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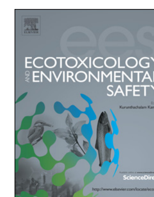

Single-cell gel electrophoresis assay in the ten spotted live-bearer fish, *Cnesterodon decemmaculatus* (Jenyns, 1842), as bioassay for agrochemical-induced genotoxicity

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ABSTRACT

The ability of two 48 percent chlorpyrifos-based insecticides (Lorsban* 48E® and CPF Zamba®), two 50 percent pirimicarb-based insecticides (Aficida® and Patton Flow®), and two 48 percent glyphosate-based herbicides (Panzer® and Credit®) to induce DNA single-strand breaks in peripheral blood erythrocytes of *Cnesterodon decemmaculatus* (Jenyns, 1842) (Pisces, *Poeciliidae*) exposed under laboratory conditions was evaluated by the single-cell gel electrophoresis (SCGE) assay. In those fish exposed to Lorsban* 48E®, CPF Zamba®, Aficida®, Patton Flow®, Credit®, and Panzer®, a significant increase of the genetic damage was observed for all formulations regardless of the harvesting time. This genotoxic effect was achieved by an enhancement of Type II-IV comets and a concomitant decrease of Type 0-I comets over control values. A regression analysis revealed that the damage varied as a negative function of the exposure time in those Lorsban* 48E®- and Aficida®-treated fish. On the other hand, a positive correlation between damage increase and exposure time was achieved after Patton Flow® and Credit® treatment. Finally, no correlation was observed between increase in the genetic damage and exposure time after treatment with CPF Zamba® or Panzer®. These results highlight that all agrochemicals inflict primary genotoxic damage at the DNA level at sublethal concentrations, regardless of the exposure time of the aquatic organisms under study, at least within a period of 96 h of treatment.

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1. Introduction

Pesticides have become ubiquitous on the planet since they are employed to control or eliminate a variety of agricultural and household pests that can directly or indirectly damage crops and livestock, thus reducing their productivity. Although there are many benefits of the use of pesticides in crop fields and they have made a significant contribution to the lifestyles we have come to expect, pesticides can also be hazardous if not used appropriately, and many of them may represent potential hazards to the environment due to the contamination of food, water, and air (WHO-FAO, 2009). Specially, anthropogenic activities are continuously introducing daily extensive amounts of these compounds into the environment, regardless of their persistence, bioaccumulation, and toxicity, mainly on croplands and pastures.

Most of the agrochemicals in the aquatic environments exert their effects through genotoxic and metabolically toxic mechanisms causing, simultaneously, genotoxic damage, disease syndrome, and carcinogenesis as well (Könen and Cavaş, 2008; Vera-Candioti et al., 2010a). Accordingly, current awareness of the real/potential hazards of pollutants in the aquatic environment has a high interest in the use of aquatic organisms as indicators for monitoring pollutant-induced environmental genotoxicity and cytotoxicity. Notwithstanding, it is well known that pesticides not only affect target organisms, but concomitantly exert negative side effects on nontarget organisms (www.epa.gov/pesticides).

In epidemiological as well as in experimental genotoxic and cytotoxic studies, there is an increasing interest in biomonitoring markers to provide measurements as well as estimations of biological exposure to genotoxic pollutants. To achieve this goal, several end points for testing both cytotoxicity and genotoxicity have been employed on aquatic organisms to assess the impact of pollution on contaminated areas (*in situ* assays) (Cavaş and Ergene-Gözükara, 2005; de Lemos et al., 2008; Pantaleão et al., 2006) as well as for screening different compounds after direct or indirect exposure (*in vivo* assays) (Barsiene et al., 2006; Cavaş, 2008; Cavaş and Könen, 2007).

The single-cell gel electrophoresis assay (SCGE) has been proposed as a very sensitive indicator of DNA damage as well as

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a biomarker in DNA kinetic repair studies. Thus, this assay is a reliable bioassay for monitoring exposure to hundreds of xenobiotics in a wide variety of *in vitro* and *in vivo* short-term studies as a biomarker of exposure, or biological dosimeter (Valverde and Rojas, 2009). The sensitivity of this biomarker has enabled genetic toxicologists to monitor low-level, short- and long-term exposure to chemicals, thus predicting genetic damage at an early stage (Valverde and Rojas, 2009). Analysis of micronucleus (MN) frequency and the induction of DNA single-strand breaks by SCGE assay are the most frequently recommended and employed end points for detecting DNA damage in circulating blood cells (Ali et al., 2008a; Cavaş, 2011; Cavaş and Könen, 2007; Mohanty et al., 2011; Vera-Candioti et al., 2010b, 2013b, 2013c; Yin et al., 2009).

Fish are suitable aquatic vertebrates to be employed as reliable environmental genotoxicity bioindicator organisms due to both their role in the aquatic trophic chain and their sensitivity to low concentrations of genotoxic compounds, characteristic of polluted aquatic environments. Besides, the ability of fish to efficiently metabolize and accumulate chemical pollutants it is well documented (Cavaş and Ergene-Gözükara, 2005; Frenzilli et al., 2009).

The ten spotted live-bearer fish, *Cnesterodon decemmaculatus* (Jenyns, 1842) (Pisces, *Poeciliidae*), is an endemic species with an extensive distribution in Neotropical America that attains high densities in a large variety of water bodies within the whole La Plata River and other South American basins (Menni et al., 1996). This is a small ovoviviparous, microomnivorous, benthic–pelagic, nonmigratory fish that 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 (Menni et al., 1996). Furthermore, several reports found this species suitable as a test organism in acute and chronic toxicity bioassays (de la Torre et al., 2007; Di Marzio et al., 2005; Menéndez-Helman et al., 2012; Vera-Candioti et al., 2010b, 2013b).

Recently, we have been using *C. decemmaculatus* as a laboratory-exposed target species to evaluate the lethal and sublethal toxicity of several agrochemical commercial formulations. Among them, two 48 percent chlorpyrifos-based insecticides (Lorsban* 48E® and CPF Zamba®) (Vera-Candioti et al., 2013a), two 50 percent pirimicarb-based insecticides (Aficida® and Patton Flow®) (Vera-Candioti et al., 2010b, 2013c), and two 48 percent glyphosate-based herbicides (Panzer® and Credit®) (Vera-Candioti et al., 2013b) were included. These agrochemicals were chosen because they represent the commercial formulations used most widely for cereal and leguminous crop production as well as garden control, not only in Argentina but also worldwide. In all cases, whereas LC_{50} estimation was employed as a biomarker for lethality, induction of micronuclei and alterations in the erythrocyte/erythroblast frequencies were employed as biomarkers of genotoxicity and cytotoxicity, respectively. However, no attempts have been made to elucidate whether these commercial formulations were able to exert other genotoxic damage into the DNA of exposed *C. decemmaculatus* organisms. In the current study, the induction of DNA single-strand breaks in peripheral blood erythrocytes of fish exposed under laboratory conditions was evaluated by SCGE assay to further characterize the genotoxic effects of the aforementioned pesticide technical formulations.

2. Materials and methods

2.1. Chemicals

Agrochemicals included (1) two liquid commercial formulations of 48 percent chlorpyrifos-based (*O*,*O*-diethyl *O*-3,5,6-trichloropyridin-2-yl phosphorothioate; CAS 2921-88-2) commercial formulations, Lorsban* 48E® and Chlorpyrifos Zamba® purchased from Dow AgroSciences Argentina S.A. (Buenos Aires, Argentina) and Nidera S.A. (Buenos Aires, Argentina), respectively, and (2) two liquid 50 percent pirimicarb-based (2-dimethylamino-5,6-dimethylpyrimidin-4-yl dimethylcarbamate; CAS 23103-98-2) commercial formulations, Patton Flow® (purchased from Gleba S.A., Buenos Aires, Argentina) and Aficida® (purchased from Syngenta Agro S.A., Buenos Aires, Argentina). Finally, (3) two 48 percent isopropylamine salt of glyphosate-based [*N*-(phosphonomethyl) glycine; CAS 1071-83-6] commercial formulations, Panzer® (Dow AgroSciences Argentina S.A.) and Credit® (Nufarm S.A., Buenos Aires, Argentina), were used. Cyclophosphamide (CAS 6055-19-2) was purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals and solvents were of analytical grade.

2.2. Quality control

Methods for detecting the concentration levels of pirimicarb, chlorpyrifos, and glyphosate in the test solutions have been described in detail elsewhere (Vera-Candioti et al., 2010b, 2013a,b,c). Briefly, concentration levels pesticides in the test solutions were analyzed by QV Chem Laboratory (La Plata, Buenos Aires, Argentina). Pirimicarb and glyphosate levels were detected by high performance liquid chromatography (Agilent 1100) whereas chlorpyrifos level was determined by gas chromatograph with electron capture detector (Hewlett Packard, HP 6890). Detection limit for pirimicarb and glyphosate was 0.2 mg/L whereas for chlorpyrifos the value was 0.01 μ/L. Active ingredient samples from test solutions correspond to values obtained immediately after preparation (0 h) and 24 h thereafter. Results of 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 percent recovery). Concentrations assessed throughout the study represent the nominal concentrations of active ingredient present within technical formulations.

2.3. Test organisms

Specimens of *C. decemmaculatus* were collected from a permanent pond free of pluvial runoff from agricultural areas, in the vicinity of La Plata, Buenos Aires, Argentina. Adults were transported to the laboratory and then acclimatized for at least 20 d to a 16/8 h light/dark cycle in aquaria at 21 ± 1 °C in dechlorinated tap water (pH 7.6–8.3; hardness, 143 mg/L $CaCO_3$) and artificial aeration. Since the species is sexually dimorphic (Menni et al., 1996), males and females were maintained separately and fed *ad libitum* daily with commercially available fish food (TetraMin®, Tetra Werke, Germany) until 24 h before the beginning of the experimental procedures, as reported previously (Vera-Candioti et al., 2010b). Organisms with an average weight of 0.26 ± 0.1 g and total length of 29.5 ± 2.7 mm were selected for the experiments.

2.4. Single-cell gel electrophoresis assay

Concentrations assessed throughout the study represent the nominal concentrations of active ingredients present within pesticide-based formulations. Experiments were carried out following recommendations proposed by the U.S. Environmental Protection Agency for standardized methods for acute toxicity tests (USEPA, 1975, 2002). Each experiment was conducted using five males and five nongravid females maintained in a 1 L glass container and exposed to a concentration of the test compound equivalent to 25 percent of the corresponding LC_{50} (96 h) values reported previously (Vera-Candioti et al., 2010b, 2013a,b,c). To achieve these concentrations, fish were exposed to 0.008 mg/L Lorsban* 48E®, 0.052 mg/L Chlorpyrifos Zamba®, 22 mg/L Aficida®, 25 mg/L Patton Flow®, 3.9 mg/L Panzer®, and 22.9 mg/L Credit®, respectively. All test solutions were prepared immediately before each experiment. Fish were treated during 96 h, with test solutions replaced every 24 h. A negative control group consisted of ten organisms kept in dechlorinated tap water (pH 7.6–8.3; hardness, 143 mg/L $CaCO_3$), and a positive control group consisted of ten fishes treated with 10 mg/L cyclophosphamide, for which experiments were conducted and run simultaneously with pesticide-exposed fish. Experiments were performed in triplicate and run simultaneously for each experimental point. The SCGE assay was performed following the alkaline procedure described by Singh et al. (1988) with minor modifications in fifteen specimens harvested either at 48 and in fifteen specimens at 96 h after initial treatment, respectively. At the end of each experiment, fish sleepiness was induced by immersion in ice-cold water. Fish were killed by severing the spinal column behind the opercula and two drops of peripheral blood from each specimen were collected.

Blood samples were diluted with 1 mL phosphate-buffered saline, centrifuged (2000 rpm, 8 min), and resuspended in a final volume of 50 μL of phosphate-buffered saline. An aliquot of 30 μL of the diluted samples was mixed with 70 μL of 0.5 percent low-melting-point agarose, and 50 μL were then layered on a slide precoated with 100 μL of 0.5 percent 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 100 μL of 0.5 percent low-melting-point agarose. After solidification, the coverslip was removed, and slides were immersed in ice-cold freshly prepared lysis solution (1 percent sodium sarcosinate, 2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, pH 10.0, 1 percent Triton X-100, and 10 percent 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 15 min at 4 °C to allow the cellular DNA to unwind, followed by electrophoresis in the same buffer and temperature for 10 min at 25 V and 250 mA. Finally, the slides were neutralized with a solution comprising 0.4 M Tris–HCl, pH 7.5, stained with propidium iodide (Fluoroshield™, Sigma-Aldrich, St. Louis, MO). 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 as recommended elsewhere (Azqueta et al., 2011; Collins et al., 2008; Marlin et al., 2004; Poletta et al., 2011). DNA damage was classified in four classes (0-I, undamaged; II, minimum damage; III, medium damage; IV, maximum damage), as suggested previously (Fig. 1) (Cavaş, 2011). Data are expressed as the mean number of damaged cells (sum of Classes II–IV) and the mean comet score for each treatment group. The genetic damage index (GDI) was calculated for each test compound using the formula GDI = [(I) + 2(II) + 3(III) + 4(IV)]/N(I–IV), where I–IV represent the nucleoid type, and NI–NIV the total number of nucleoids scored according to Pitarque et al. (1999).

2.5. Statistical analyses

Statgraphics Centurion XV software was used for statistical analyses. After assessing the normality of distribution of the data by the Shapiro–Wilk W test, even after logarithmic transformation, nonparametric tests were used to detect differences. The one-tailed Mann–Whitney U test for independent samples was applied to assess differences between treated and control groups. The level of significance was $\alpha = 0.05$ unless indicated otherwise.

3. Results

3.1. Chlorpyrifos-based insecticide-treated specimens

In those fish exposed to Lorsban* 48E® and CPF Zamba®, a significant increase of the GDI was observed both at 48 h ($P < 0.001$) and 96 h of treatment ($P < 0.001$) (Table 1). Statistical analyses revealed that the GDI increase induced by both formulations was due to an enhancement over negative control values in the frequency of Types II–IV comets ($P < 0.001$) and a concomitant decrease of Type 0-I comets ($P < 0.001$). Results revealed a decrease in the GDI in those Lorsban* 48E®-treated fish during 96 h in relation to those exposed during 48 h ($P < 0.05$). On the other hand, no alteration in the GDI was observed between those CPF Zamba®-exposed fish regardless of the exposure time ($P > 0.05$) (Table 1).

3.2. Pirimicarb-based insecticide-treated specimens

A significant increase of the GDI was observed in those fish exposed to both Aficida® and Patton Flow® regardless of the treatment period ($P < 0.001$) (Table 1). Statistical analyses revealed that the GDI increase induced by both formulations was due to an enhancement over negative control values in the frequency of Types II–IV comets ($P < 0.001$) and a concomitant decrease of Type 0-I comets ($P < 0.001$). Results revealed a decrease in the GDI in those Aficida®-treated fish during 96 h in relation to those exposed during 48 h ($P < 0.01$). An increase in the GDI was observed in those 96 h Patton Flow®-exposed fish in relation to those treated during 48 h ($P < 0.05$) (Table 1).

3.3. Glyphosate-based herbicide-treated specimen

In those fish exposed to Panzer® and Credit® a significant increase of the GDI was observed both at 48 h ($P < 0.001$) and 96 h of treatment ($P < 0.001$) (Table 1). Statistical analyses revealed that the GDI increase induced by both formulations was due to an enhancement over negative control values in the frequency of Types II–IV comets ($P < 0.001$) and a concomitant decrease of Type 0-I comets ($P < 0.001$). Results revealed an increase in the GDI in those Credit®-treated fish during 96 h in relation to those exposed during 48 h ($P < 0.001$). On the other hand, no alteration in the GDI was observed between those Panzer®-exposed fish regardless of the exposure time ($P > 0.05$) (Table 1).

4. Discussion

The results of the SCGE assay, using *C. decemmaculatus* as a test system, highlight the ability of the agrochemicals Lorsban* 48E®, CPF Zamba®, Aficida®, Patton Flow®, Panzer®, and Credit® to inflict DNA single-strand breaks at sublethal concentrations at least within a period of 96 h of treatment.

Very few studies have previously employed the SCGE bioassay in the piscine model to demonstrate the ability of chlorpyrifos to induce

Table 1
Analysis of DNA damage measured by comet assay in *Cnesterodon decemmaculatus* circulating blood cells exposed to different pesticides.

Commercial formulation	Concentration (mg/L)[a]	Time (h)	Number of animals analyzed	Number of cells analyzed	Damaged nuclei (%)				Damaged cells % ± SE (II+III+IV)	GDI ± SE[c]
					Type 0-I	Type II	Type III	Type IV		
Negative control		48	15	1500	78.66	14.07	3.87	3.40	21.34 ± 1.85	1.31 ± 0.03
		96	15	1500	76.47	15.13	4.53	3.87	23.53 ± 1.91	1.35 ± 0.03
Lorsban*48E®	0.008	48	14	1400	7.00	41.25	21.25	30.50	93.00 ± 1.47*	2.75 ± 0.06*
		96	13	1300	13.26	41.87	19.07	25.80	86.73 ± 1.34*	2.57 ± 0.04*1;#
CPF Zamba®	0.052	48	14	1400	16.57	44.57	17.57	21.29	83.43 ± 2.59*	2.43 ± 0.09*
		96	15	1500	15.67	39.80	18.80	25.73	84.33 ± 1.40*	2.54 ± 0.06*
Aficida®	25.00	48	13	1300	10.77	43.15	19.85	26.23	89.23 ± 1.37*	2.62 ± 0.05*
		96	12	1200	25.00	39.42	15.50	20.08	75.00 ± 2.47*	2.31 ± 0.07*2;##
Patton flow®	22.00	48	14	1400	10.71	39.00	14.86	35.43	89.29 ± 0.95*	2.75 ± 0.03*
		96	13	1300	8.30	32.62	18.62	40.46	91.69 ± 1.09*	2.91 ± 0.07*1;#
Panzer®	3.90	48	15	1500	12.07	39.13	21.00	27.80	87.93 ± 1.58*	2.65 ± 0.08*
		96	14	1400	17.22	34.14	17.21	31.43	82.79 ± 2.37*	2.63 ± 0.08*
Credit®	22.90	48	14	1400	17.51	37.14	18.71	26.64	82.50 ± 2.36*	2.55 ± 0.06*
		96	13	1300	8.47	32.92	18.69	39.92	91.54 ± 0.91*	2.90 ± 0.06*3;###
CP[b]	10.00	48	15	1500	21.54	42.00	18.13	18.33	78.47 ± 3.77*	2.33 ± 0.07*
		96	15	1500	13.16	27.67	22.75	36.42	86.83 ± 2.17*	2.81 ± 0.09*3;###

[a] Equivalent to 25% of LC_{50} 96 h.
[b] Cyclophosphamide (CP) was used as positive control.
[c] GDI: Genetic damage index.
* $P < 0.001$; significant differences with respect to negative control values.
# $P < 0.05$.
$P < 0.01$.
$P < 0.001$; significant differences between exposure time.

genotoxicity in the aquatic biota. An enhancement of DNA damage was observed in lymphocytes and gill cells of *Channa punctatus* after *in vivo* exposure of Tricel, a 20 percent chlorpyrifos-based formulation (Ali et al., 2008b, 2009). Our current observations clearly extend the concept that not only do the damaging effects of these latter commercial formulations include the induction MN (Vera-Candioti et al., 2013a), but that they are also capable of introducing primary lesions into the DNA of peripheral blood cells in fish at least when *C. decemmaculatus* is employed.

Available data on pirimicarb-induced genotoxicity in *in vivo* biomonitoring are scarce. We demonstrated previously that Aficida® and Patton Flow® induced an increase in the frequency of MNs within circulating blood cells of *C. decemmaculatus* (Vera-Candioti et al., 2010b, 2013c). Accordingly, our current results represent the first evidence that the insecticide is able to exert genotoxic damage through inflicting primary DNA-strand breaks evaluated by the SCGE assay, at least in those erythrocytes of *C. decemmaculatus* exposed to the pirimicarb-based formulations Aficida® and Patton Flow®.

Review of the genotoxicity studies of the herbicide glyphosate was published recently (Kier and Kirkland, 2013). Although discordant results have been reported, they demonstrate the ability of the herbicide glyphosate and several glyphosate-based products to induce DNA single-strand breaks evaluated by the SCGE bioassay in several fish. Positive results have been reported in circulating erythrocytes after laboratory exposure of *Carassius auratus* when not only the MN but also the comet assay was employed as an end point (Cavaş and Könen, 2007). Furthermore, it has been reported a high rate of DNA damage revealed by the comet assay in blood and hepatic cells of *Corydoras paleatus* (de Castilhos Ghisi and Cestari, 2013), and in erythrocytes and gill cells of *Prochilodus lineatus* (Cavalcante et al., 2008). It has been also demonstrated that not only Roundup™, but also its surfactant POEA (polyethoxylated tallow amine) as well as its active ingredient are able introduce DNA primary lesions in erythrocytes (Guilherme et al., 2012b) and in addition to gill and liver cells of *Anguilla anguilla* (Guilherme et al., 2012a). Our current observations clearly demonstrate that both commercial formulations are able to induce not only an enhancement of MN frequency (Vera-Candioti et al., 2013b), but also primary DNA lesions revealed by the SCGE assay.

Our data also indicate that no differences in either CPF Zamba®- or Panzer®-induced DNA damage were observed in relation to the sampling time. On the other hand, a significant decrease in GDI was achieved in those fish exposed to Lorsban* 48E® and Aficida® during 96 h in relation to those 48 h-treated fish. Such alteration could be explained by the presence of cytotoxic potential exerted by the insecticide and inhibitory effects of the concentrations tested due to alterations in blood cell kinetics and erythrocyte replacement (Cavaş and Ergene-Gözükara, 2003; Polard et al., 2011; Vera-Candioti et al., 2013b). Then, alteration in circulating blood cell populations may results from the dynamic balance between the formation and elimination of red cells, as suggested previously (Polard et al., 2011). It is well documented that defective erythrocytes in fish exposed to xenobiotics undergo passage from the kidney into the peripheral blood, from which they are removed by the spleen (Udroiu, 2006). One possible explanation for the decrease in the frequency of damaged nucleoids found in those fish exposed to Lorsban* 48E® or Aficida® for 96 h could be an inhibition of the erythropoiesis, resulting in a cell-cycle delay. Whether or not cytotoxicity is exerted, erythropoiesis could become stimulated, and therefore a large amount of nondamaged erythrocytes could become present in the bloodstream. Therefore, damaged circulating blood cells within peripheral blood become diluted, resulting in an accumulation of nondamaged blood cells as observed at 96 h of treatment and giving a false negative result. However, there is a possibility that stimulated splenic erythrocatheretic activity could also contribute to diminishing damaged nucleoid frequency through damaged cell elimination (Polard et al., 2011). Furthermore, the possibility of a repair process of the damage reducing the level of the injury introduced into the DNA of blood cells upon increasing pesticide exposure cannot be ruled out. Previous reports in aquatic organisms, especially agrochemical-exposed fish support this concept (Ali et al., 2009; Mohanty et al., 2011; Saleha Banu et al., 2001). Finally, the possibility of activation of the microsomal cytochrome P450 family of enzymes system, very well known to participate in the detoxification process of environmental pollutants, also cannot be disregarded (Uno et al., 2012). So far, we do not have any experimental evidence explaining the reason for this particular finding, but rather some plausible hypotheses explaining the observation. Further experiments should be conducted to elucidate whether this observation is related to any of the aforementioned possibilities or whether it is the result of several independent processes occurring simultaneously.

The current observations demonstrate a significant increase in GDI achieved in 96 h-exposed fish in relation to those treated for 48 h with the pirimicarb-based insecticide Patton Flow® as well as for those exposed to the glyphosate-based herbicide Credit®. Although speculative, such observation could be explained by a toxic and inhibitory effect induced by the pesticides tested due to alterations in blood cell kinetics and erythrocyte replacement as mentioned above (Cavaş and Ergene-Gözükara, 2003; Polard et al., 2011), or by inhibition of DNA repair during the exposure time (Cavaş and Könen, 2007). Finally, numerous xenobiotics, including pesticides, can produce reactive oxygen species (ROS) via several mechanisms, e.g., inactivation of antioxidant enzymes, depletion of nonenzymatic antioxidants, and membrane lipid peroxidation, among others (Kaya and Yigit, 2012). Increased levels of ROS may result in DNA oxidation and elevated steady-state levels of unrepaired DNA resulting in an even higher negative impact into DNA (Azqueta et al., 2011; Collins et al., 2008). It has been demonstrated that chlorpyrifos and glyphosate could mediate detrimental effects on several cellular targets associated with ROS generation (Ali et al., 2009; Lee and Steinert, 2003; Lushchak et al., 2009; Mohanty et al., 2011; Saleha Banu et al., 2001). Further studies are therefore necessary in order to confirm whether the deleterious effect induced by these agrochemicals into DNA blood cells of *C. decemmaculatus* is due to the generation of ROS.

Previous results demonstrated that concentrations of 0.03 and 0.21 mg/L were determined for *C. decemmaculatus* as the LC_{50} (96 h) values for Lorsban* 48E® and CPF Zamba®, respectively, indicating that the chlorpyrifos-based insecticide Lorsban* 48E® was nearly sevenfold more toxic than CPF Zamba® (Vera-Candioti et al., 2013a). Similarly, reported recently for the same species were mean LC_{50} (96 h) values of 15.68 and 91.73 mg/L for the glyphosate-based formulations Panzer® and Credit®, respectively, pinpointing that the former glyphosate-based herbicide was nearly sixfold more toxic than Credit® (Vera-Candioti et al., 2013b). Thus, the presence of xenobiotic(s) in the formulations Lorsban* 48E® and Panzer® exerting a toxic effect themselves or an additive and/or synergistic outcome with the active ingredient has been suggested (Vera-Candioti et al., 2013a, 2010b). Our SCGE results demonstrate that a 6.5 times lower amount of Lorsban* 48E® or a 5.9 times lower concentration of Panzer® is required to induce an even higher or nearly equal level of DNA damage than that caused by CPF Zamba® or Credit®, respectively, which is in agreement with the previous remarks. To dissect out the real effects of the active ingredient from those caused by other ingredient(s) present within formulations, further experiments should be conducted using pure chlorpyrifos and glyphosate.

It is worth mentioning that the lowest chlorpyrifos concentration tested when Lorsban* 48E® was assayed was as low as 0.008 mg/L and might be considered environmentally realistic. Available data indicate that chlorpyrifos detected in surface waters is usually at

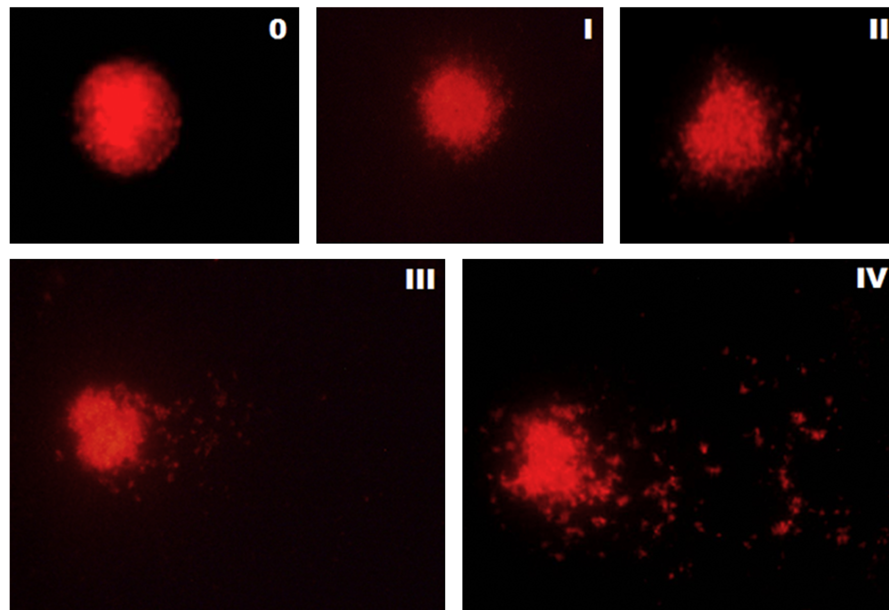

Fig. 1. Digitized comet images showing undamaged (0-I) and damaged nucleoids (II–IV) of circulating blood cells from *Cnesterodon decemmaculatus*. They represent classes 0-IV as used for visual scoring (0-I: undamaged, II: minimum damage, III: medium damage, IV: maximum damage). Cells were stained with propidium iodide and capture at fluorescent microscope. Magnification (1000 ×).

concentrations below 0.0001 mg/L, with the majority of measurements being below 0.00001 mg/L (Commission, 2005; USEPA, 1998). In the pampasic Argentinean water streams, where *C. decemmaculatus* is commonly found, concentration values between <0.002 and 0.011 mg/L has been reported (Marino and Ronco, 2005). Thus, the concentration employed in this research can be expected to be present in cultivated crop fields or in their vicinity. On the other hand, both the lowest insecticide pirimicarb (22 mg/L of Patton Flow®) and the lowest herbicide glyphosate treatments employed in this study (3.9 mg/L of Panzer®) represent a relatively high end of the environmental threshold values reported previously. Pirimicarb has been reported to be found at concentrations of 0.004 mg/L in surface water bodies (TOXNET, 2010). Unfortunately, there is no available information on the insecticide concentrations found in Argentinean pampasic habitats of *C. decemmaculatus*. For glyphosate, previous studies have reported that the maximum concentration for this herbicide found in water bodies can reach values of 3.7 mg/L (Giesy et al., 2000). Furthermore, Peruzzo et al. (2008) reported values between 0.10 to 0.70 mg/L found in pampasic Argentinean water streams. Thus, the concentrations of pirimicarb and glyphosate employed in this investigation would be expected to be rare in the environment, perhaps only observed when specific events occurred, e.g., a direct application adjacent to surface waters in creeks, ponds, and drainage ditches by accidental discharge or spills, among others. Although, we cannot rule out that fish populations and also occupationally exposed human workers could be exposed accidentally to these agrochemicals at this range of concentrations.

Changes in population dynamics in several fish species, with the phenomenon being in most cases associated with pollution of agricultural areas or water reservoirs with pesticides, are known worldwide. Furthermore, other factors, e.g., overexploitation, diseases, changes in reproductive patterns, and/or habitat loss, can also be the result of this situation. Among others, negative effects exerted by these factors against wild populations of *Gambusia holbrooki* (Edwards et al., 2010), *Thunnus thynus* (Dickhut et al., 2009), *Merluccius merluccius* (Bodiguel et al., 2009), *Oryzias latipes* (Zhang et al., 2008), *Solea solea* (Dierking et al., 2009), and *Acipenser transmontanus* (Gundersen et al., 2008), have been reported.

Finally, the induction of genetic injury into DNA after chronic exposure to agrochemicals is, perhaps, the most relevant biological effect. The results we obtained with *C. decemmaculatus* exposed under laboratory conditions to chlorpyrifos-, pirimicarb-, and glyphosate-based pesticides highlight that this native species provides a suitable and useful experimental model for biomonitoring aquatic ecosystems.

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References

Ali, D., El-Shehawi, A.M., Seehy, M.A., 2008a. Micronucleus test in fish genome: a sensitive monitor for aquatic pollution. Afr. J. Biotechnol 7, 606–612.
Ali, D., Nagpure, N.S., Kumar, S., Kumar, R., Kushwaha, B., 2008b. Genotoxicity assessment of acute exposure of chlorpyrifos to freshwater fish *Channa punctatus* (Bloch) using micronucleus assay and alkaline single-cell gel electrophoresis. Chemosphere 71, 1823–1831.
Ali, D., Nagpure, N.S., Kumar, S., Kumar, R., Kushwaha, B., Lakra, W.S., 2009. Assessment of genotoxic and mutagenic effects of chlorpyrifos in freshwater fish *Channa punctatus* (Bloch) using micronucleus assay and alkaline single-cell gel electrophoresis. Food Chem. Toxicol. 47, 650–656.
Azqueta, A., Meier, S., Priestley, C., Gutzkow, K.B., Brunborg, G., Sallette, J., Soussaline, F., Collins, A.R., 2011. The influence of scoring method on variability in results obtained with the comet assay. Mutagenesis 26, 393–399.
Barsiene, J., Dedonyte, V., Rybakovas, A., Andreikenaite, L., Andersen, O.K., 2006. Investigation of micronuclei and other nuclear abnormalities in peripheral blood and kidney of marine fish treated with crude oil. Aquat. Toxicol. 78S, S99–S104.
Bodiguel, X., Loizeau, V., Le Guellec, A.M., Roupsard, F., Philippon, X., Mellon-Duval, C., 2009. Influence of sex, maturity and reproduction on PCB and *p,p′*DDE concentrations and repartitions in the European hake (*Merluccius merluccius*, L.) from the Gulf of Lions (N.W. Mediterranean). Sci. Total Environ. 408, 304–311.
Cavalcante, D.G., Martinez, C.B., Sofia, S.H., 2008. Genotoxic effects of Roundup on the fish *Prochilodus lineatus*. Mutat. Res. 655, 41–46.
Cavaş, T., 2008. *In vivo* genotoxicity of mercury chloride and lead acetate: micronucleus test on acridine orange stained fish cells. Food Chem. Toxicol. 46, 352–358.

Cavaş, T., 2011. *In vivo* genotoxicity evaluation of atrazine and atrazine-based herbicide on fish *Carassius auratus* using the micronucleus test and the comet assay. Food Chem. Toxicol. 49, 1431–1435.

Cavaş, T., Ergene-Gözükara, S., 2003. Evaluation of the genotoxic potential of lambda-cyhalothrin using nuclear and nucleolar biomarkers on fish cells. Mutat. Res. 534, 93–99.

Cavaş, T., Ergene-Gözükara, S., 2005. Micronucleus test in fish cells: a bioassay for *in situ* monitoring of genotoxic pollution in the marine environment. Environ. Mol. Mutagen 46, 64–70.

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

Collins, A.R., Oscoz, A.A., Brunborg, G., Gaivão, I., Giovannelli, L., Kruszewski, M., Smith, C.C., Stetina, R., 2008. The comet assay: topical issues. Mutagenesis 23, 143–151.

Commission, E., 2005. Chlorpyrifos. SANCO/3059/99—Rev. 1.5. European Commission. Health and Consumer Protection Directotrate-General. Food Safety: Production and Distribution Chain. Unit D.3—Chemicals, Contaminants and Pesticides.

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

de la Torre, F.R., Salibián, A., Ferrari, L., 2007. Assessment of the pollution impact on biomarkers of effect of a freshwater fish. Chemosphere 68, 1582–1590.

de Lemos, C.T., Iranço, F.d.A., de Oliveira, N.C., de Souza, G.D., Fachel, J.M., 2008. Biomonitoring of genotoxicity using micronuclei assay in native population of *Astyanax jacuhiensis* (*Characiformes*: *Characidae*) at sites under petrochemical influence. Sci. Total Environ. 406, 337–343.

Di Marzio, W., Sáenz, M., Alberdi, J., Tortorelli, M., Galassi, S., 2005. Risk assessment of domestic and industrial effluents unloaded into a freshwater environment. Ecotoxicol. Environ. Saf. 61, 380–391.

Dickhut, R.M., Deshpande, A.D., Cincinelli, A., Cochran, M.A., Corsolini, S., Brill, R.W., Secor, D.H., Graves, J.E., 2009. Atlantic bluefin tuna (*Thunnus thynnus*) population dynamics delineated by organochlorine tracers. Environ. Sci. Technol. 43, 8522–8527.

Dierking, J., Wafo, E., Schembri, T., Lagadec, V., Nicolas, C., Letourneur, Y., Harmelin-Vivien, M., 2009. Spatial patterns in PCBs, pesticides, mercury and cadmium in the common sole in the NW Mediterranean Sea, and a novel use of contaminants as biomarkers. Mar. Pollut. Bull. 58, 1605–1614.

Edwards, T.M., Toft, G., Guillette, L.J.J., 2010. Seasonal reproductive patterns of female *Gambusia holbrooki* from two Florida lakes. Sci. Total Environ. 408, 1569–1576.

Frenzilli, G., Nigro, M., Lyons, B.P., 2009. The comet assay for the evaluation of genotoxic impact in aquatic environments. Mutat. Res. 681, 80–92.

Giesy, J.P., Dobson, S., Solomon, K.R., 2000. Ecotoxicological risk assessment for Roundup herbicide. Rev. Environ. Contam. Toxicol. 167, 35–120.

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

Guilherme, S., Santos, M.A., Barroso, C., Gaivão, I., Pacheco, M., 2012b. Differential genotoxicity of Roundup® formulation and its constituents in blood cells of fish (*Anguilla anguilla*): considerations on chemical interactions and DNA damaging mechanisms. Ecotoxicology 21, 1381–1390.

Gundersen, D.T., Webb, M.A., Fink, A.K., Kushner, L.R., Feist, G.W., Fitzpatrick, M.S., Foster, E.P., Schreck, C.B., 2008. Using blood plasma for monitoring organochlorine contaminants in juvenile white sturgeon, *Acipenser transmontanus*, from the lower Columbia River. Bull. Environ. Contam. Toxicol. 81, 225–229.

Kaya, A., Yigit, E., 2012. Interactions among glutathione s-transferase, glutathione reductase activity and glutathione contents in leaves of *Vicia faba* L. subjected to flurochloridone. Fresenius Environ. Bull 21, 1635–1640.

Kier, L.D., Kirkland, D.J., 2013. Review of genotoxicity studies of glyphosate and glyphosate-based formulations. Crit. Rev. Toxicol., 1–33. (Early online).

Könen, S., Cavaş, T., 2008. Genotoxicity testing of the herbicide trifluralin and its commercial formulation Treflan using the piscine micronucleus test. Environ. Mol. Mutagen 49, 434–438.

Lee, R.F., Steinert, S., 2003. Use of the single cell gel electrophoresis/comet assay for detecting DNA damage in aquatic (marine and freshwater) animals. Rev. Mutat. Res. 544, 43–64.

Lushchak, O.F., Kubrak, O., Storey, J., Storey, K.B., Lushchak, V.I., 2009. Low toxic herbicide Roundup induces mild oxidative stress in goldfish tissues. Chemosphere 76, 932–937.

Marino, D., Ronco, A., 2005. Cypermethrin and chlorpyrifos concentration levels in surface water bodies of the Pampa Ondulada, Argentina. Bull. Environ. Contam. Toxicol. 75, 820–826.

Marlin, D.J., Johnson, L., Kingston, D.A., Smith, N.C., Deaton, C.M., Mann, S., Heaton, P., Van Vugt, F., Saunders, K., Kydd, J., Harris, P.A., 2004. Application of the comet assay for investigation of oxidative DNA damage in equine peripheral blood mononuclear cells. J. Nutr. 134, 2133S–2140S.

Menéndez-Helman, R.J., Ferreyroa, G.V., dos Santos Afonso, M., Salibián, A., 2012. Glyphosate as an acetylcholinesterase inhibitor in *Cnesterodon decemmaculatus*. Bull. Environ. Contam. Toxicol. 88, 6–9.

Menni, R.C., Gómez, S.E., López Armengol, F., 1996. Subtle relationships: freshwater fishes and water chemistry in southern South America. Hydrobiologia 328, 173–197.

Mohanty, G., Mohanty, J., Nayak, A.K., Mohanty, S., Dutta, S.K., 2011. Application of comet assay in the study of DNA damage and recovery in rohu (*Labeo rohita*) fingerlings after an exposure to phorate, an organophosphate pesticide. Ecotoxicology 20, 283–292.

Pantaleão, S.d.M., Alcântara, A.V., Alves Jdo, P., Spanó, M.A., 2006. The piscine micronucleus test to assess the impact of pollution on the Japaratuba river in Brazil. Environ. Mol. Mutagen. 47, 219–224.

Peruzzo, P.J., Porta, A.A., Ronco, A.E., 2008. Levels of glyphosate in surface waters, sediments and soils associated with direct sowing soybean cultivation in north pampasic region of Argentina. Environ. Pollut. 156, 61–66.

Pitarque, M., Creus, A., Marcos, R., Hughes, J.A., Anderson, D., 1999. Examination of various biomarkers measuring genotoxic endpoints from Barcelona airport personnel. Mutat. Res. 440, 195–204.

Polard, T., Jean, S.,L.,G., Laplanche, C., Merlina, G., Sánchez-Pérez, J.M., Pinelli, E., 2011. Mutagenic impact on fish of runoff events in agricultural areas in south-west France. Aquat. Toxicol. 101, 126–134.

Poletta, G.L., Kleinsorge, E., Paonessa, A., Mudry, M.D., Larriera, A., Siroski, P.A., 2011. Genetic, enzymatic and developmental alterations observed in *Caiman latirostris* exposed *in ovo* to pesticide formulations and mixtures in an experiment simulating environmental exposure. Ecotoxicol. Environ. Saf. 74, 852–859.

Saleha Banu, B., Danadevi, K., Rahman, M.F., Ahuja, Y.R., Kaiser, J., 2001. Genotoxic effect of monocrotophos to sentinel species using comet assay. Food Chem. Toxicol. 39, 361–366.

Singh, N.P., McCoy, M.T., Tice, R.R., Schneider, E.L., 1988. A simple technique for quantitation of low levels of DNA damage in individual cells. Exp. Cell Res. 175, 184–191.

TOXNET, 2010. Pirimicarb. Toxicological Data Network. Hazardous Substances Databank Number: 7005.

Udroiu, I., 2006. The micronucleus test in piscine erythrocytes. Aquat. Toxicol. 79, 201–204.

Uno, T., Ishizuka, M., Itakura, T., 2012. Cytochrome P450 (CYP) in fish. Rev. Art. Environ. Toxicol. Pharmacol 34, 1–3.

USEPA, 1975. Methods for Acute Toxicity Tests with Fish, Macroinvertebrates, and Amphibians. United States Environmental Protection Agency, Corvallis, Oregon. (EPA-660/3-75-009, 67).

USEPA, 1998. Drinking Water Assessment of Chlorpyrifos. US Environmental Protection Agency, Office of Prevention, Pesticides and Toxic Substances, Washington, DC, USA.

USEPA, 2002. Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms. United States Environmental Protection Agency p. 266. (Report No. EPA 821-R-02-012).

Valverde, M., Rojas, E., 2009. Environmental and occupational biomonitoring using the Comet assay. Mutat. Res. 681, 93–109.

Vera-Candioti, J., Natale, G., Soloneski, S., Ronco, A.E., Larramendy, M.L., 2010a. Sublethal and lethal effects on *Rhinella arenarum* (Anura, *Bufonidae*) tadpoles exerted by the pirimicarb-containing technical formulation insecticide Aficida®. Chemosphere 78, 249–255.

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

Vera-Candioti, J., Soloneski, S., Larramendy, M.L., 2013a. Chlorpyrifos-based insecticides induced genotoxic and cytotoxic effects in the ten spotted live-bearer fish, *Cnesterodon decemmaculatus* (Jenyns, 1842). Environ. Toxicol, http://dx.doi.org/10.1002/tox.21869, (In Press).

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

Vera-Candioti, J., Soloneski, S., Larramendy, M.L., 2013c. Pirimicarb-based formulation-induced genotoxicity and cytotoxicity on the fresh water fish *Cnesterodon decemmaculatus* (Jenyns, 1842) (Pisces, *Poeciliidae*). Toxicol. Ind. Health, http://dx.doi.org/10.1177/0748233713486958, (In Press).

WHO-FAO, Pesticides Residues in Food. FAO Plant Production and Protection Paper Vol. 196. World Health Organization and Food and Agriculture Organization of the United Nations, Rome, 2009, pp. 1-426.

Yin, X., Zhu, G., Li, X.B., Liu, S., 2009. Genotoxicity evaluation of chlorpyrifos to amphibian Chinese toad (*Amphibian*: Anura) by comet assay and micronucleus test. Mutat. Res. 680, 2–6.

Zhang, Z., Hu, J., Zhen, H., Wu, X., Huang, C., 2008. Reproductive inhibition and transgenerational toxicity of triphenyltin on medaka (*Oiyzias latipes*) at environmentally relevant levels. Environ. Sci. Technol. 42, 8133–8139.