Chlorpyrifos-Based Insecticides Induced Genotoxic and Cytotoxic Effects in the Ten Spotted Live-Bearer Fish, Cnesterodon decemmaculatus (Jenyns, 1842)

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ABSTRACT: Mortality, genotoxicity, and cytotoxicity of the 48% chlorpyrifos (CPF)-based formulations Lorsban* 48E® and CPF Zamba® were evaluated on *Cnesterodon decemmaculatus* (Jenyns, 1842) (Pisces, Poeciliidae) under laboratory conditions. Induction of micronucleus (MN) and alterations in the erythrocyte/erythroblast frequencies were employed as end points for genotoxicity and cytotoxicity, respectively. For Lorsban* 48E®, mean values of 0.13 and 0.03 mg/L were determined for LC_{50} at 24 and 96 h, respectively, and these concentrations reached mean values of 0.40 and 0.21 mg/L for CPF Zamba®. Mortality values increased as a positive linear function of the CPF Zamba® concentrations, but not for Lorsban* 48E® concentrations. There was no significant relationship between mortality and exposure time within the 0–96 h period for both formulations. LC_{50} values indicated that the fish were seven fold more sensitive to Lorsban* 48E® than to CPF Zamba®. Lorsban* 48E® within the concentration range of 0.008–0.025 mg/L increased MN frequency at both 48 and 96 h of treatment. Similar results were also observed when fish were exposed to 0.052–0.155 mg/L of CPF Zamba®, regardless of the exposure time. Cellular cytotoxicity was found after Lorsban* 48E® and CPF Zamba® treatments for all concentrations and time exposures, estimated by a decrease in the frequency of mature erythrocytes and a concomitant enhanced frequency of erythroblasts in circulating blood. Furthermore, our results demonstrated that Lorsban* 48E® and CPF Zamba® should be considered as CPF-based commercial formulations with marked genotoxic and cytotoxic properties. Environ Toxicol 29: 1390–1398, 2014.
Keywords: mortality; micronucleus; erythrocyte:erythroblast frequency; Poeciliidae; Lorsban* 48E®; Chlorpyrifos Zamba®

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).

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Anthropogenic 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 also concomitantly exert negative side effects on nontarget organisms (www.epa.gov/pesticides).

Organophosphates have become the most widely used class of insecticides worldwide, replacing the persistent and problematic organochlorine compounds (Ramsden, 1996). Chlorpyrifos (CPF; *O*,*O*-diethyl *O*−3,5,6-trichloropyridin-2-yl phosphorothioate) is a broad-spectrum organophosphate insecticide. CPF is the active ingredient in Dursban and Lorsban insecticides, which are among the most widely used insect control products globally to control pests in agricultural and domestic situations (USEPA, 2006). It was introduced in 1965 by Dow Chemical Company (www.dow.com/search.aspx?q=chlorpyrifos&sa), and although originally used primarily to kill mosquitoes, it is no longer registered for this use (www.chlorpyrifos.org). Products containing CPF have been used with confidence for more than three decades and are registered for use in nearly 100 nations with more than 300 registered formulations worldwide (www.chlorpyrifos.com/product-benefits.htm). To avoid insecticide exposure to children and pregnant women, because CPF is a known developmental neurotoxicant (Li et al., 2012), most nonagricultural uses, such as residential control of insect pests such as cockroaches and termites, and animal uses including flea and tick control, were phased out in the USA in 2001 (USEPA, 2006) and in the European Union in 2005 (European Union, 2007). CPF is effective in controlling cutworms, corn rootworms, cockroaches, grubs, flea beetles, flies, termites, fire ants, and lice. It is used as an insecticide on grain, cotton, fields, and fruit, nut, and vegetable crops, as well as on lawns and ornamental plants. It is also registered for direct use on sheep and turkeys, and for treatment of horse sites, dog kennels, domestic dwellings, farm buildings, storage bins, and commercial establishments. CPF acts on pests primarily as a contact poison, with some action as a stomach poison, and is available as granules, wettable, or dustable powder, and an emulsifiable concentrate (www.dow.com/search.aspx?q=chlorpyrifos&sa=).

CPF is effective by contact, ingestion, and vapour action. Like other organophosphates, CPF's insecticidal activity is caused by the inhibition of the acetylcholinesterases, which results in the accumulation of the neurotransmitter acetylcholine at the nerve endings. This results in excessive transmission of nerve impulses, which causes mortality in the target pest (Li et al., 2012). However, the plasma butyryl-cholinesterase, or pseudocholinesterase, seems to be the most sensitive among acetylcholinesterases (Li et al., 2012). Available data provide strong evidence that, with respect to CPF, significant depression of brain acetylcholinesterase is required to cause toxicity in mammals (Li et al., 2012).

So far, CPF has been ranked as a Class II chemical (moderately hazardous) by the World Health Organization (WHO) and as moderately toxic (Class 2) by the US Environmental Protection Agency (USEPA), although it has not been included as a carcinogen by the International Agency for Research on Cancer (IARC) (http://www.pesticideinfo.org). However, the latter emphasizes that CPF should be considered as a highly toxic compound for aquatic organisms, especially to both cold water and warm water fish species (USEPA, 2006). Overall, while negative responses have been reported for the insecticide-induced mutagenicity in bacterial and yeast systems (Eaton et al., 2008), CPF has elicited a number of other effects including hepatic (Slotkin, 2011) and endocrine dysfunction (Betts, 2010), immunological abnormalities (Noworyta-Glowacka et al., 2012), teratogenicity (McCollum et al., 2011; Uggini et al., 2012), neurochemical and neurobehavioral changes (Turgeman et al., 2011), embryo toxicity (Tian et al., 2005), and genotoxicity (Ali et al., 2009; Li et al., 2011) in *in vitro* and *in vivo* studies employing different biotic matrices.

There is an increasing interest in biomonitoring markers to provide a measurement as well as an estimation of biological exposure to genotoxic pollutants. Their effects can be monitored employing a broad range of both *in vivo* and *in vitro* bioassays. To achieve this goal, several end points for testing both genotoxicity and cytotoxicity have been employed on aquatic organisms, including fishes. Analysis of micronucleus (MN) frequency and the induction of DNA single-strand breaks by single-cell gel electrophoresis assay are the most frequently recommended and employed end points for detecting DNA damage in circulating blood erythrocytes (Cavaş and Könen, 2007; Ali et al., 2008a; Vera-Candioti et al., 2010b, 2013).

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, for example, 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 (Di Marzio et al., 2005; de la Torre et al., 2007; Menéndez-Helman et al., 2012; Vera-Candioti et al., 2010b, 2013).

In the current study, attempts have been made to characterize the lethal and sublethal toxicity of Lorsban* 48E® and CPF Zamba®, two CFP-based insecticides, on *C. decemmaculatus* exposed under laboratory conditions. While LC_{50} estimation was employed as a biomarker for lethality, induction of MN and alterations in the erythrocyte/erythroblast frequency were employed as biomarkers of genotoxicity and cytotoxicity, respectively.

MATERIALS AND METHOD

Chemicals

Two liquid commercial formulations of CPF, namely, Lorsban* 48E® (48% a.i.; excipients q.s.; Dow Agrosciences Argentina S.A., Buenos Aires, Argentina) and CPF Zamba® (48% a.i.; excipients q.s.; Nidera S.A., Buenos Aires, Argentina) 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.

Quality Control

Concentration levels of CPF in test solutions were analyzed by passing them through C18 columns (Agilent; solid phase extraction) and frozen until analysis. Extracts were eluted from C18 columns with 2 mL hexane followed by 2 mL dichloromethane. The sample extracts were injected into a gas chromatograph with electron capture detector (Hewlett Packard, HP 6890) equipped with a 30 m $\times$ 0.25 mm HP1 column, N_2 carrier, and ramp and detector temperatures of 190–250 and 320°C, respectively. Recovery from the C18 columns was tested by passing through a solution of known concentration. The C18 columns showed a 97 $\pm$ 5.0% CPF recovery. CPF samples from test solution correspond to 0 and 24 h thereafter. The detection limit was 0.01 μg/L.

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 a 16/8 h light/dark cycle in aquaria at 21 $\pm$ 1°C in dechlorinated tap water (pH 7.6–8.3; hardness, 143 mg/L $CaCO_3$) and artificial aeration. As 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®, 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 $\pm$ 0.1 g and total length of 29.5 $\pm$ 2.7 mm were selected for the experiments.

Determination of LC_{50}

Concentrations assessed throughout the study represent the nominal concentrations of active ingredient present within Lorsban* 48E® and CPF Zamba® CPF-based formulations. Experiments were carried out for toxicity assessment following recommendations proposed by the USEPA standardized methods for acute toxicity tests (USEPA, 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 one of 10 different concentrations of Lorsban* 48E® (0.0001, 0.001, 0.01, 0.025, 0.05, 0.1, 1.0, 5.0, 50.0, and 100.0 mg/L) or CPF Zamba® (0.025, 0.05, 0.1, 0.15, 0.25, 0.35, 0.45, 0.55, 1.0, and 1.5 mg/L) during 96 h, with test solutions replaced every 24 h. While the negative control group consisted of 10 organisms kept in 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), the positive control group consisted of 10 fishes treated with 21.4 mg Cr(VI)/L as reported previously (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.

Determination of MN Frequency

Each experiment was conducted using five fish following the same experimental design described in Determination of LC_{50} section, 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 0.008, 0.017, and 0.025 mg/L or 0.052, 0.104, and 0.155 mg/L of Lorsban* 48E® and CPF Zamba®, respectively. Negative (dechlorinated tap water; see Test Organisms section) and positive controls (5 mg/L cyclophosphamide) were conducted and run simultaneously with Lorsban* 48E®- and CPF Zamba®-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, 2013). 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).

Frequency of Circulating Erythrocyte/Erythroblast

The circulating erythrocyte/erythroblast frequency was determined as described previously for xenobiotic-exposed aquatic organisms (Vera-Candioti et al., 2010a,b). In brief, the criteria employed for erythrocyte and erythroblast identification in peripheral circulating blood were as follows: An erythrocyte has a cell nucleus oval to rounded oval (Claver and Quaglia, 2009) and compact chromatin that stains purple in preparations stained with aqueous Giemsa (Rey Vázquez and Guerrero, 2007). Moreover, an erythroblast cell has a circular or slightly ovoid basophilic cytoplasm and round to slightly ovoid nucleus containing diffuse chromatin of variable density that represents 40–60% of cell volume (Peters and Schwarzer, 1985). The 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 expressed as the total number of erythrocytes and erythroblasts per 1000 cells.

Statistical Analyses

Data of lethality tests were analyzed using the USEPA Probit Analysis, version 1.5, statistical software (http://www.epa.gov/nerleerd/stat2.htm) and based on Finney's (1971) method. Statgraphics Centurion XV software was used for statistical analyses. After assessing the normality of the distribution of the data by the Shapiro–Wilk W test, even after logarithmic transformation, nonparametric tests were used 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 frequencies with insecticide concentrations was evaluated by simple linear regression analyses. The level of significance was $\alpha = 0.05$.

RESULTS

Mortality Assays

Results of chemical analyses showed no differences between both used and measured CPF concentrations or during the 24 h interval renewals of the testing solutions for both commercial formulations evaluated (0.001, 0.01, 0.05, 0.1 and 0.025, 0.05, 0.1, 0.15 mg/L, for Lorsban* 48E® and CPF Zamba®, respectively). While no mortality was observed in those dechlorinated tap water maintained specimens (negative control), an increase in mortality rate was achieved in those 21.4 mg Cr(VI)/L-treated fish (positive control) during the 96 h experiment.

Probit analysis of the mortality experiment allowed determination of the LC_{50} values of Lorsban* 48E® after 24, 48, 72, and 96 h of exposure, with mean values of 0.13 mg/L (range, 0.09–0.19 mg/L), 0.05 mg/L (range, 0.04–0.07 mg/L), 0.04 mg/L (range, 0.03–0.05 mg/L), and 0.03 mg/L (range, 0.03–0.05 mg/L), respectively. Overall, Lorsban* 48E® treatments induced a nonsignificant concentration-dependent increase ($r = 0.43$, $p > 0.05$) as well as a nonsignificant time-dependent decrease ($r = -0.84$, $p > 0.05$) in mortality rate when the exposure time increased from 24 to 96 h.

Mortality experiments revealed the LC_{50} values of CPF Zamba® after 24, 48, 72, and 96 h of exposure, with mean values of 0.40 mg/L (range, 0.34–0.47 mg/L), 0.32 mg/L (range, 0.27–0.37 mg/L), 0.25 mg/L (range, 0.21–0.29 mg/L), and 0.21 mg/L (range, 0.18–0.24 mg/L), respectively. Overall, all treatments induced a significant concentration-dependent increase in mortality rate ($r = 0.81$, $p < 0.01$). In contrast, no significant time-dependent decrease was observed in mortality rate when the exposure time increased from 24 to 96 h ($r = -0.78$, $p > 0.05$).

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 ($p < 0.01$) or 96 h ($p < 0.01$) of treatment in Lorsban* 48E® experiments (Table I), as well as at 48 h ($p < 0.05$) or 96 h ($p < 0.05$) of treatment in CPF Zamba® experiments (Table II).

Table I summarizes the results of the analysis of Lorsban* 48E®-induced MN in circulating erythrocytes of exposed fish. At both 48 and 96 h of treatment, a significant increase in MN frequency was observed in fish treated with Lorsban* 48E® concentrations of 0.008 mg/L ($p < 0.001$), 0.017 mg/L ($p < 0.001$), and 0.025 mg/L ($p < 0.001$) compared to negative control values (Table I). Overall, a regression analysis revealed that the increase in MN frequency was not affected by Lorsban* 48E® concentrations either at 48 h ($r = -0.14$, $p > 0.05$) or at 96 h of exposure ($r = 0.12$, $p > 0.05$; Table I). On the other hand, MN frequency induced by Lorsban* 48E® increased as a function of the exposure time ($r = 0.23$, $p < 0.05$).

The results of the analysis of CPF Zamba®-induced MN in circulating erythrocytes of exposed fish are summarized in Table II. At 48 h of treatment, a significant increase in MN frequency was observed in fish treated with CPF Zamba® concentrations of 0.052 mg/L ($p < 0.05$), 0.104 mg/L ($p < 0.01$), and 0.155 mg/L ($p < 0.01$) compared to negative control values. Similarly, when the analysis was performed after 96 h of treatment, a significant increase in MN

TABLE I. Incidence of micronuclei (MN) and erythrocyte/erythroblast frequencies in peripheral blood cells of *Cnesterodon decemmaculatus* exposed to different concentrations of Lorsban* 48E®

			MN Induction		Erythrocyte/Erythroblast		
Exposure Time (h)	Dosage (mg/L)	Number of Fishes Analyzed	Number of Cells Analyzed	Frequency MN (‰ ± S.E.)	Number of Erythrocytes Analyzed	Number of Erythroblats Analyzed	Frequency Erythroblasts (‰ ± S.E.)
48	Control	15	22 500	0.31 ± 0.11	22 467	33	1.47 ± 0.85
	Positive control[a]	15	22 500	0.98 ± 0.14**	21 449	1051	46.71 ± 19.32**
	0.008	16	24 000	0.71 ± 0.23***	23 781	219	9.13 ± 3.70
	0.017	16	24 000	0.38 ± 0.12***	23 251	749	31.21 ± 13.50*
	0.025	16	24 000	0.42 ± 0.25***	23 074	926	38.58 ± 15.84*
96	Control	15	22 500	0.18 ± 0.08	22 500	0	0.00 ± 0.00
	Positive control[a]	15	22 500	0.84 ± 0.22**	20 146	2354	104.62 ± 26.64***
	0.008	16	24 000	0.71 ± 0.23***	23 968	32	1.33 ± 0.74*
	0.017	16	24 000	1.08 ± 0.28***	23 765	235	9.79 ± 5.60**
	0.025	16	24 000	1.00 ± 0.20***	24 000	0	0.00 ± 0.00

[a]Ciclophosphamide, 5 mg/L.
*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; significant differences with respect to control values.

frequency was observed in fish treated with concentrations of 0.052 mg/L ($p < 0.01$), 0.104 mg/L ($p < 0.001$), and 0.155 mg/L ($p < 0.001$) compared to negative control values (Table II). A regression analysis revealed the absence of a relationship between MN frequency and CPF Zamba® concentrations after treatment period ($r = 0.11$, $p > 0.05$ and $r = 0.21$, $p > 0.05$ for 48 and 96 h of treatment, respectively). Similarly, MN frequency induced by CPF Zamba® did not increase as a function of the exposure time ($r = 0.15$, $p > 0.05$) (Table II).

Erythrocyte/erythroblast frequencies in specimens of *C. decemmaculatus* exposed to cyclophosphamide in both Lorsban* 48E® and CPF Zamba® experiments are summarized in Tables I and II, respectively. In those Lorsban* 48E®-exposed fish, a significant decrease and a concomitant increase in the frequency of erythrocytes and erythroblasts, respectively, were observed in the blood of those specimens exposed to either 0.017 mg/L ($p < 0.05$) or 0.025 mg/L ($p < 0.05$) concentrations. However, when the analysis was performed after 96 h of treatment, such effect was observed in fish treated with concentrations of 0.008 mg/L ($p < 0.05$) and 0.017 mg/L ($p < 0.01$). Overall, a regression analysis revealed that the alterations in the erythrocyte/erythroblast frequencies found in those Lorsban* 48E®-treated fish were not dependent on the concentration of the formulation employed, either at 48 h ($r = -0.24$, $p > 0.05$) or at 96 h of

TABLE II. Incidence of micronuclei (MN) and erythrocyte/erythroblast frequencies in peripheral blood cells of *Cnesterodon decemmaculatus* exposed to different concentrations of Clorpirifos Zamba®

			MN Induction		Erythrocyte/Erythroblast		
Exposure Time (h)	Dosage (mg/L)	Number of Fishes Analyzed	Number of Cells Analyzed	Frequency MN (‰ ±S.E.)	Number of Erythrocytes Analyzed	Number of Erythroblats Analyzed	Frequency Erythroblasts (‰ ± S.E.)
48	Control	15	22 500	0.36 ± 0.13	22 478	22	0.98 ± 0.55
	Positive control[a]	15	22 500	0.93 ± 0.16*	21 306	1194	53.07 ± 18.88**
	0.052	15	22 500	0.93 ± 0.19*	21 974	526	23.38 ± 5.32***
	0.104	15	22 500	1.20 ± 0.26**	22 053	447	19.87 ± 5.58***
	0.155	15	22 500	1.16 ± 0.17**	21 939	561	24.93 ± 6.14***
96	Control	15	22 500	0.27 ± 0.09	22 356	144	6.40 ± 4.15
	Positive control[a]	15	22 500	0.98 ± 0.28*	20 161	2339	103.96 ± 26.82***
	0.052	15	22 500	1.02 ± 0.22**	22 321	179	7.96 ± 2.31*
	0.104	15	22 500	1.56 ± 0.27***	22 357	143	6.36 ± 1.79**
	0.155	15	22 500	1.51 ± 0.23***	22 432	68	3.02 ± 0.92*

[a]Ciclophosphamide, 5 mg/L.
*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; significant differences with respect to control values.

exposure ($r = 0.04$, $p > 0.05$), but were dependent on the exposure time ($r = 0.30$, $p < 0.01$; Table I).

The results of the analysis of CPF Zamba®-induced alterations in the erythrocyte/erythroblast frequencies are summarized in Table II. At 48 h of treatment, a significant decrease and a concomitant increase in the frequency of erythrocytes and erythroblasts, respectively, was observed with CPF Zamba® concentrations of 0.052 mg/L ($p < 0.001$), 0.104 mg/L ($p < 0.001$), and 0.155 mg/L ($p < 0.001$) compared to negative control values. Similarly, when the analysis was performed at 96 h of treatment, such alterations were observed in fish treated with concentrations of 0.052 mg/L ($p < 0.05$), 0.104 mg/L ($p < 0.01$), and 0.155 mg/L ($p < 0.05$) compared to negative control values. Overall, a regression analysis revealed that the alterations in the erythrocyte/erythroblast frequencies found in those CPF Zamba®-treated fish were not dependent on the concentration of the formulation employed ($r = 0.03$, $p > 0.05$ and $r = 0.29$, $p > 0.05$, for 48 and 96 h, respectively), but were dependent on the exposure time ($r = 0.47$, $p < 0.01$; Table II).

DISCUSSION

The acute lethal toxicity, genotoxicity, and cytotoxicity of the 48% CPF-containing technical formulation insecticides Lorsban* 48E® and CPF Zamba® were evaluated on specimens from *C. decemmaculatus* exposed under laboratory conditions. Concerning the acute lethal toxicity values, both formulated insecticides can be classified as very toxic compounds (Category I) following the European Union (Mazzatorta et al., 2002) or United Nations directives (UN, 2011). Furthermore, according to the ecotoxicological scoring employed by the WHO (Smrchek et al., 1993; Wagner et al., 1995), both Lorsban* 48E® and CPF Zamba® can be ranked as "high concern" xenobiotics. Reviews on the safety of CPF, including major CPF-based insecticides, have been conducted by several regulatory international agencies who have reached the conclusion that there is no indication of any human concern, with the USEPA classifying the active ingredient in 1993 as a Class E agent (USEPA, 2005), supporting the same status proposed by the Agency for Toxic Substances and Disease Registry (www.atsdr.cdc.gov/). However, CPF is currently under review by the European Commission and the USEPA (http://www.fao.org/agriculture/crops/en/). Nevertheless, it has recently been clearly demonstrated that this insecticide is not as safe as previously believed, as there is now experimental evidence that the insecticide may contribute to or be involved in CPF-induced carcinogenesis (Josephson, 2005; Karunanayake et al., 2012). Our current findings are in concordance with the aforementioned classification and verify and extend the latter observations.

Comparing the LC_{50} values at 96 h that we observed for *C. decemmaculatus* exposed to two CPF-based insecticides with those published for several CPF-based pesticide-treated fish species, *C. decemmaculatus* can be ranged between the most and least sensitive fish reported so far. The LC_{50} 96 h values found were greater than those reported for *Gambusia yucatana* (Cyprinodontiformes, Poeciliidae), with an LC_{50} 96 h value of 0.01 mg/L CPF-based Lorsban 480EM (Rendón-von Osten et al., 2005), and lower than the high LC_{50} 96 h value of 0.88 mg/L CPF-based unspecified formulation in *Lepomis macrochirus* (Perciformes, Centrarchidae) (USEPA, 2000). Furthermore, when comparing the LC_{50} 96 h value for *C. decemmaculatus* exposed to Lorsban* 48E® with those from the literature reported for active ingredient-treated fish, it showed a higher sensitivity than *Cyprinus carpio* (Cyprinniformes, Cyprinidae) (Ramesh and David, 2009), *Gambusia affinis* (Cyprinodontiformes, Poeciliidae) (USEPA, 2006), and *Poecilia reticulata* (Cyprinodontiformes, Poeciliidae) (Selvi et al., 2005), but not *L. macrochirus* (Perciformes, Centrarchidae) (USEPA, 2006), with LC_{50} 96 h values of 0.16, 0.59, 1.79, and 0.002 mg/L, respectively. Finally, in terms of the LC_{50} 96 h value obtained for *C. decemmaculatus* exposed to CPF Zamba®, it resulted more toxic for freshwater species we evaluated than the active ingredient did on *G. affinis* (USEPA, 2006) and *P. reticulata* (Selvi et al., 2005), but not when *C. carpio* (Ramesh and David, 2009) and *L. macrochirus* (Perciformes, Centrarchidae) were used as target organisms.

To the best of our knowledge, very few studies have demonstrated the ability of CPF-based products to induce MN in erythrocytes of both fish and tadpoles *in vivo* (Ali et al., 2008a, 2009; Kumar, 2012; Özkan et al., 2011). Our results are in accordance with these latter observations demonstrating the ability of Lorsban* 48E® and CPF Zamba® to increase the MN frequency in *C. decemmaculatus*. Furthermore, our current observations emphasize the importance of the MN assay as an early biological marker of fish exposure to genotoxic pollutants in the aquatic environment.

Previous results indicate that the maximum MN frequency in peripheral erythrocytes of xenobiotic-exposed fish occurs between 1 and 5 days of treatment (Al-Sabti and Metcalfe, 1995). However, in most fish species, the high peak of incidence appears after 2–3 days of exposure (Udroiu, 2006). Experiments with *Channa punctatus* (Perciformes, Channidae) exposed to CPF-based formulations revealed that the increase in MN frequency in erythrocytes could require from 96 h (Ali et al., 2008b) up to 14–25 days of treatment to reach the maximum level (Ali et al., 2009; Kumar, 2012). Our results demonstrate that only 2 days of exposure to Lorsban* 48E® and CPF Zamba® are required to induce the maximum increase in MN frequency in *C. decemmaculatus*. In agreement with this observation, it was reported recently for the same species that a 2 days exposure time is a period long enough to observe the maximum induction of MN after a glyphosate-based herbicide treatment (Vera-Candioti et al., 2013).

In terms of cytotoxic effects, both Lorsban* 48E® and CPF Zamba® were capable of inducing alterations in erythrocyte/erythroblast frequencies. For both insecticides, and regardless of the concentrations assayed, a clear biphasic behavior in the erythrocyte/erythroblast frequencies occurred with incubation time, namely, an increase in the frequency of erythroblasts accompanied by a subsequent decrease in the proportion of erythrocytes within 48 h of treatment, and a time-dependent decrease of these cells thereafter. Such alteration could be explained by the presence of cytotoxic potential exerted by the insecticides and inhibitory effects of the highest 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., 2013). Then, alteration in the erythrocyte/erythroblast frequency in peripheral blood results from the dynamic balance between the formation and elimination of red cells as suggested elsewhere (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 erythrocytes could be that a higher concentration of either Lorsban* 48E® or CPF Zamba® causes an inhibition of the erythropoiesis, resulting in a decrease in the production of 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, that is, the stimulation of the erythropoietic process. Whether or not 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, resulting in an accumulation of erythrocytes as observed at 96 h of treatment and giving a false negative result. Finally, the possibility that stimulated splenic erythrocatheretic activity could also contribute to diminishing erythrocyte frequency through damaged cell elimination cannot be ruled out (Polard et al., 2011). So far, we do not have any experimental evidence explaining the reason for this particular finding.

Overall, concentrations of 0.03 and 0.21 mg/L were determined as the LC_{50} values obtained at 96 h for Lorsban* 48E® and CPF Zamba® respectively. Accordingly, Lorsban* 48E® was nearly seven fold more toxic than CPF Zamba®. We cannot rule out the presence of xenobiotic(s) in the formulation Lorsban* 48E® exerting a toxic effect on themselves or an additive and/or synergistic effect with the active ingredient. Previous reports have shown that the toxicity of several commercial formulations is higher than that of the active ingredients which must be taken into consideration for risk assessment studies (Lin and Garry, 2000; Zeljezic et al., 2006; Soloneski et al., 2008; Soloneski and Larramendy, 2010). In order to dissect out the real effects jeopardized by the active ingredient from that caused by other ingredient(s) present within the technical formulations, further experiments should be conducted using pure CPF in addition to the commercial CPF-based formulations.

It is worth mentioning that the lowest Lorsban* 48E® concentration tested (0.008 mg/L CFP) might be considered environmentally realistic. Available information revealed that CPF detected in surface waters is usually at concentrations below 0.0001 mg/L and with a maximum concentration of 0.0004 mg/L, whereas it was detected in groundwater in less than 1% of the wells tested, with the majority of measurements being below 0.00001 mg/L (USEPA, 1998; European Commission, 2005). Although the *in vivo* CPF treatments in this study covered a wide range of concentrations (range, 0.008–0.155 mg/L), it should be noted that they represent concentrations determined in pampasic Argentinean water streams, where *C. decemmaculatus* is commonly found, with threshold values between <0.002 and 0.011 mg/L CFP, as reported elsewhere (Marino and Ronco, 2005). Thus, the range of concentrations employed in this research can be expected to be present in cultivated crop fields or in their vicinity. Considering the worldwide use of CPF for agricultural and nonagricultural purposes, the present results are complementary to those reported by other research groups concerning the environmental risk of this insecticide.

REFERENCES

Al-Sabti K, Metcalfe CD. 1995. Fish micronuclei for assessing genotoxicity in water. Mutat Res 343:121–135.

Ali D, El-Shehawi AM, Seehy MA. 2008a. Micronucleus test in fish genome: A sensitive monitor for aquatic pollution. Afr J Biotechnol 7:606–612.

Ali D, Nagpure NS, 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 NS, Kumar S, Kumar R, Kushwaha B, Lakra WS. 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.

Betts KS. 2010. Endocrine damper?: Flame retardants linked to male hormone, sperm count changes. Environ Health Perspect 118:A130.

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, 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.

Claver JA, Quaglia AIE. 2009. Comparative morphology, development, and function of blood cells in nonmammalian vertebrates. J Exotic Pet Med 18:87–97.

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.

Eaton DL, Daroff RB, Autrup H, Bridges J, Buffler P, Costa LG, Coyle J, McKhann G, Mobley WC, Nadel L, Neubert D, Schulte-Hermann R, Spencer PS. 2008. Review of the toxicology of chlorpyrifos with an emphasis on human exposure and neurodevelopment. Crit Rev Toxicol 38:1–125.

European Commission. 2005. Chlorpyrifos. SANCO/3059/99-rev. 1.5. European Commission Health and Consumer Protection Directorate-General Food Safety: Production and Distribution Chain Unit D3—Chemicals, Contaminants and Pesticides.

European Union. 2007. Official Journal of the European Union Legislation 50 (part 216):17–21.

Fenech M, Chang WP, Kirsch-Volders M, Holland N, Bonassi S, Zeiger E. 2003. HUMN project: Detailed description of the scoring criteria for the cytokinesis-block micronucleus assay using isolated human lymphocyte cultures. Mutat Res 534:65–75.

Finney DJ. 1971. Probit Analysis. London: Cambridge University Press, 333 p.

Josephson J. 2005. New chlorpyrifos link?. Environ Health Perspect 113:A158.

Karunanayake CP, Spinelli JJ, McLaughlin JR, Dosman JA, Pahwa P, McDuffie HH. 2012. Hodgkin lymphoma and pesticides exposure in men: A Canadian case-control study. J Agromedicine 17:30–39.

Kumar AP. 2012. Micronucleus assay: A sensitive indicator for aquatic pollution. Int J Res BioSci 1:32–37.

Li R, Li S, Liu J. 2011. Studies of hemocytes DNA damage by two pesticides acetamiprid and chlorpyrifos in predaceous spiders of Pardosa astrigera Koch. Acta Ecol Sinica 31:3156–3162.

Li AA, Lowe KA, McIntosh LJ, Mink P. 2012. Evaluation of epidemiology and animal data for risk assessment: Chlorpyrifos developmental neurobehavioral outcomes. J Toxicol Environ Health B Crit Rev 15:109–184.

Lin N, Garry VF. 2000. In vitro studies of cellular and molecular developmental toxicity of adjuvants, herbicides, and fungicides commonly used in Red River Valley, Minnesota. J Toxicol Environ Health 60:423–439.

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.

Mazzatorta P, Benfenati E, Neagu D, Gini G. 2002. The importance of scaling in data mining for toxicity prediction. J Chem Inf Comput Sci. 42:1250–1255.

McCollum CW, Ducharme NA, Bondesson M, Gustafsson JA. 2011. Developmental toxicity screening in zebrafish. Birth Defects Res C Embryo Today: Rev 93:67–114.

Menéndez-Helman RJ, Ferreyroa GV, dos Santos Afonso M, Salibián A. 2012. Glyphosate as an acetylcholinesterase inhibitor in Cnesterodon decemmaculatus. Bull Environ Contam Toxicol 88:6–9.

Menni RC, Gómez SE, López Armengol F. 1996. Subtle relationships: Freshwater fishes and water chemistry in southern South America. Hydrobiologia 328:173–197.

Noworyta-Glowacka J, Bankowski R, Siennicka J, Wiadrowska B, Ludwicki JK. 2012. Influence of chlorpyrifos on the profile of subpopulations of immunoactive cells and their phagocytic activity in an experimental in vivo model. Ann Agric Environ Med 19:483–486.

Özkan F, Gündüz SG, Berköz M, Hunt AÖ, Yalm S. 2011. Effects of dietary supplementation of vitamin C on level of protein carbonyl in liver and nuclear abnormalities in peripheral erythrocytes of Oreochromis niloticus. Fresenius Environ Bull 20:1520–1524.

Peters G, Schwarzer R. 1985. Changes in hemopoietic rainbow trout tissue under stress. Dis Aquat Organ 1:1–10.

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

Ramesh H, David M. 2009. Respiratory performance and behavioral responses of the freshwater fish, Cyprinus carpio (Linnaeus) under sublethal chlorpyrifos exposure. J Basic Clin Physiol Pharmacol 20:127–139.

Ramsden EN. 1996. A Level Chemistry—Chemistry of the Environment. Surrey, UK: Stanley Thornes. 168 p.

Rendón-von Osten J, Ortíz-Arana A, Guilhermino L, Soares AMVM. 2005. In vivo evaluation of three biomarkers in the mosquitofish (Gambusia yucatana) exposed to pesticides. Chemosphere 58:627–636.

Rey Vázquez G, Guerrero GA. 2007. Characterization of blood cells and hematological parameters in Cichlasoma dimerus (Teleostei, Perciformes). Tissue Cell 39:151–160.

Selvi M, Sarikaya R, Erkoç F, Koçak O. 2005. Investigation of acute toxicity of chlorpyrifos-methyl on guppy Poecilia reticulata. Chemosphere 60:93–96.

Slotkin TA. 2011. Does early-life exposure to organophosphate insecticides lead to prediabetes and obesity?. Reprod Toxicol 31:297–301.

Smrchek JC, Clements R, Morcock R, Robert W. 1993. Assessing ecological hazards under TSCA: Methods and evaluation of data. In: Landis WA, Hughes JS, Landis MA, editors. Environmental Toxicology and Risk Assessment ASTM STP 1179. Philadelphia, PA: ASTM International. pp 22–39.

Soloneski S, Larramendy ML. 2010. Sister chromatid exchanges and chromosomal aberrations in Chinese hamster ovary (CHO-K1) cells treated with the insecticide pirimicarb. J Hazard Mater 174:410–415.

Soloneski S, Reigosa MA, Molinari G, González NV, Larramendy ML. 2008. Genotoxic and cytotoxic effects of carbofuran and Furadan® on Chinese hamster ovary (CHOK1) cells. Mutat Res 656:68–73.

Tian Y, Ishikawa H, Yamaguchi T, Yamauchi T, Yokoyama K. 2005. Teratogenicity and developmental toxicity of chlorpyrifos: Maternal exposure during organogenesis in mice. Reprod Toxicol 20:267–271.

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

Turgeman G, Pinkas A, Slotkin TA, Tfilin M, Langford R, Yanai J. 2011. Reversal of chlorpyrifos neurobehavioral teratogenicity

in mice by allographic transplantation of adult subventricular zone-derived neural stem cells. J Neurosci Res 89:1185–1193.

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

Uggini GK, Patel PV, Balakrishnan S. 2012. Embryotoxic and teratogenic effects of pesticides in chick embryos: A comparative study using two commercial formulations. Environ Toxicol 27:166–174.

UN. 2011. Peligros para el medio ambiente. Naciones Unidas Parte 4:229–258.

USEPA. 1975. Methods for Acute Toxicity Tests with Fish, Macroinvertebrates, and Amphibians. Corvallis, OR: United States Environmental Protection Agency, EPA-660/3-5-009:67.

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

USEPA. 2000. Pesticide Ecotoxicity Database (Formerly: Environmental Effects Database (EEDB). Washington, DC 344: US Environmental Protection Agency.

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

USEPA. 2005. Guidelines for Carcinogen Risk Assessment (Final) US Environmental Protection Agency.

USEPA. 2006. Reregistration Eligibility Decision (RED) for Chlorpyrifos. Washington, DC: US Environmental Protection Agency, EPA-HQ-OPP-2006-0618:1–259.

Vera-Candioti J, Natale G, Soloneski S, Ronco AE, Larramendy ML. 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 ML. 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 ML. 2011. Acute toxicity of chromium on Cnesterodon decemmaculatus (Pisces: Poeciliidae). Theoria 20:85–93.

Vera-Candioti J, Soloneski S, Larramendy ML. 2013. 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.

Wagner PM, Nabholz JV, Kent RJ. 1995. The new chemicals process at the Environmental Protection Agency (EPA): Structure-activity relationships for hazard identification and risk assessment. Toxicol Lett 79:67–73.

WHO-FAO. 2009. Pesticides Residues in Food. Rome, Italy: World Health Organization and Food and Agriculture Organization of the United Nations. pp 1–426.

Zeljezic D, Garaj-Vrhovac V, Perkovic P. 2006. Evaluation of DNA damage induced by atrazine and atrazine-based herbicide in human lymphocytes in vitro using a comet and DNA diffusion assay. Toxicol In Vitro 20:923–935.