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# Genotoxicity by long-term exposure to the auxinic herbicides 2,4-dichlorophenoxyacetic acid and dicamba on *Cnesterodon decemmaculatus* (Pisces: Poeciliidae)☆

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ABSTRACT

Long-term genotoxic effects of two auxinic herbicide formulations, namely, the 58.4% 2,4-dichlorophenoxyacetic acid (2,4-D)-based DMA® and the 57.7% dicamba (DIC)-based Banvel® were evaluated on *Cnesterodon decemmaculatus*. Primary DNA lesions were analyzed by the single-cell gel electrophoresis methodology. Two sublethal concentrations were tested for each herbicide corresponding to 2.5% and 5% of the $LC50_{96h}$ values. Accordingly, fish were exposed to 25.2 and 50.4 mg/L or 41 and 82 mg/L for 2,4-D and DIC, respectively. Fish were continuously exposed for 28 days with replacement of test solutions every 3 days. Genotoxicity was evaluated in ten individuals from each experimental point at the beginning of the exposure period (0 day) and at 7, 14, 21 and 28 days thereafter. Results demonstrated for first time that 2,4-D-based formulation DMA® induced primary DNA strand breaks after 7–28 days exposure on *C. decemmaculatus* regardless its concentration. On the other hand, DIC-based formulation Banvel® exerted its genotoxic effect after exposure during 7–14 days and 7 days of 2.5 and 5% $LC50_{96h}$, respectively. The present study represents the first evidence of primary DNA lesions induced by two widely employed auxinic herbicides on *C. decemmaculatus*, namely 2,4-D and DIC, following long-term exposure.



## 1. Introduction

The increase in pollution due to the complexity of anthropized environments makes the analysis of aquatic environments a continuous challenge. The presence of pesticides and their detrimental consequences on aquatic biota has been largely documented (Larramendy, 2017). According to the Food and Agriculture Organization (FAO) of the United Nations, the worldwide production of formulated pesticides has been increasing continuously since the 1950's, where the herbicides were the chemical group that expanded the most, followed by insecticides and fungicides (FAO, 2017).

Auxinic compounds were the first selective herbicides developed. They are among the most employed pesticides, either alone or in combination with other active ingredients, in commercial formulations. These herbicides mimic the properties of natural phytohormones. Among them, indole-3-acetic acid constitutes the most common class of auxins (USEPA, 2006). At low concentrations, these phytochemicals stimulate cell division and growth, whereas at higher concentrations they cause several growth abnormalities such as leaf epinasty, stem curvature, growth inhibition of root and shoot, foliar senescence and chloroplast damage with progressive chloroplast chlorosis, leading, consequently, to plant death (Grossmann, 2010). In spite of continuing to be an important group of agrochemicals, their molecular mechanism is not yet well characterized. In plants, these chemicals emulate the action of auxins mainly through manipulating the plant phytohormones responses (Gleason et al., 2011). However, no such mimicking of hormonal action has been reported in other living species (Osterloh et al., 1983).

Among the synthetic plant-growth regulators, 2,4-dichlorophenoxyacetic acid (2,4-D) and dicamba (DIC; 3,6-dichloro-2-methoxybenzoic acid) are two of the most applied herbicides worldwide, falling within the top ten agrochemicals in

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Argentina (CASAFE, 2017). 2,4-D belongs to the phenoxy alkanoic acid group of auxinic herbicides, whereas DIC is included in the benzoic acid chemical family (USEPA, 2005, 2006).

2,4-D has been classified by the World Health Organization (WHO) as a class II member (WHO, 2009) and it has been included in the category II-III by the United States Environmental Protection Agency (U.S. EPA). Furthermore, the International Agency for Research on Cancer (IARC) recently ranked 2,4-D as a Group 2B member (2017). Since large amounts of this chemical are released into the environment daily, exposure of the general population and living species may result from the presence of 2,4-D through agricultural use, food products, or through its use for control of aquatic weeds and woody vegetation. As an example, the presence of 2,4-D through conifer release in forests and site preparation has been well demonstrated (USEPA, 2005). For drinking water, the maximum contaminant level of 70 μg 2,4-D/L was established by the U.S. EPA (USEPA, 2005). However, levels of 2,4-D have been detected in surface, ground and potable water supplies, with overall concentrations ranging from 0 to 14.4 μg/L (Loos et al., 2010; Glozier et al., 2012; Félix-Cañedo et al., 2013). In Argentina, only one study reports a 2,4-D concentration of 0.99 μg/L found in El Crespo River in Buenos Aires Province (Pérez et al., 2017). In aquatic environment, 2,4-D is most commonly found as free anion (Halter, 1980). In surface waters, 2,4-D half-lives varied widely, ranging from 7 to 21 days under aerobic conditions. However, under anaerobic conditions, it can exceed 120 days (USEPA, 2005). For soil, the half-life of 2,4-D has been reported to be between 7 and 13 days and it has a residuality of 1–4 weeks; thus, it is not considered to be a persistent chemical (USEPA, 2005).

Genotoxic properties of 2,4-D have been confirmed in several biotic matrices, *in vitro* and *in vivo,* including non-mammalian systems, mammalian and human cells, as well as occupationally-exposed humans (IARC, 2017). The genotoxic effect of 2,4-D has demonstrated the ability to induce chromosomal aberrations in mouse bone marrow and spermatogonial cells, human lymphocytes and kidney cells of fish *Channa punctatus* (Amer and Aly, 2001; Garaj-Vrhovac and Zeljezic, 2002; Farah et al., 2006), sister chromatid exchanges (SCEs) in CHO cells (Madrigal-Bujaidar et al., 2001; Arias, 2007; Soloneski et al., 2007), micronuclei formation in fish such as *Clarias batrachus* and *Cnesterodon decemmaculatus* (Ateeq et al., 2002; Ruiz de Arcaute et al., 2016), DNA damage evaluated by the single-cell gel electrophoresis (SCGE) assay in CHO-K1 and SHE cells, *in vitro* human lymphocytes, epithelioma papillosum cyprini (EPC) cell line and in fish blood cells (Martínez-Tabche et al., 2004; Ateeq et al., 2005; González et al., 2005; Sandal and Yilmaz, 2011; Bokán et al., 2013). Additionally, mutagenic effects where observed when employing the dominant-lethal assay in freshwater snails *Biomphalaria glabrata* (Estevam et al., 2006). Whereas no induction of micronuclei in human lymphocytes was observed for genotoxic studies, the generation of chromosomal aberrations was reported in workers who employed 2,4-D (IARC, 2017). Additionally, in human lymphocytes from workers exposed to a pesticide mixture including 2,4-D, the induction of chromosomal aberrations, micronuclei formation, SCEs as well as DNA single strand breaks were also reported (IARC, 2017). Finally, several studies suggested a positive association between 2,4-D exposition and carcinogenesis development, including the occurrence of non-Hodgkin's lymphoma in occupational workers (IARC, 2017).

DIC is a selective herbicide classified by WHO (www.who.int/ipcs/publications/pesticides_hazard/en/), as a class II member and by U.S. EPA as a type III compound (2006). DIC is employed to eradicate broad-leaved plants growing in numerous urban and peri-urban areas including home grass, farms, golf courses, rights-of-way along roadsides and railways, among others. According to U.S. EPA (2006) the maximum contaminant level of 200 μg DIC/L was established for drinking water. The half-life of DIC in soil is between 1 and 6 days and the herbicide has high water solubility (USEPA, 2006). It has been detected in surface waters with concentrations ranging from 0.89 ng/L to 50 μg/L (Cessna and Elliot, 2004; Donald et al., 2007; Woudneh et al., 2007; Glozier et al., 2012; Ensminger et al., 2013; Farenhorst et al., 2015), drinking-water reservoirs with concentrations ranging from to 1.04–16 ng/L (Donald et al., 2007; Ensminger et al., 2013) and in wastewater treatment plants in concentrations of 185 ng/L (Westlund and Yargeau, 2017). As far as we know, there is no information available on the environmental concentrations of DIC in Argentina.

Although DIC is considered the third-most commonly used broadleaf herbicide, with a toxicity higher than the herbicide glyphosate, knowledge about its toxicity on living species is limited (USEPA, 2006). In fact, a few positive results have been reported on the genotoxicity of DIC, such as the induction of SCEs in CHO-K1 cells and human lymphocytes (Perocco et al., 1990; González et al., 2006), high level of DNA damage evaluated by SCGE assay as well as micronuclei induction in CHO-K1 cells and in *C. decemmaculatus* (González et al., 2007; Ruiz de Arcaute et al., 2014b). To the best of our knowledge and according to U.S. EPA (2006), the genotoxicity and the carcinogenicity potential of DIC have never been studied on occupational workers. In some epidemiologic studies, neither association between exposure to DIC and childhood acute lymphoblastic leukemia nor non-Hodgkin lymphoma risk was observed in residential exposure areas (Hartge et al., 2005; Metayer et al., 2013). Similarly, in the evaluation of cancer occurrence from workers exposed to a pesticide mixture including DIC, no apparent risk for non-Hodgkin lymphoma was reported. However, a low association between DIC exposure and lung and colon cancer was observed (Samanic et al., 2006).

The alkaline SCGE assay represents a rapid and highly sensitive technique frequently used and recommended as an end point for the detection of DNA single-strand breaks (Bony et al., 2010; Ruiz de Arcaute et al., 2014b; 2016). Fish genotoxicity analyzed by the SCGE assay after pesticide exposure is extensively documented throughout the literature, including *C. decemmaculatus* (Ali et al., 2009; Cavaş, 2011; Vera-Candioti et al., 2013; Ruiz de Arcaute et al., 2014b; Rodrigues et al., 2016; Ruiz de Arcaute et al., 2016; Martins and Costa, 2017). This Neotropical small-fish species has a wide range of tolerance, and it is easy to handle and adapt to laboratory conditions, which makes it a suitable test organism in lethal and sublethal toxicity bioassays (de la Torre et al., 2007; Ruiz de Arcaute et al., 2014b; Vera-Candioti et al., 2015; Ruiz de Arcaute et al., 2016 and references therein).

We have previously analyzed the short-term effects of the 58.4% 2,4-D-based DMA® and the 57.7% DIC-based Banvel® herbicide formulations on the toxicity, as well as the genotoxic effects, in adult *C. decemmaculatus* exposed during 96 h under semi-static laboratory conditions (Ruiz de Arcaute et al., 2014b, 2016). In these studies, where LC50 estimation was used as end point for acute lethal toxicity, both the primary DNA damage induction revealed by SCGE assay and the frequency of micronuclei, as well as other nuclear abnormalities detection, were used as end points for genotoxicity. Results highlighted that both herbicides induced genomic instability by increasing both DNA single-strand breaks, as well as the micronucleus, blebbed, lobed and notched nuclei frequencies. Furthermore, other sublethal effects, such as behavioral alterations, were also reported as a consequence of both 2,4-D and DIC exposure (Ruiz de Arcaute et al., 2014b, 2016).

This study aimed to assess the long-term genotoxicity of two auxinic-based herbicidal-formulated products, DMA® (58.4% 2,4-D) and Banvel® (57.7% DIC) on *C. decemmaculatus*, exposed under controlled laboratory conditions employing a semi-static exposure

procedure lasting 28 days. The frequency of primary breaks introduced into DNA on blood cells of exposed fish was used as the genotoxicological end point after a prolonged treatment employing these auxinic herbicides.

## 2. Materials and methods

### 2.1. Experimental species

*Cnesterodon decemmaculatus* is an ovoviviparous teleost, a member of the Poeciliidae family, extensively distributed in Neotropical America. Adult specimens were collected from an unpolluted stream, near La Plata city (Buenos Aires Province, Argentina). After collection, fish were immediately transported to laboratory and placed in aquaria with dechlorinated tap water (pH $7.55 \pm 0.1$; dissolved oxygen, $6.3 \pm 0.3$ mg/L; ammonium ($NH_4^+$) < 0.2 mg/L; hardness, $143 \pm 23.5$ mg $CaCO_3$/L) and artificial aeration. Acclimation was carried out for at least 20 days to 16/8 h light/dark cycle, at $20 \pm 1$ °C. Fish were fed daily *ad libitum* with fish food flakes (TetraMin®, TetraWerke, Melle, Germany).

### 2.2. Chemicals and quality control

Herbicides included 2,4-D (CAS 94-75-7) commercial-grade trade formulation DMA® (58.4% 2,4-D; Dow AgroSciences Argentina S.A) and DIC (CAS, 1918-00-9) commercial-grade trade formulation Banvel® (57.7% DIC; Syngenta Agro S.A.). All other chemicals of analytical grade were purchased from Sigma Chemical Co. (St. Louis, MO).

Concentration analyses of 2,4-D and DIC in the test solutions were verified by QV Chem Laboratory (La Plata, Buenos Aires, Argentina). Active ingredient samples from test solutions correspond to values obtained immediately after preparation (0 h) and every 24 h thereafter up to the replacement. Concentrations assessed throughout the study represent the nominal concentrations of the analyte present within the formulations Banvel® and DMA®.

### 2.3. Experimental design

Specimens of *C. decemmaculatus* (weight $0.26 \pm 0.1$ g, length $29.5 \pm 2.7$ mm) were exposed for 28 days to two different concentrations of each commercial formulation, DMA® and Banvel®, equivalent to 2.5% and 5% of the $LC50_{96h}$. The $LC50_{96h}$ for both herbicides were reported in previous studies performed in our laboratory (Ruiz de Arcaute et al., 2014b, 2016). The test concentrations were 25.2 and 50.4 mg/L 2,4-D for DMA® and 41 and 82 mg/L DIC for Banvel® (Ruiz de Arcaute et al., 2014b, 2016). Fifty specimens for each test concentration were selected at random and maintained in 4 aquaria of 5 L with continuous aeration. Negative control (dechlorinated tap water) was conducted and run simultaneously with 2,4-D- and DIC-exposed fish. Herbicide solutions were replaced every 3 days. Fish were fed 1 h prior to pesticide replacement. Weekly, from day 0 up to 28 days thereafter, 10 individuals were randomly taken from each experimental point, *i.e.*, herbicide- and non-herbicide (negative control)-exposed fish. Fish were anaesthetized by immersion in ice water (Summerfelt and Smith, 1990; Ackerman et al., 2005). Afterwards, blood samples were obtained for the SCGE assay following recommendations previously reported (Ruiz de Arcaute et al., 2014b, 2016). Briefly, after severing the spinal column behind the opercula, two drops of peripheral blood from each specimen were collected and processed according to the procedure described in detailed elsewhere (Ruiz de Arcaute et al., 2016).

### 2.4. Single-cell gel electrophoresis assay

The alkaline SCGE assay was performed following the recommendations of Singh (1996), with adjustments reported for the species elsewhere (Vera-Candioti et al., 2013; Ruiz de Arcaute et al., 2016). Nucleoids were classified in five classes (0–I, undamaged nucleoids; II-IV, damaged nucleoids) as suggested previously (Çavaş and Könen, 2007). Data are expressed as the mean number of damaged cells (sum of nucleoid classes II, III, and IV) and the mean comet score for each treatment group. The genetic damage index (GDI) was estimated for each experimental point according to the algorithm of Pitarque et al. (1999).

### 2.5. Statistical analysis

The proportion of individuals affected per experimental point was calculated. Each proportion was angular-transformed and a two-way ANOVA with Tukey's test was performed (Zar, 1999). ANOVA assumptions were corroborated with Barlett's test for homogeneity of variances and a $\chi^2$ test for normality. Cases in which an assumption of normality was not performed underwent a Kruskal-Wallis test. A simple linear regression and correlation analyses were made to evaluate relationships between concentration and GDI results. The level of significance chosen was 0.05 unless indicated otherwise.

## 3. Results

*T*-tests between chemical analyses showed no significant changes ($P > 0.05$) in the concentration of the pure analyte in treatments during the interval renewals of the testing solutions (concentration range, $97 \pm 5\%$ recovery).

### 3.1. DNA damage

Results of the SCGE assay obtained from individuals exposed to the 2,4-D are presented in Table 1 and in Fig. 1. Table 1 summarizes the frequency of damaged nucleoids and GDI, whereas the different categories of nucleoids found for each experimental point are depicted in Fig. 1. Overall, in treated specimens employing either 2.5% or 5% of 2,4-D, significant increases in the frequency of damaged cells were observed in exposed fish sampled at 7, 14, 21 and 28 days of treatment, irrespective of the control values ($P < 0.001$) (Table 1). After either 2.5% or 5% of 2,4-D treatments, and regardless of the exposure time, the percentage of damaged nucleoids was significantly increased with respect to control values ($P < 0.001$). For both treatments, the lowest DNA damage was observed on day 7, and there was gradual increase in damage up to day 21, where the maximum level of damage was observed (90.05% and 89.67% for 2.5% and 5%, respectively). However, DNA damage started to decline from the 21 day up to the 28 day of exposure (74.49% and 71.99% for 2.5% and 5%, respectively), although it remained significant when compared to the control values ($P < 0.001$). For both 2,4-D concentrations assayed, the DNA damage was found to be time-independent, showing a *r* value of −0.48 ($P > 0.05$) and −0.41 ($P > 0.05$) for 2.5% and 5%, respectively. Tukey's test demonstrated that, at 7 days 2,4-D-exposed fish, an enhanced frequency of type II, III and IV nucleoids was observed ($P < 0.001$). When fish were exposed for 14 days, an enhancement of type II and III nucleoids was registered ($P < 0.001$). In addition, an increased frequency of type IV nucleoids in individuals treated with 2.5% of 2,4-D for 14 days was achieved ($P < 0.01$) (Fig. 1). In fish exposed for 21 days, the variation in the frequency of nucleoids population was due to an increased frequency of type II, III and IV nucleoids ($P < 0.001$). Finally, when fish were analyzed at day 28, an increased

**Table 1**
Analysis of DNA damage measured by SCGE assay in *Cnesterodon decemmaculatus* cells exposed to 2,4-D-based herbicide DMA® and dicamba-based herbicide Banvel®.

| Compound | Exposure dosage (mg/L) | Exposure time (days) | Number of animals observed | Number of nucleoids analyzed | % of damaged nucleoids (II + III + IV) | GDI[a] ± SE |
|---|---|---|---|---|---|---|
| Negative Control | | 7 | 10 | 1027 | 10.42 | 0.69±0.05 |
| | | 14 | 10 | 1003 | 12.76 | 0.77±0.07 |
| | | 21 | 10 | 1010 | 11.09 | 0.72±0.11 |
| | | 28 | 10 | 1048 | 13.93 | 0.74±0.07 |
| DMA® | 2.5% $LC50_{96h}$ | 7 | 10 | 1078 | 83.67 | 2.36±0.09*** |
| | | 14 | 10 | 1046 | 88.81*** | 2.30±0.06*** |
| | | 21 | 10 | 1256 | 90.05*** | 2.60±0.12*** |
| | | 28 | 10 | 1184 | 74.49*** | 2.14±0.06*** |
| | 5% $LC50_{96h}$ | 7 | 9 | 976 | 81.56*** | 2.39±0.10*** |
| | | 14 | 10 | 1135 | 84.85*** | 2.13±0.04*** |
| | | 21 | 10 | 1220 | 89.67*** | 2.61±0.11*** |
| | | 28 | 10 | 989 | 71.99*** | 1.97±0.09*** |
| Banvel® | 2.5% $LC50_{96h}$ | 7 | 10 | 1177 | 43.16*** | 1.38±0.11*** |
| | | 14 | 10 | 1163 | 58.30*** | 1.66±0.08*** |
| | | 21 | 10 | 1018 | 21.32 | 1.11±0.04* |
| | | 28 | 10 | 1112 | 21.04 | 0.99±0.09 |
| | 5% $LC50_{96h}$ | 7 | 10 | 1013 | 77.20*** | 2.07±0.06*** |
| | | 14 | 10 | 1064 | 30.92 | 1.15±0.14 |
| | | 21 | 10 | 1048 | 20.04 | 0.99±0.08 |
| | | 28 | 9 | 947 | 23.02 | 0.95±0.06 |

*$P < 0.05$.
***$P < 0.001$; significant differences with respect to negative control values.
[a] GDI: Genetic damage index.

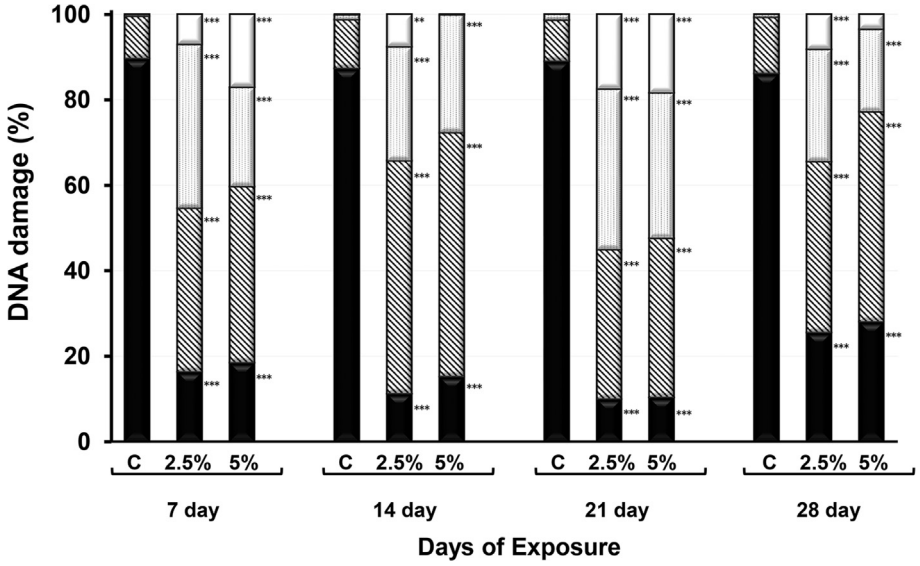


**Fig. 1.** DNA damage induced by 2,4-D-based herbicide formulation DMA® measured by single-cell gel electrophoresis assay in circulating blood cells from *Cnesterodon decemmaculatus* (Pisces: Poeciliidae). The frequencies of undamaged type 0-I nucleoids (black bar), type II (stripped bar), type III (dotted bar), and type IV (white bar) were determined by analysing 100 nucleoids from each specimen. Results are presented as percentages of pooled data from three independent experiments. Negative control (untreated fish) was conducted and run simultaneously with DMA® exposed fish. **, $P < 0.01$; ***, $P < 0.001$; significant differences with respect to control values.

frequency of type II-IV and II-III nucleoids was observed in fish exposed to 2.5% and 5%, respectively ($P < 0.001$). Finally, a concomitant decrease of type 0–I nucleoids was observed in all four exposure times ($P < 0.001$) (Fig. 1). Likewise, a significant increase of GDI was observed in both 2.5% and 5% treatments for all exposure times ($P < 0.001$) (Table 1). For both 2,4-D concentrations, the maximum GDI value was at 21 days of exposure.

Results of the SCGE assay obtained from specimens exposed to the DIC are presented in Table 1 and in Fig. 2. Table 1 summarizes the frequency of damaged nucleoids and GDI, whereas the different categories of nucleoids found for each experimental point are depicted in Fig. 2. Overall, in specimens exposed to 2.5% of DIC, significant increases in the frequency of damaged cells were observed in fish sampled at 7 and 14 days of treatment ($P < 0.001$). In specimens exposed to 5% of DIC significant increases in the frequency of damaged cells were observed only in fish sampled at 7 days of treatment ($P < 0.001$) (Table 1). The maximum induction of DNA damage was observed on day 14 (58.30%) at the lowest

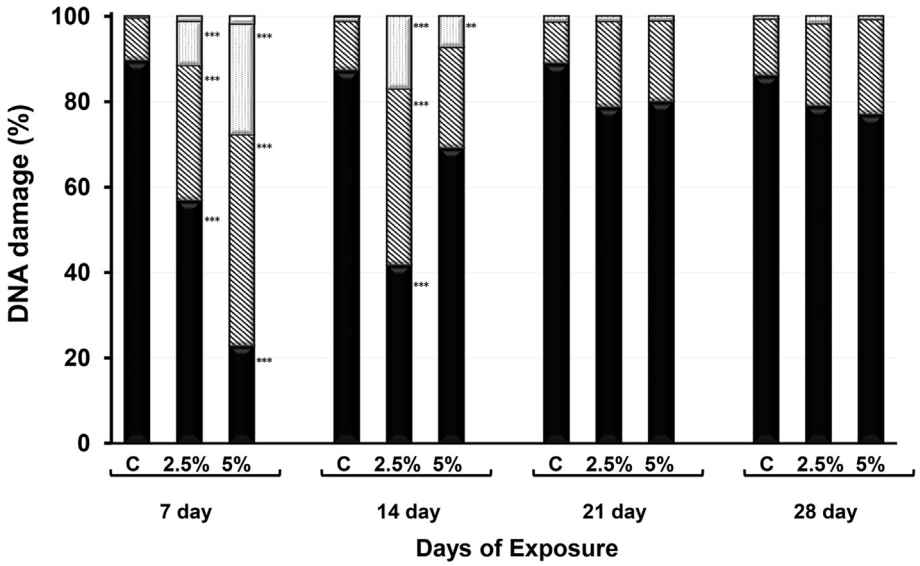


**Fig. 2.** DNA damage induced by DIC-based herbicide formulation Banvel® measured by single-cell gel electrophoresis assay in circulating blood cells from *Cnesterodon decemmaculatus* (Pisces: Poeciliidae). The frequencies of undamaged type 0-I nucleoids (black bar), type II (stripped bar), type III (dotted bar), and type IV (white bar) were determined by analysing 100 nucleoids from each specimen. Results are presented as percentages of pooled data from three independent experiments. Negative control (untreated fish) was conducted and run simultaneously with Banvel® exposed fish. **, $P < 0.01$; ***, $P < 0.001$; significant differences with respect to control values.

concentration employed (2.5% DIC), whereas the DNA induction for 5% DIC was highest on day 7 (77.20%). For both concentrations assayed, the DNA damage was found to be time-dependent, showing an $r$ value of −0.74 ($P < 0.05$) and an $r$ value of −0.84 ($P < 0.01$) for 2.5% and 5% DIC, respectively. For both concentrations assayed, in 7 days herbicide-exposed fish, a Tukey's test revealed an enhanced frequency of type II and III nucleoids ($P < 0.001$). When fish were exposed for 14 days, an enhancement of type III nucleoids was observed ($0.01 > P < 0.001$) (Fig. 2). In addition, an increased frequency of type II nucleoids in individuals treated with 2.5% DIC for 14 days was observed ($P < 0.001$). Finally, a concomitant decrease of type 0–I nucleoids was observed in 7 and 14 days of exposure in fish treated with 2.5% DIC, and after 7 days of treatment with concentration equivalent to 5% DIC ($P < 0.001$) (Fig. 2). In DIC-exposed specimens, a significant increase of GDI was observed in fish exposed to 2.5% DIC at 7–21 days of treatment ($0.05 > P < 0.001$) (Table 1). In addition, in fish exposed to 5% DIC, such increment was only observed at 7 days of treatment ($P < 0.001$). The maximum GDI value was achieved at day 14 for 2.5% DIC and at day 7 for 5% DIC treatment ($P < 0.001$).

## 4. Discussion

In the present work, long-term sublethal effects of the 2,4-D-based herbicide DMA® (58.4% 2,4-D) and the DIC-based herbicide formulation Banvel® (57.7% DIC) were analyzed on *C. decemmaculatus* exposed under laboratory conditions using a semi-static experimental model for toxicity testing.

Under the experimental design employed, results showed that exposure to concentrations of 25.2 and 50.4 mg/L 2,4-D, equivalent to 2.5% and 5% of the $LC50_{96h}$ values reported for the species, increased the frequencies of breaks into DNA estimated by alkaline SCGE in those fish exposed at 7, 14, 21 and 28 days. However, exposure to concentrations of 41 and 82 mg/L DIC, equivalent to 2.5% and 5% of the $LC50_{96h}$ values reported for the species, increased the frequencies of breaks into DNA only in those fish exposed at 7 and 14 days and 7 days for 2.5% and 5% DIC, respectively. These results are in agreement with previous studies performed in our laboratory, demonstrating that after an acute exposure to 2,4-D and DIC within the 252–756 and 410–1229 mg/L concentration range lasting up to 96 h, respectively, an increased frequency of DNA single-strand breaks was achieved, regardless of the length of treatment (Ruiz de Arcaute et al., 2014b, 2016). Furthermore, in these studies, the frequency of micronucleus was employed as a another genotoxicity end point whereas the estimation of mortality and behavioral parameters were employed as lethal and other sublethal end points (Ruiz de Arcaute et al., 2014b, 2016). The results demonstrated that 2,4-D contained in the formulated product DMA® increased the frequency of micronucleus after exposure within the 252–756 mg/L 2,4-D concentration range (Ruiz de Arcaute et al., 2016), as well as the frequency of micronucleus on DIC-based commercial formulation Banvel®-exposed-fish to 1229 mg/L DIC for 48 h (Ruiz de Arcaute et al., 2014b).

Nowadays, due to the extensive use of agrochemicals worldwide, the employment of biomarkers to qualitatively and quantitatively estimate the exposure of the biota to pollutants is of growing concern. Among these, the SCGE assay became one of the most employed biomarkers for detecting DNA damage, allowing an early assessment on the response in the biota, *e.g.*, in aquatic environment (Lee and Steinert, 2003). A variety of organisms within this aquatic compartment, including fish, have been tested, and induction of DNA damage was reported after exposure to several xenobiotics, including agrochemicals (Martins and Costa, 2017 and references therein).

Additionally, in agreement with our previous observations on the lethal and sublethal effects exerted by 2,4-D and DIC when employing *C. decemmaculatus* as the test organism, acute DNA damage analyzed by the SCGE assay has been reported in the rainbow trout *Oncorhynchus mikiss* (Martínez-Tabche et al., 2004) and in the catfish *C. batrachus* (Ateeq et al., 2005). Similarly, DNA single-strand breaks were induced by 2,4-D or its commercial formulation DMA® in a carp cell line *in vitro* (Bokán et al., 2013). To the best of our knowledge, no other studies addressing the generation of DNA damage in fish using the herbicide DIC have been reported so far.

Long-term toxicity studies in aquatic biota are relevant in order to identify those plausible xenobiotics that could introduce DNA damage in a greater extent after prolonged exposure, increasing the

potential appearances of chronic diseases, including carcinogenesis. In this study, a significant increase in the generation of DNA single-strand breaks was detected in fish exposed for up to 28 days to two sublethal concentrations of two auxinic herbicides, 2,4-D and DIC. The frequency of damaged cells observed in *C. decemmaculatus* after long-term exposure to 2,4-D concentrations, equivalent to 2.5% and 5% of the $LC50_{96h,}$ were significantly higher than that of non-exposed group at all sampling times (7–28 days). These findings are in concordance with previous studies reported for the herbicide. Long-term toxicity of 2,4-D has been reported in the common fruit fly *Drosophila melanogaster* by increasing the frequency of large single and total spots when the wing-spot test was employed (Kaya et al., 1999). In rats and mice, Charles et al. (1996) reported low long-term toxicity and alterations in the oncogenic pattern following chronic intake of 2,4-D. Similarly, alterations on germinal cells from rats chronically exposed to the 2,4-D-based commercial formulation, Tordon 75D®, and testicular damage and sperm cell reduction in their offspring were observed (Oakes et al., 2002). To the best of our knowledge, no studies on 2,4-D- and DIC-long-term exposed fish have been reported so far. Thus, our study demonstrated for the first time the genotoxic effect of these two herbicides in an aquatic organism, like *C. decemmaculatus*, after exposure conditions for up to 28 days.

According to our experimental design, results revealed that a period as short as 7 days is required for the induction of breaks in DNA after both 2,4-D concentrations assayed. On the other hand, the time required for the induction of the maximum level of DNA damage was the same for both 2,4-D concentrations tested, 21 days of exposure. At 28 days of exposure, the damage decreased in relation to that observed at day 21, but never reached control values. However, the level of DNA damage observed in blood cells of *C. decemmaculatus* was not time-dependent for both 2,4-D concentrations assayed from 7 to 28 days.

Similarly, results also revealed a significant increase in the induction of DNA damage observed in *C. decemmaculatus* specimens long-term exposed to the two sublethal concentrations of DIC assayed. The frequency of damaged cells observed in *C. decemmaculatus* after exposure to concentration equivalent to 2.5% DIC $LC50_{96h}$ values were significantly higher than that from the negative group at 7 and 14 days of treatment. On the other hand, the frequency of damaged cells was only significantly increased at 7 days of treatment in fish exposed to the concentration equivalent to 5% DIC $LC50_{96h}$. Likewise 2,4-D, results revealed that a period as short as 7 days is required for the induction of breaks in DNA after both DIC concentrations assayed. However, the time required for the induction of the maximum level of DNA damage varied depending upon the DIC concentration. When 5% DIC was employed, this level was observed on day 7, whereas for a concentration of 2.5% DIC, the highest level of DNA damage was found on day 14. After the maximum peak of DNA damage, the damage decreased in relation to that observed at day 14, reaching control values. Overall, for both auxinic herbicides, the frequency of DNA damage over the 28 days of exposition showed, then, a biphasic induction pattern. This observation could indicate that 2,4-D, as well DIC, would influence the blood cell turn-over. However, other plausible explanations to our observation could be suggested. The results obtained in our study could also be in agreement with those previous long-term investigations reported by others in *in vitro* and *in vivo* biotic matrices exposed to pesticides, where an adaptive mechanism of tolerance has been observed (Çavaş and Könen, 2007; Chang et al., 2009; Mezzelani et al., 2018). In the present study, the genotoxicity exerted by the herbicides on the DNA is notably evident and tends to increase after long-term exposure conditions, thus possibly indicating their potential biotransformation into genotoxic breakdown products by *C. decemmaculatus*. However, this hypothesis could be ruled out since no plausible explanation for the decrease in DNA damage observed at the end of the exposure period could be suggested. Another plausible hypothesis of our findings might be an adaptive mechanism of tolerance to chemical stress in *C. decemmaculatus* haematopoietic organs, resulting in an enhancement in resistance under long-term exposure conditions. It is known that a continuous exposure to environmental xenobiotics might increase the resistance of the biota, even to lethal concentrations (Campana et al., 1999; de Lemos et al., 2001; Mezzelani et al., 2018). Some authors have demonstrated that after long-term treatments the frequency of DNA damage in fish blood cells, as well as in mussel haemocytes, declines after 15 and 60 days of exposure, respectively (Campana et al., 1999; de Lemos et al., 2001; Mezzelani et al., 2018). These differences seem to be related to the continuous cellular replacement of damaged cells in order to preserve normal physiological conditions (Mersch et al., 1996; Çavaş and Könen, 2007). Another putative explanation could be related to the perturbation of the enzymatic process toward detoxification (Kumar et al., 2012), or the gene activation of metabolizing enzymes, such as P450, in various tissues that provides a defensive mechanism against the formation of DNA damage after auxinic exposure (Espandiari et al., 1995, 1999). Toxic compounds such as pesticides and metals are known to decrease different free-radical scavenging enzymes, *e.g.*, glutathione reductase, catalase and glutathione peroxidases (Lobo et al., 2010). A possible explanation may be that DNA damage in blood cells decreases with elevated activity of glutathione reductase during the DNA damage declining period, which could activate the reduced glutathione toward detoxification of the herbicides. Oxidative enzyme profiles in *C. decemmaculatus* at acute and chronic exposures of the herbicide are required to address these possibilities. Finally, it is worth mentioning that the time to recover a basal level of genetic damage can vary greatly depending upon the species. Recovery periods as short as 72 h for fish (Mohanty et al., 2011), of 11–14 days for amphibians (Mouchet et al., 2015; Pérez-Iglesias et al., 2018), and up to 21 days for rats (Prado-Ochoa et al., 2016) have been reported. Our current results demonstrated that a period of 14–21 days is enough to recover the basal level of DIC-induced primary DNA damage. According to Mohanty et al. (2011), the explanation for this recovery process is associated with a selective loss of damaged cells, as previously stated, and/or the onset of the DNA repair process. In this sense, several authors have suggested that the rapid recovery from the genetic damage induced by pesticides is related to the onset of DNA repair systems by DNA repair enzymes through the process of base excision repair and/or nucleotide excision repair, acting in several aquatic organisms (Mohanty et al., 2011; Guilherme et al., 2014; Michel and Vincent-Hubert, 2015; Mouchet et al., 2015; Pérez-Iglesias et al., 2018). Further studies are required employing *C. decemmaculatus* as fish model to solve this scientific conundrum.

In plants, the auxin-like mode of action of 2,4-D has been well established by several investigations (IARC, 2017), but the mechanism by which 2,4-D induces toxicity in animals, including humans, remains poorly understood (Bukowska, 2006). Some authors have demonstrated that 2,4-D contributes to the generation of reactive oxygen species as well as the induction of oxidative damage in different organisms, including fish (Oruc et al., 2004; Tayeb et al., 2012). Genotoxic compounds have the ability to induce reactive oxygen species by imbalances of free radicals, and then interact with DNA to cause single- and double-breaks and other damage inside the DNA molecule. For DIC, it has been described as a peroxisome proliferator inducer and hepatocarcinogenesis promoter in rats (Espandiari et al., 1995, 1999). In plants, DIC induces tissue damage and cell death in *Galium aparine* by generation of lipid peroxidation (Grossmann et al., 2001). Additionally, DIC was

suggested to cause decreases in loss of food intake and body weight and to induce abnormal and dead cells in the liver of 90-day-exposed rats (USEPA, 1984). Further toxicity studies are required on *C. decemmaculatus* to obtain comprehensive knowledge of the precise mechanism(s) involved in genotoxic process exerted by the auxinic herbicides 2,4-D and DIC.

In agroecosystems, pesticides are commonly employed as formulated products, where the active ingredient is combined with organic solvents and emulsifying and wetting agents, in order to enhance the pesticide penetration and performance (WHO, 1990). These additives may alter the toxicity of the active ingredient and their toxic effects can go beyond those of the pure compound. Several authors have proved and agree in demonstrating that the excipients present in pesticide commercial products, including herbicides, are able to induce both toxicity and cellular damage by themselves rather than the pure compound either *in vitro* or *in vivo* (Mann and Bidwell, 1999; Vera-Candioti et al., 2010; Molinari et al., 2013; Nikoloff et al., 2014; Pérez-Iglesias et al., 2014; Ruiz de Arcaute et al., 2014a; Mansano et al., 2016, 2018). 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". Thus, unfortunately the identities of the additive compounds present in the commercial herbicide formulations DMA® and Banvel® were not made available to us. Further studies should be required to reveal whether the sublethal damage exerted by both auxinic formulations assayed in our study results from the presence of xenobiotic(s) with genotoxic properties included in the formulated products.

Xenobiotics with a significant toxicity pattern can cause undesirable chronic consequences on the biota at concentrations well below those that produce acute effects (Aronzon et al., 2011). The present investigation shows that the use of SCGE assay in blood cells could detect DNA lesions at lower auxinic herbicide concentrations after long-term conditions. Finally, the current study emphasizes that the SCGE assay is a sensitive biological biomarker for evaluating the long-term genotoxic properties of the auxinic herbicides, namely 2,4-D and DIC, especially in the aquatic freshwater biota, when employing a small fish like *C. decemmaculatus* as the experimental model.

## Conflict of interest statement

The authors declare that there are no conflicts of interest.

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