-based herbicide formulation Credit® (Vera-Candioti et al., 2013b), 88 mg/L for the pirimicarb-based insecticide formulation Patton Flow® (Vera-Candioti et al., 2015), 67.4 mg/L for the paraquat-based insecticide formulation Osaquat® (Di Marzio et al., 1998), 15.68 mg/L for the glyphosate-based herbicide formulation Panzer® (Vera-Candioti et al., 2013b) and values as low as 0.43 mg/L for the insecticide cypermethrin (a.i.) (Carriquiriborde et al., 2007), 0.21 and 0.03 mg/L mg/L for the chlorpyrifos-based insecticide formulations Chorpyrifos Zamba® and Lorsban*48E®, respectively (Vera-Candioti et al., 2013a) as well as 0.005 mg/L for the endosulfan-based insecticide formulation Brometan® (Mugni et al., 2012b). Accordingly, this guppy is nearly 0.6 times more sensitive to 2,4-D than dicamba, the least toxic agrochemical reported so far for the species but more sensitive to any other pesticide assayed so far on the species. Finally, and in agreement with our previous observations (Ruiz de Arcaute et al., 2014), it could be suggested that auxinic herbicides (regardless whether they belong to the benzoic, *e.g.*, dicamba, or to the phenoxyalkanoic, *e.g.*, 2,4-D, acid groups are less toxic compounds to *C. decemmaculatus* than any other agrochemical analyzed so far. Further toxicity studies are required on the target species employing the remaining two members of the auxinic herbicide family of herbicides, *i.e.*, quinolinecarboxylic and pyrimidinecarboxylic acids, to verify this hypothesis.

Recently, the International Agency for Research on Cancer (2015) evaluated the risk exposure of some organochlorine insecticides and some chlorophenoxy herbicides, i.e., DDT, lindane and 2,4-D. As a result, the genotoxic potential of 2,4-D has been strongly endorsed and confirmed by diverse *in vivo* and *in vitro* assays including the ability of induce chromosomal and DNA damage in different biotic matrices (for review see IARC (2015), Soloneski and Larramendy (2011), and references therein). In agreement, the results of the current study demonstrate that 2,4-D can be considered a deleterious agent with genotoxic effects at both chromosomal and DNA levels for *C. decemmaculatus*. Additionally, our observations revealed that SCGE assay was as sensitive than the MN test in detecting early DNA damage when the same 2,4-D concentrations were assayed for fish exposure. To the best of our knowledge, among them the walking catfish *Clarias batrachus* (Siluriformes, Clariidae) (Ateeq et al., 2002), the air-breathing spotted snakehead *Channa punctata* (Perciformes, Channidae) (Farah et al., 2003, 2006) and the mollie *Poecilia vivípara* (Cyprinodontiformes, Poeciliidae) (Vigário and Sabóia-Morais, 2014) can be included. Finally, our current results demonstrate that other nuclear abnormalities rather than MNs were also induced after 2,4-D exposure, regardless of the exposure time. It has been previously reported that these morphological nuclear abnormalities, *e.g.*, blebbed and notched nuclei, can be considered indicators of genetic instability (Cavaş and Ergene-Gözükara, 2005; Gökalp Muranli and Güner, 2011). Our results accord well with this assumption since such nuclear abnormalities were only found as a consequence of exposure to 2,4-D as well as cyclophosphamide, an antineoplastic agent employed as positive control.

Very little is known in relation with 2,4-D-induced primary DNA lesions in fish cells. Positive induction of DNA breaks evaluated with the comet assay have been reported in the catfish *C. batrachus* (Ateeq et al., 2005) and in the rainbow trout *Oncorhynchus mikiss* (Salmoniformes, Salmonidae) (Martínez-Tabche et al., 2004) after 2,4-D exposure. Similar results have been also observed after *in vitro* treatment of Epithelioma Papillosum Cyprini (EPC) cells, a fish-derived cell line derived from the common carp *Cyprinus carpio* (Cypriniformes, Cyprinidae) (Bokán et al., 2013). Genotoxicity associated with pesticide exposure using the SCGE test in piscine cells is well documented (Cavaş, 2011, 2013; De Castilhos Ghisi and Cestari, 2013; Gholami-Seyedkolaei et al., 2013; Mohanty et al., 2011, 2013; Ruiz de Arcaute et al., 2014; Selvi et al., 2013; Vera-Candioti et al., 2013c). Accordingly, our current results constitute the first experimental evidence of the genotoxic effect at the DNA level exerted by 2,4-D on the Neotropical fish *C. decemmaculatus* exposed under laboratory conditions.

It is known that most of the pesticides in aquatic environments exert their side effects through genotoxic and metabolically toxic

mechanisms triggering, simultaneously, genotoxic damage, disease, and even carcinogenesis (Könen and Cavaş, 2008; Ruiz de Arcaute et al., 2014; Vera-Candioti et al., 2010, 2013a, 2013b, 2013c, 2015). 2,4-D can be introduced into the environment through effluents and spills resulting from its manufacture and transport or directly through its application as a herbicide agent. The herbicide does not bioaccumulate in aquatic or terrestrial organisms but some algae because of its rapid degradation (WHO, 2003). Residues of 2,4-D can enter ponds and streams by several manners including direct application or accidental drift, inflow of herbicide previously deposited in dry streambeds, pond bottoms or irrigation channels, runoff from soils as well as by leaching through the soil column (Norris, 1981). Groundwater contribution of 2,4-D residues into ponds and streams is dependent upon soil type, with coarse-grained sandy soils with low organic content expected to leach 2,4-D into groundwater (Ghassemi et al., 1981; Norris, 1981). Therefore, 2,4-D, whether inefficiently diluted or degraded after applied in the soil, may reach concentration levels that could pose genotoxic effects in aquatic biota. To the best of our knowledge, no data regarding to the concentration level of the herbicide in different environments have been reported for Argentina. Information on concentrations of 2,4-D in environmental compartments will be necessary to complete exposure profiles to be included in future risk assessments studies when employing a native fish as *C. decemmaculatus* as experimental model.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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