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Evaluation of the genotoxic and cytotoxic effects of glyphosate-based herbicides in the ten spotted live-bearer fish *Cnesterodon decemmaculatus* (Jenyns, 1842)

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ABSTRACT

Mortality, genotoxicity, and cytotoxicity of the 48% glyphosate-based formulations Panzer and Credit® were evaluated on *Cnesterodon decemmaculatus* (Jenyns, 1842) (Pisces, Poeciliidae) under laboratory conditions. Induction of micronuclei (MN) and alterations in the erythrocytes:erythroblasts ratio were employed as end points for genotoxicity and cytotoxicity, respectively. For Panzer®, mean values of 16.70 and 15.68 mg/L were determined for LC_{50} at 24 and 96 h, respectively, and these concentrations reached mean values of 98.50 and 91.73 mg/L for Credit®. LC_{50} values decreased as a negative linear function of Panzer® exposure time within the 0–96 h period, but not for Credit®. LC_{50} values indicated that the fish were more sensitive to Panzer® than to Credit®. Both 3.9 and 7.8 mg/L of Panzer® increased MN frequency at 48 and 96 h of treatment. When fish were exposed to Credit®, an increased frequency of MN over control values was found after 96 h for all concentrations assayed, but not after 48 h. No cellular cytotoxicity was found after Panzer® and Credit® treatment, regardless of both the concentration and the sampling time. Furthermore, our results demonstrated that Panzer® and Credit® should be considered as glyphosate-based commercial formulations with genotoxic but not cytotoxic effect properties.

1. Introduction

Pesticides are ubiquitous on the planet, and they are employed to control or eliminate a variety of agricultural and household pests that can damage crops and livestock and reduce productivity. Despite the many benefits of the use of pesticides in crop fields and their 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 due to the contamination of food, water, and air (WHO-FAO, 2009). Anthropic activities are continuously introducing extensive amounts of these compounds into the environment regardless of their persistence, bioaccumulation, and toxicity. However, it is well known that pesticides not only affect target organisms, but concomitantly exert side effects on nontarget organisms (www.epa.gov/pesticides).

Glyphosate [*N*-(phosphonomethyl) glycine], usually employed in the form of a glyphosate isopropylamine salt, is a nonselective and postemergent broad-spectrum herbicide used for inhibition of unwanted weeds and grasses in agricultural, industrial, urban, and forest areas. It is also used for aquatic weed control in fish ponds, lakes, canals, and slow-running water reservoirs (Tsui and Chu, 2008). Worldwide, glyphosate is the most widely used herbicide, and it is considered as one of the world's leading agrochemicals reported so far. Furthermore, it is the second most commonly used herbicide around and in homes and gardens (Monsanto, 2005b). According to Monsanto (2005b) Company, there are no more approved uses for glyphosate than for any other herbicide. So far, the available information reported by the United States Environmental Protection Agency (EPA) indicates that more than 400 formulated products containing glyphosate as an active ingredient have been registered in more than 100 countries worldwide (EPA, 2009). Its major formulation is Roundup®, in which glyphosate is presented as isopropylamine salt, and a surfactant, polyoxyethylene amine (POEA), is added to enhance the herbicidal efficacy (Franz, 1985). These glyphosate-based herbicides are major pollutants of rivers and surface waters (Hanke et al., 2010). They exert toxic effects on organisms, including humans, and contaminate food and ecosystems (Jan et al., 2009; Low et al., 2004). Their use and presence within the food chain are further enhanced by the fact that more than 75% of genetically altered edible plants have been designed to tolerate high levels of these commercialized formulations (Wilson et al., 2011). The main breakdown product of glyphosate in plants, soil, and water is aminomethylphosphonic acid (AMPA) (EPA, 1993).

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Glyphosate inhibits plant growth through a competitive inhibition of the 5-enolpyruvil shikimate-3-phosphate synthase, essential for the synthesis of certain essential aromatic amino acids in plants as well as in bacteria. This enzyme is specifically responsible for the biosynthesis of chorismate, an intermediate in phenylalanine, tyrosine, and tryptophan biosynthesis (Sharps, 1984). This biosynthetic shikimate pathway is not shared by members of the animal kingdom, so the herbicide is claimed to be, then, selectively toxic to plants but relatively nontoxic to animals (Bolognesi et al., 1997). Connections between aerial spraying with glyphosate added to a surfactant solution and genetic alterations in an Ecuadorian population (Paz y Miño et al., 2007) and cancer incidence, including non-Hodgkin's lymphoma, among glyphosate-exposed pesticide users (De Roos et al., 2003; Eriksson et al., 2008) have been reported. At the cellular level, an increase in chromosomal aberrations and sister chromatid exchanges as well as cytotoxicity measured by cell viability and mitotic activity have been observed in mammalian cells *in vitro* (Lioi et al., 1998; Mañas et al., 2009), demonstrating the ability of glyphosate to exert both cytotoxic and genotoxic effects in mammal cells. However, negative results have been also reported for the same end points (Li and Long, 1988). On the other hand, glyphosate has been reported to introduce DNA damage in a variety of cells and organisms, including humans and other animal and plant species (Álvarez-Moya et al., 2011; Cavalcante et al., 2008; Cavaş and Könen, 2007; Mañas et al., 2009; Mladinic et al., 2009; Mohamed, 2011; Poletta et al., 2009, 2011).

The ten spotted live-bearer, *Cnesterodon decemmaculatus* (Jenyns, 1842) (Pisces, Poeciliidae), is an endemic fish 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, benthicpelagic, 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 (Carriquiriborde et al., 2007; de la Torre et al., 2007; Di Marzio et al., 2005; Menéndez-Helman et al., 2012; Vera-Candioti et al., 2010b).

Therefore, attempts have been made in the current study to characterize the lethal and sub-lethal toxicity of Panzer® and Credit®, two glyphosate-based herbicides, on *C. decemmaculatus* exposed under laboratory conditions. Whereas LC_{50} estimation was employed as biomarker for lethality, induction of micronuclei (MN) and alterations in the eryrthrocyte:erythroblast ratio were employed as biomarkers of genotoxicity and cytotoxicity, respectively.

2. Materials and methods

2.1. Chemicals

Two commercial formulations of glyphosate namely Panzer® (Dow Agrosciences Argentina S.A., Buenos Aires, Argentina) and Credit® (Nufarm S.A., Buenos Aires, Argentina) containing isopropylamine salt of glyphosate at 480 g L^{-1} as active ingredient (equivalent to 48% of glyphosate; excipients c.s.) were used. Cyclophosphamide (CAS 6055-19-2) was purchased from Sigma Chemical Co. (St. Louis, MO) whereas $K_2Cr_2O_7$ [Cr(VI)] (CAS 7778-50-9) was obtained from Merck KGaA (Darmstadt, Germany). All other chemicals and solvents were of analytical grade.

2.2. Quality control

Concentration levels of glyphosate in test solutions were analyzed by QV Chem Laboratory (La Plata, Buenos Aires, Argentina) according to OSHA Analytical Method PV2067. Glyphosate levels were analyzed by high performance liquid chromatography (HPLC) using an ultraviolet detector (UV) and derivatization with fluorenylmethyloxycarbonyl (FMOC). Glyphosate samples from test solution correspond to the initial time and 24 h after treatment. The detection limit for glyphosate was 0.2 mg/L.

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 days to 16/8 h light/dark cycles in aquaria at 21 ± 1 °C in dechlorinated tap water (pH 7.6–8.3; hardness, 143 mg/L $CaCO_3$) and artificial aeration. Male and females were maintained separately and fed *ad libitum* daily with commercially available fish food (TetraMin®, TetraWerke, 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. Determination of LC_{50}

Concentrations assessed throughout the study represent the nominal concentrations of active ingredient present within Panzer® and Credit® glyphosate-based formulations. Experiments were carried out for toxicity assessment following recommendations proposed by the EPA standardized methods for acute toxicity tests (EPA, 1975, 2002). Briefly, for each experimental point, 10 specimens (five males and five nongravid females) were maintained in a 1 L glass container and exposed to 10 different concentrations of Panzer® (0.1, 1.0, 10.0, 12.0, 14.0, 16.0, 20.0, 40.0, 60.0, and 100.0 mg/L) or Credit® (0.1, 1.0, 10.0, 40.0, 70.0, 80.0, 85.0, 100.0, 150.0, and 200.0 mg/L) during 96 h, with test solutions replaced every 24 h. While negative control group consisted of 10 organisms kept in dechlorinated tap water (pH 7.55 ± 0.1; dissolved oxygen 6.3 ± 0.3 mg/L; ammonium (NH_4^+) < 0.2 mg/L and hardness 143 ± 23.5 mg $CaCO_3$/L), positive control group consisted of 10 fishes treated with 21.4 mg Cr(VI)/L as previously reported (Vera-Candioti et al., 2011). All treatments were performed in triplicate. Fish were not fed throughout the experiment. A lethal effect was determined as the toxicity end point. Fish were visually examined daily and considered dead when either no respiratory movements were observed or there was a lack of sudden swimming in response to gentle touching compared to control organisms.

2.5. Determination of MN frequency

Each experiment was conducted using five fish following the same experimental design described in Section 2.4, with specimens exposed to three different concentrations of test compound equivalent to 25%, 50%, and 75% of the corresponding LC_{50} 96 h value. To achieve these concentrations, fish were exposed to 3.9, 7.8, and 11.8 mg/L or 22.9, 45.9, and 68.8 mg/L Panzer® or Credit®, respectively (see Section 3.1). Negative (dechlorinated tap water; see Section 2.3) and positive controls (5 mg cyclophosphamide/L) were conducted and run simultaneously with Panzer®- and Credit®-exposed fish as reported previously (Vera-Candioti et al., 2010b). The frequency of MN was determined in peripheral mature erythrocytes at 48 and 96 h after initial treatment. Experiments were performed in triplicate and run simultaneously.

Fish were killed by severing the spinal column behind the opercula. Two drops of peripheral blood from each specimen were smeared onto precleaned slides. Afterward, slides were air dried, fixed with 100% (v/v) cold methanol (4 °C), and stained with 5% Giemsa solution. Slides were coded and blind-scored at 1000× magnification. The frequency of MN was determined by analyzing 1500 mature erythrocytes from each fish as suggested previously (Cavaş and Könen, 2007; Vera-Candioti et al., 2010b) and is expressed as the total number of MN per 1000 cells. The frequency of MN was determined following previously reported examination criteria (Cavaş and Könen, 2007; Vera-Candioti et al., 2010b). Briefly, criteria for MN identification in erythrocytes were as follows: diameter smaller than one-third of the main nuclei diameter, nonrefractability, staining intensity similar to or lighter than that of the main nuclei, no connection or link with the main nuclei, no overlapping with the main nuclei, and an MN boundary distinguishable from the main nuclei boundary (Fenech et al., 2003).

2.6. Analysis of erythrocytes:erythroblasts ratio

The circulating erythrocyte:erythroblast ratio was determined as described previously for xenobiotic-exposed aquatic organisms (Vera-Candioti et al., 2010a,b). Briefly, frequencies of mature erythrocytes and erythroblasts were blind-determined by one researcher at 1000× magnification by analyzing a total of 1500 erythrocyte:erythroblast cells from each fish specimen in those slides employed for MN analysis, and they are expressed as the total number of erythrocytes and erythroblasts per 1000 cells.

2.7. Statistical analyses

Data of lethality tests were analyzed using the EPA Probit Analysis, Version 1.5 statistical software (http://www.epa.gov/nerleerd/stat2.htm#tsk) and based on Finney's method (Finney, 1971). 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, non-parametric tests were used in order to detect differences. The Kruskal-Wallis test and the one-tailed Mann-Whitney *U* test for independent samples were applied to assess differences between treated and control groups. The relationship between mortality, MN frequency, and circulating erythrocyte:erythroblast ratios with herbicide concentrations was evaluated by simple linear regression analyses. The level of significance was $\alpha = 0.05$.

3. Results

3.1. Mortality assays

Results of chemical analyses showed no differences between both used and measured glyphosate concentrations or during the 24 h interval renewals of the testing solutions for both commercial formulations evaluated.

Probit analysis of the mortality experiment allowed determination of the LC_{50} values of Panzer® after 24, 48, 72, and 96 h of exposure, with mean values of 16.70 mg/L (range, 15.97–17.60 mg/L), 16.15 mg/L (range, 15.50–16.93 mg/L), 16.07 mg/L (range, 15.42–16.83 mg/L), and 15.68 mg/L (range, 15.02–16.45 mg/L), respectively. Overall, all treatments induced a significant concentration-dependent increase in mortality rate ($r = 0.76$, $p < 0.01$). In contrast, no significant time-dependent decrease was observed in mortality rate when the exposure time increased from 24 to 96 h ($r = -0.77$, $p > 0.05$).

Mortality experiments revealed the LC_{50} values of Credit® after 24, 48, 72, and 96 h of exposure, with mean values of 98.50 mg/L (range, 93.60–105.80 mg/L), 93.73 mg/L (range, 89.10–100.20 mg/L), 91.73 mg/L (range, 86.80–98.00 mg/L), and 91.73 mg/L (range, 86.80–98.00 mg/L), respectively. A concentration-dependent increase was observed in mortality rate ($r = 0.92$, $p < 0.001$). On the other hand, no significant time-dependent decrease was registered in mortality rate when the exposure time increased from 24 to 96 h ($r = -0.83$, $p > 0.05$).

3.2. Genotoxicity and cytotoxicity assays

No mortality was registered during the experiments. The frequency of MN in cyclophosphamide-exposed specimens of *C. decemmaculatus* was significantly increased compared to negative control values when the analysis was performed at 48 h ($p < 0.05$) or 96 h ($p < 0.01$) of treatment in Panzer® experiments (Fig. 1), as well as at 48 h ($p < 0.01$) or 96 h ($p < 0.01$) of treatment in Credit® experiments (Fig. 2).

Fig. 1 summarizes the results of the analysis of Panzer®-induced MN in circulating erythrocytes of exposed fish. At 48 h of treatment, a significant increase in MN frequency was observed in fish treated with Panzer® concentrations of 3.9 mg/L ($p < 0.05$) and 7.8 mg/L ($p < 0.05$), but not in specimens treated with 11.8 mg/L ($p > 0.05$), compared to negative control values. Similarly, the results demonstrate that the frequency of MN values in fish exposed for 96 h to Panzer® were enhanced compared to negative control values only after exposure with 3.9 mg/L ($p < 0.05$) and 7.8 mg/L ($p < 0.05$) (Figs. 1 and 5).

Overall, a regression analysis revealed that the increase in MN frequency was not affected by Panzer® concentrations neither at 48 h ($r = -0.02$, $p > 0.05$) nor at 96 h of exposure ($r = -0.23$, $p > 0.05$) (Fig. 1). On the other hand, MN frequency induced by Panzer® decreased as a function of the exposure time ($r = -0.21$, $p < 0.05$).

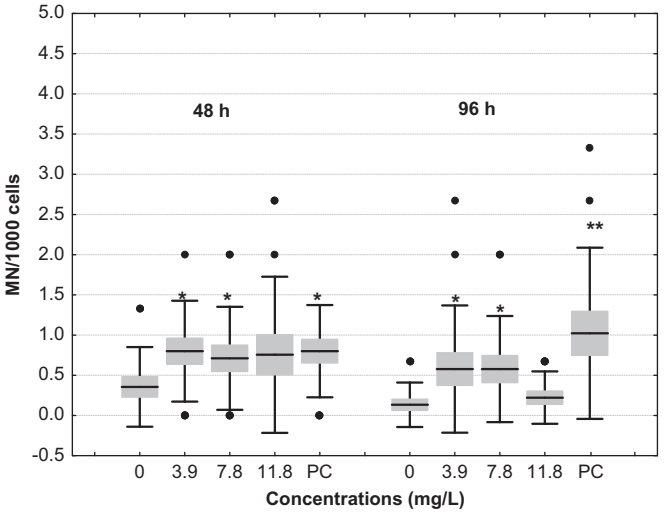

Fig. 1. Frequency of MN in circulating erythrocytes from *C. decemmaculatus* specimens exposed to different Panzer® concentrations. The frequency of MN was determined at 48 and 96 h after initial treatment by analyzing 1500 erythrocytes from each treated fish. The data are displayed as box plots, where the number of MN/1000 cells (*y*-axis) is plotted against Panzer® concentration (*x*-axis). Each box encloses 50% of the data, with the mean value of the variable displayed as a line. The top and the bottom of the box mark the limits ± 25% of the variable population. The lines extending from the top and the bottom of each box mark the minimum and maximum values that fall within an acceptable range. Any value outside this range is displayed as an individual point (black circles).

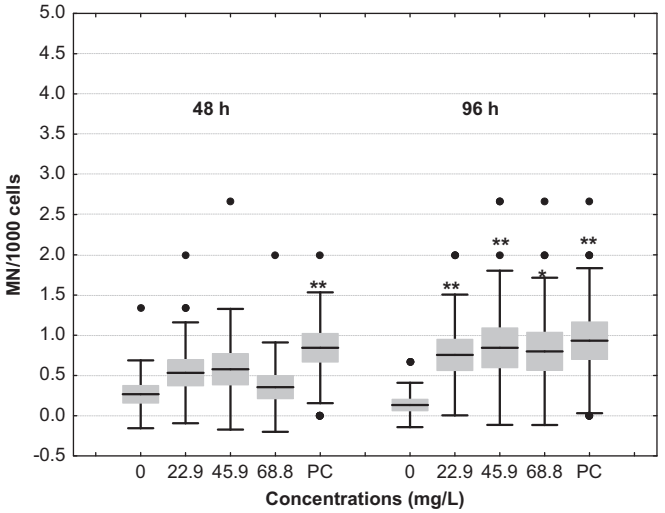

Fig. 2. Frequency of MN in circulating erythrocytes from *C. decemmaculatus* specimens exposed to different Credit® concentrations. The frequency of MN was determined at 48 and 96 h after initial treatment by analyzing 1500 erythrocytes from each treated fish. The data are displayed as box plots, where the number of MN/1000 cells (*y*-axis) is plotted against Credit® concentration (*x*-axis). Each box encloses 50% of the data, with the mean value of the variable displayed as a line. The top and the bottom of the box mark the limits ± 25% of the variable population. The lines extending from the top and the bottom of each box mark the minimum and maximum values that fall within an acceptable range. Any value outside this range is displayed as an individual point (black circles). Cyclophosphamide (5 mg/L) was used as positive control (PC). (*), $p < 0.05$; (**), $p < 0.01$.

The results of the analysis of Credit®-induced MN in circulating erythrocytes of exposed fish are summarized in Fig. 2. Only after 96 h of treatment a significant increase in MN frequency was observed in fish treated with concentrations of 22.9 mg/L ($p < 0.01$), 45.9 mg/L ($p < 0.01$), and 68.8 mg/L ($p < 0.05$) compared to negative control values. A regression analysis revealed

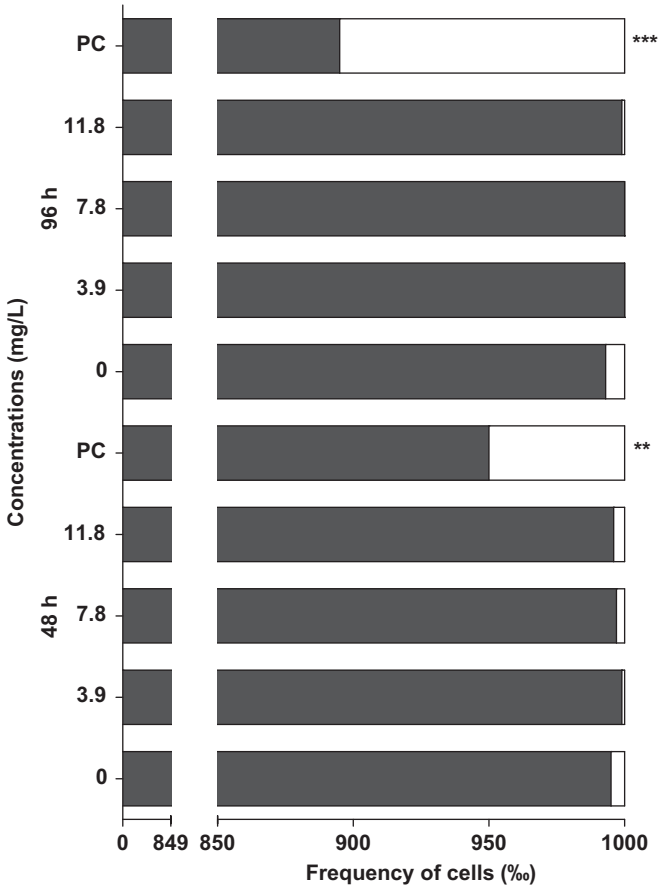

Fig. 3. Frequency of circulating erythrocytes (gray bar areas) and erythroblasts (white bar areas) from *C. decemmaculatus* specimens exposed to different Panzer® concentrations. The frequency of erythrocytes and erythroblasts was determined at 48 and 96 h after initial treatment. The erythrocyte and erythroblast frequencies were determined by analyzing 1500 cells from each fish and are expressed as the total number of erythrocytes/erythroblasts in 1000 cells. Cyclophosphamide (5 mg/L) was used as positive control (PC). (**), $p < 0.01$; (***), $p < 0.001$.

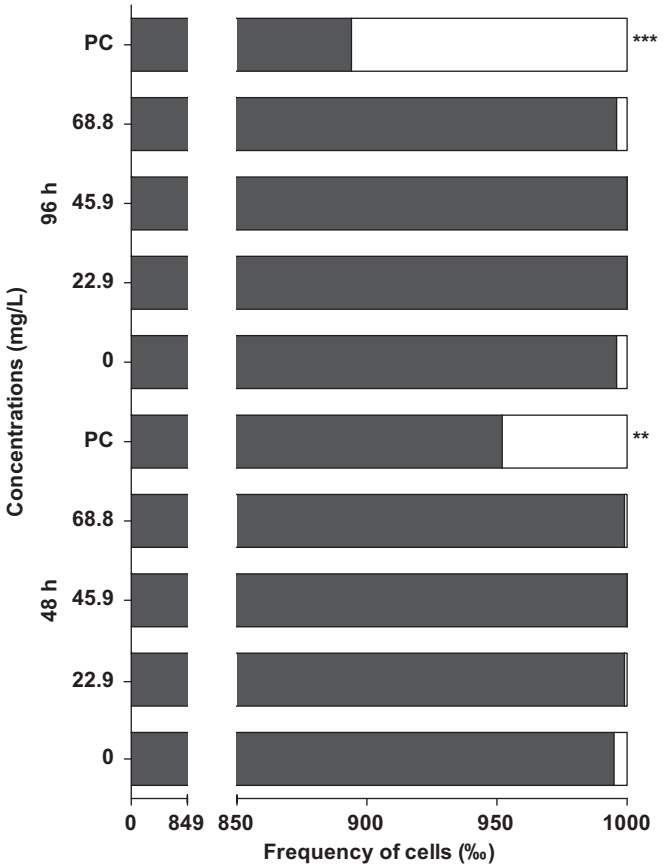

Fig. 4. Frequency of circulating erythrocytes (gray bar areas) and erythroblasts (white bar areas) from *C. decemmaculatus* specimens exposed to different Credit® concentrations. The frequency of erythrocytes and erythroblasts was determined at 48 and 96 h after initial treatment. The erythrocyte and erythroblast frequencies were determined by analyzing 1500 cells from each fish and are expressed as the total number of erythrocytes/erythroblasts in 1000 cells. Cyclophosphamide (5 mg/L) was used as positive control (PC). (**), $p < 0.01$; (***), $p < 0.001$.

the absence of a relationship between MN frequency and Credit® concentrations after 96 h of treatment period ($r = 0.02$, $p > 0.05$). On the other hand, no alteration in the basal frequency of MN was observed in those fish treated during 48 h, regardless of Credit® concentration ($p > 0.05$) (Figs. 2 and 5).

Erythrocyte:erythroblast ratios in specimens of *C. decemmaculatus* exposed to cyclophosphamide in both Panzer® and Credit® experiments are summarized in Figs. 3 and 4, respectively. A significant decrease and a concomitant increase in the frequency of erythrocytes and erythroblasts, respectively, were observed in the blood of those fish exposed for 48 h ($p < 0.01$) and 96 h ($p < 0.001$) in both Panzer® (Fig. 3) and Credit® experiments (Fig. 4). On the other hand, the results showed that neither Panzer® (Fig. 3) nor Credit® (Fig. 4) modified the frequency of erythrocytes and erythroblasts compared to negative controls, regardless of both the concentration and the sampling time ($p > 0.05$).

4. Discussion

The acute lethal toxicity, genotoxicity, and cytotoxicity of the 48% glyphosate-containing technical formulation herbicides Panzer® and Credit® were evaluated on specimens from *C. decemmaculatus* (Pisces, Poeciliidae) exposed under laboratory conditions. Regarding the LC_{50} 96 h values obtained in the present study, Panzer® and Credit® can be classified as slightly and practically nontoxic agrochemicals, respectively, according the scoring used by the Office of Pollution Prevention and Toxics of the EPA (Wagner et al., 1995). However, according the scoring used by the World Health Organization (Smrchek et al., 1993; Wagner et al., 1995), both Panzer® and Credit® can be classified as toxic compounds. Reviews on the safety of glyphosate, including major glyphosate-based marketed herbicides, have been conducted by several regulatory agencies (*e.g.*, the EPA, WHO-FAO, and European Union), who have concluded that there is no indication of any human health concern, with the EPA even classifying glyphosate as a Class E agent with evidence of non-carcinogenicity for humans (Monsanto, 2005a). Nevertheless, recent investigations have clearly indicated that this herbicide is not as safe as previously reported (Çağlar and Kolankaya, 2008; Isenring, 2004). Our current observations are in agreement with this classification and verify these latter observations.

The LC_{50} values at 96 h in the current study were 15.68 and 91.73 mg/L for Panzer® and Credit®, respectively. These findings demonstrated that Panzer® was more toxic than Credit® to *C. decemmaculatus*. In other words, *C. decemmaculatus* was found to be more sensitive to Panzer® than to Credit®. For both glyphosate-based formulations, mortality was found to be concentration-dependent, thus accounting for differences in LC_{50} values at 24–96 h obtained at different concentrations and times of exposure.

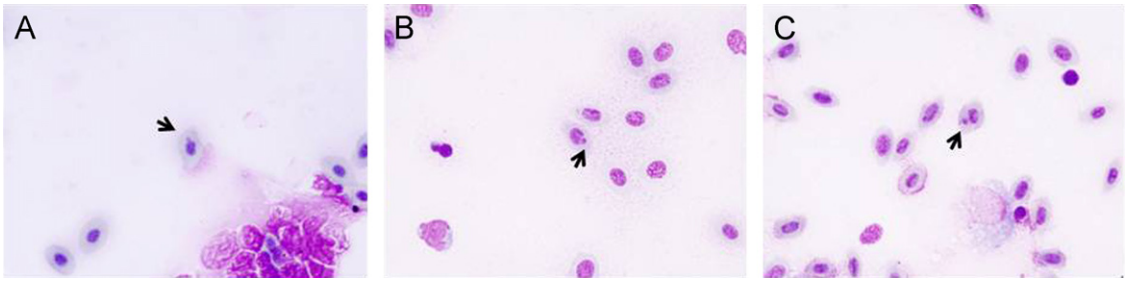

Fig. 5. Photomicropraphs from blood smear of *C. decemmaculatus* showing mature erythrocytes exhibiting micronucleus (arrow). Cells were stained with 5% Giemsa and viewed at 1000 times magnification. Cells are approximately 10 μm along the long axis.

Comparing the LC_{50} values at 96 h that we observed for *C. decemmaculatus* exposed to two glyphosate-based herbicides with those from the literature for several glyphosate-treated fish species in standardized toxicity tests, *C. decemmaculatus* is within the range between the most and least sensitive fish. The LC_{50} values found were greater than those reported for the silver catfish *Rhamdia quelen* (Siluriformes, Heptapteridae), with an LC_{50} 96 h value of 7.3 mg glyphosate/L (Kreutz et al., 2008), and lower than the fairly high LC_{50} 96 h value of 620 mg glyphosate/L obtained for the common carp *Cyprinus carpio* (Cypriniformes, Cyprinidae) (Neskovic et al., 1996).

Comparing the LC_{50} 96 h value for *C. decemmaculatus* exposed to Panzer® with those from the literature for other glyphosate-containing formulated product-treated fish, the small Poeciliidae showed similar sensitivity of *Prochilodus lineatus* (Langiano and Martinez, 2008), *Oreochromis niloticus* (Jiraungkoorskul et al., 2002), and *Gambusia yucatana* (Rendón-von Osten et al., 2005), with LC_{50} 96 h values of 13.7, 16.8, and 17.8 mg/L, respectively. Finally, in terms of the LC_{50} 96 h value obtained for *C. decemmaculatus* exposed to Credit®, the freshwater species we evaluated could be considered the least sensitive fish, with the highest LC_{50} 96 h value reported so far.

It is worth mentioning that the lowest Panzer® concentration tested in the present study (3.9 mg glyphosate/L) might be considered environmentally realistic. Previous studies have reported that the maximum concentration for this herbicide found in water bodies was 3.7 mg glyphosate/L (Giesy et al., 2000). Although the *in vivo* glyphosate treatments in this study covered a wide range of concentration, the 3.9–68.8 mg/L range represents the relatively high end of the threshold values between 0.10 and 0.70 mg glyphosate/L found in pampasic Argentinean water streams reported elsewhere (Peruzzo et al., 2008) where *C. decemmaculatus* is commonly found. Thus, the range of concentrations employed in this research would be expected to be rare in the environment, perhaps only being observed when specific events could occur, *e.g.*, when humans are exposure by occupational working or a direct application adjacent to surface waters by accidental discharge/spills, among others proceedings.

The acute toxicity of glyphosate is considered to be very low by the WHO-FAO (1997). However, several reports agree in demonstrating that commercial glyphosate formulations are more acutely toxic than the pure herbicide (Cedergreen and Streibig, 2005; Peixoto, 2005; Pereira et al., 2009; Sobrero et al., 2007). Furthermore, it has also been observed that formulations containing trimethylsulfonium salt of glyphosate, *e.g.*, Avans, are more toxic to than those in which glyphosate is present as an isopropylamine salt, *e.g.*, Roundup® (Pettersson and Ekelund, 2006). It should be noted that coadyuvants and surfactants are common ingredients always included in formulations to enhance the adsorption of glyphosate and improve the effectiveness of the herbicide. The surfactant POEA is the principal toxic component in the glyphosate-based formulation Roundup®, and several reports demonstrate that this product may have a toxicity several times higher than glyphosate itself, making the formulated mixture of greater toxicity than the active ingredient (Bolognesi et al., 1997; Folmar et al., 1979; Tsui and Chu, 2003). The observations reported by Folmar et al. (1979) should be considered as some of the most comprehensive. These authors compared the acute toxicity of technical-grade glyphosate acid, Roundup®, isopropylamine salt of glyphosate, and POEA to several freshwater species, including invertebrates and fish such as daphnids (*Daphnia magna*), scuds (*Gammarus pseudolimnaeus*), midge larvae (*Chironomous plumosus*), mayfly nymphs (*Ephemerella walkeri*), rainbow trout (*Salmo gairdneri*), fathead minnows (*Pimephales promelas*), channel catfish (*Ictalurus punctatus*), and bluegills (*Lepomis macrochirus*), and concluded that the relatively high toxicity of Roundup® was mostly attributable to the presence of POEA within its formulation (Folmar et al., 1979). Finally, it is worth mentioning that, not only for glyphosate-based herbicides but also for several other agrochemicals, it has been reported that commercial formulations exert higher toxic effects than their active ingredients both *in vivo* and *in vitro*. Among them, the atrazine-based herbicide Gesaprim® (Zeljezic et al., 2006), the dicamba-based herbicide Banvel® (González et al., 2011), the carbofuran-based insecticide Furadan® (Soloneski et al., 2008), the pirimicarb-based insecticide Aficida® (Soloneski and Larramendy, 2010), the bifenthrin-based insecticide Talstar® (Beggel et al., 2010), and the fipronil-based insecticide Termidor® (Beggel et al., 2010) can be included. Our results could suggest that the MN induction exerted by both formulations is most probably due to a deleterious effect(s) induced by xenobiotics included in the composition of the excipient of the glyphosate-based herbicides. Unfortunately, the origin and identities of the additive compounds present in both commercial formulations Panzer® and Credit® were not made available to us by the manufacturers.

The MN assay has been widely used in studies for the evaluation of genotoxic effects caused by different xenobiotics on aquatic organisms (Ali et al., 2008; Campana et al., 2003; Cavaş, 2011; Mouchet et al., 2006; Pamplona et al., 2011; Polard et al., 2011; Poletta et al., 2009; Udroiu, 2006; Vera-Candioti et al., 2010a,b; Villela et al., 2006). Previous reports have demonstrated the ability of pure glyphosate and several glyphosate-based products to induce MN *in vivo*, *in vitro*, and *in ovo*, depending upon the end point, the compound assayed, as well as the cellular target (Bolognesi et al., 1997; Cavalcante et al., 2008; Cavaş and Könen, 2007; Clements et al., 1997; Mañas et al., 2009; Poletta et al., 2009, 2011; Williams et al., 2000). Our results are in accord with these observations demonstrating the ability of Panzer® and Credit® to induce MN in circulating erythrocytes of *C. decemmaculatus*.

In the present study, the MN assay revealed a significant increase in DNA damage in circulating erythrocytes of *C. decemmaculatus* exposed to the two lowest Panzer® concentrations after 48 and 96 h of treatment. However, the highest concentration was

unable to induce MN in fish treated for 48 and 96 h. Furthermore, Panzer®-induced MN decreased as a function of the exposure time. The lack of MN induction by the highest concentration of Panzer® is consistent with other studies demonstrating such a finding in *C. carpio* exposed to mercury (Nepomuceno et al., 1997), *Cheirodon interruptus interruptus* exposed to lambda-cyhalothrin (Campana et al., 1999), *Astyanax bimaculatus* treated with cyclophosphamide (Matsumoto and Cólus, 2000), *O. niloticus* exposed to textile effluents (Cavaş and Ergene-Gözükara, 2003), *Channa punctatus* exposed to chlorpyrifos (Ali et al., 2008), and *Salmo trutta*, *Anguilla anguilla*, and *Phoxinus phoxinus* exposed to cyclophosphamide, colchicine, and cadmium (Rodriguez-Cea et al., 2003), among others. As pointed out by these authors, such decreases could be explained by a toxic and inhibitory effect of the highest concentrations tested due to alterations in blood cell kinetics and erythrocyte replacement (Cavaş and Ergene-Gözükara, 2003; Polard et al., 2011). Then, MN frequency in peripheral erythrocytes results from the dynamic balance between the formation and elimination of micronucleated cells as suggested elsewhere (Polard et al., 2011). It is well known 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 MN could be that a higher concentration of Panzer® causes an inhibition of the erythropoiesis, resulting in a decrease in the production of potentially micronucleated erythrocytes or a negative effect on cell kinetics resulting in a cell-cycle delay. However, another plausible explanation could be attributable to an antagonistic effect, *i.e.*, the stimulation of the erythropoietic process. Whether or not this effect could cause the increase in erythrocyte frequency in peripheral blood, a dilution of micronucleated erythrocytes would result among circulating blood cells (Udroiu, 2006). Finally, the possibility that stimulated splenic erythrocatheretic activity could also contribute to diminishing MN frequency through micronucleated cell elimination cannot be ruled out (Polard et al., 2011).

Exposure of the ten spotted live-bearer to Credit® induced an increase in MN frequency only after 96 h of treatment. The lack of MN induction in specimens of *C. decemmaculatus* exposed to Credit® during 48 h of treatment is in accordance with previous findings in specimens of *A. anguilla* exposed to cyclophosphamide (Pacheco and Santos, 1997) and specimens of *P. lineatus* (Cavalcante et al., 2008), *Tilapia rendalli* (Grisolia, 2002), and *Carassius auratus* (Cavaş and Könen, 2007) treated with Roundup®. These authors suggested that this effect could be due to a stimulated erythrocateretic function with a slow replacement of erythrocytes and, then, their delayed appearance in peripheral blood. Finally, this observation could also be related to the time period of treatment, which could be insufficient for the occurrence of a complete cell cycle of the erythrocytic cells and a concomitant detection of micronucleated erythrocytes in those fish treated for 48 h (Cavalcante et al., 2008). So far, we do not have any experimental evidence for explaining the reason of this particular finding.

Available data in the literature indicate that the maximum piscine MN frequency in peripheral erythrocytes occurs between 1 and 5 days of treatment to a xenobiotic (Al-Sabti and Metcalfe, 1995). However, in most fish species, the peak of incidence appears after 2–3 days of exposure (Udroiu, 2006). While experiments with *T. rendalli* (Grisolia, 2002) and *C. auratus* (Cavaş and Könen, 2007) exposed to Roundup® resulted in an increase in MN frequency after 4 days of treatment, MN induction in *A. anguilla* was found after 6 days of exposure to polycyclic aromatic hydrocarbons (Pacheco and Santos, 1997). Our results demonstrated that at least 4 days of exposure to Credit® are required to induce a significant increase in MN frequency in erythrocytes of *C. decemmaculatus*, whereas for Panzer® only 2 days are necessary to achieve the same effect.

Numerous pollutants, including pesticides, can produce reactive oxygen species via several mechanisms, *i.e.* inactivation of antioxidant enzymes, depletion of non-enzymatic antioxidants and membrane lipid peroxidation, among others (Winston and Di Giulio, 1991). Recently, it has been reported that glyphosate caused oxidative DNA damage by the induction of reactive oxygen species in both gills and liver DNA cells of *A. anguilla* exposed under laboratory conditions (Guilherme et al., 2012). Furthermore, Cattaneo et al. (2012) documented lipid peroxidation and high acetyl cholinesterase activities in the brain and muscle of *C. carpio* after a commercial glyphosate formulation exposure. Similar results have been reported by Modesto and Martinez (2010) when *P. lineatus* was exposed to glyphosate revealing a transient reduction in both superoxide dismutase and catalase activities as well as an inhibition of glutathione-S-transferase due most probably to a lipid peroxidation mechanism. Several investigations have clearly demonstrated that the generation of reactive oxygen species could be associated with DNA damage resulting in an increase of the lesions at chromosomal level (Emerit et al., 1982; Soloneski et al., 2001, 2003). At present, it seems evident that more studies are required to determine the origin of DNA damage exerted by glyphosate in *C. decemmaculatus* in order to understand and clarify the mechanisms of genotoxicity of this herbicide.

In terms of cytotoxic effects neither Panzer® nor Credit® were capable to induce alterations in erythrocyte:erythroblast ratios, at least within the concentration ranges used. This fact could be explained directly by the absence of cytotoxic potential exerted by the herbicides. However, whether cytotoxicity is exerted, erythropoiesis could become stimulated, and therefore large amount of erythrocytes could become present in the bloodstream. Therefore, erythroblasts within peripheral blood become diluted, giving a false negative result (Udroiu, 2006).

5. Conclusions

Overall, the glyphosate-based herbicides Panzer® and Credit® (48% a.i.) were found to be genotoxic but not cytotoxic agrochemicals to the fish *C. decemmaculatus*. Considering the worldwide use of glyphosate for agricultural and nonagricultural purposes, the present results are complementary to those reported by other research groups concerning the environmental risk of this herbicide.

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