Ecotoxicology and Environmental Safety 104 (2014) 120–126

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

The genotoxic effects of the imidacloprid-based insecticide formulation Glacoxan Imida on Montevideo tree frog *Hypsiboas pulchellus* tadpoles (Anura, Hylidae)

J.M. Pérez-Iglesias [a,c,1], C. Ruiz de Arcaute [a,c,1], N. Nikoloff [a,c,1], L. Dury [a], S. Soloneski [a,c], G.S. Natale [b,c], M.L. Larramendy [a,c,*]

[a] Cátedra de Citología, Facultad de Ciencias Naturales y Museo, Universidad Nacional de La Plata, Calle 64 No. 3, B1904AMA La Plata, Argentina
[b] Centro de Investigaciones del Medio Ambiente (CIMA), Facultad de Ciencias Exactas, Universidad Nacional de La Plata, La Plata, Argentina
[c] Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Buenos Aires, Argentina

ARTICLE INFO

Article history:
Received 11 December 2013
Received in revised form
28 February 2014
Accepted 1 March 2014
Available online 25 March 2014

Keywords:
Acute toxicity
Amphibian tadpoles
Comet assay
Imidacloprid
Lethality
Micronucleus

ABSTRACT

The neonicotinoid insecticide imidacloprid (IMI) affects the insect central nervous system and is successfully applied to control pests for a variety of agricultural crops. In the current study, acute toxicity and genotoxicity of the IMI-containing commercial formulation insecticide Glacoxan Imida (35 percent IMI) was evaluated on *Hypsiboas pulchellus* (Anura: Hylidae) tadpoles exposed under laboratory conditions. A lethal effect was evaluated as the end point for lethality, whereas micronucleus (MN) frequency and DNA single-strand breaks evaluated by the single cell gel electrophoresis (SCGE) assay were employed as end points for genotoxicity. Sublethal end points were assayed within the 12.5–37.5 mg/L IMI concentration range. Experiments were performed on tadpoles at stage 36 (range, 35–37) according to the classification proposed by Gosner. Lethality studies revealed an $LC_{50\ 96\ h}$ value of 52.622 mg/L IMI. Increased frequency of MNs was only observed when 25.0 mg/L was assayed for 96 h, whereas no other nuclear abnormalities were induced. Increase of the genetic damage index was observed at 48 h of treatment within the 12.5–37.5 mg/L concentration range, whereas an increased frequency of DNA damage was observed only in tadpoles treated with 37.5 mg/L IMI for 96 h. This study represents the first evidence of the acute lethal and genotoxic effects exerted by IMI on tadpoles of an amphibian species native to Argentina under laboratory conditions.

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1. Introduction

Imidacloprid (IMI; (2E)-1-[(6-Chloro-3-pyridinyl)methyl]-N-nitro-2-imidazolidinimine) is a nicotine-derived systemic insecticide belonging to a group of pesticides called neonicotinoids. These insecticides act as insect neurotoxins and belong to a class of chemicals, the chloronicotinyl nitroguanidine chemical family, that affect the insect central nervous system (Blacquière et al., 2012; Tomizawa and Casida, 2005). Neonicotinoid insecticides are successfully applied to control pests for a variety of agricultural crops (for review, see Elbert et al., 2008 and references therein); however, they may not affect only pest insects, but also nontarget organisms such as pollinators (Blacquière et al., 2012). IMI works by interfering with the transmission of stimuli in the insect nervous system. Specifically, it causes a blockage in the nicotinergic neuronal pathway. This blockage leads to the accumulation of acetylcholine resulting in the insect's paralysis and eventual death. It is effective on contact and via stomach action (http://extoxnet.orst.edu/pips/imidaclo.htm). Because IMI binds much more strongly to insect nicotinic neuron receptors than to mammal neuron ones, this insecticide is selectively more toxic to insects than mammals (Gervais et al., 2010; Tomizawa and Casida, 2005). IMI has been ranked as a Class II chemical (moderately hazardous) by the World Health Organization (WHO, 2002), whereas the U.S. Environmental Protection Agency (EPA) (NPIC, 2010) has included the insecticide in Group E, the group of compounds with no evidence of carcinogenicity, based on studies with rats and mice. Furthermore, it has not been identified as a carcinogen by the International Agency for Research on Cancer (NPIC, 2010).

Overall, studies of the adverse effects induced by IMI have revealed that the insecticide should be considered as not acutely toxic for fish and amphibians, slightly toxic for zooplankton,

Abbreviations: BL, blebbed nuclei; BN, binucleated erythrocyte; CP, cyclophosphamide; GDI, genetic damage index; IMI, imidacloprid; LB, lobed nuclei; MN, micronucleus; NT, notched nuclei; SCGE, single-cell gel electrophoresis

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

http://dx.doi.org/10.1016/j.ecoenv.2014.03.002
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moderately toxic for crustaceans, highly toxic for annelids, and very highly toxic for insects (www.pesticideinfo.org). It has been demonstrated that either IMI or some IMI-based insecticides produce adverse effects on aquatic biota. Fish, amphibians, and aquatic algae are less sensitive to IMI than certain aquatic invertebrates in terms of survival and growth (http://cfpub.epa.gov/ecotox/quick_query.htm). Among aquatic invertebrates, arthropods such as chironomid (Langer-Jaesrich et al., 2010; Stoughton et al., 2008) as well as ostracod and amphipod species (Sánchez-Bayo and Goka, 2006; Stoughton et al., 2008) are extremely sensitive to IMI exposure, with observed adverse effects on survival, growth, and reproductive success. Similarly, toxic effects have been also reported for aquatic vertebrates, namely, fish (OPP-EEDB, 2000; Sánchez-Bayo et al., 2007; Sánchez-Bayo and Goka, 2005) and amphibians including *Pelophylax nigromaculatus* and *Rana limnocharis* (Feng et al., 2004).

Amphibians have certain characteristics rendering them useful indicator species for measuring the effects of changes of the environment (Brodeur et al., 2012). However, in recent decades, amphibian populations have been reported to suffer significant decline worldwide (Brodeur et al., 2012), a phenomenon in most cases attributable to pollution of agricultural areas with the use of pesticides (Mann et al., 2009). However, other factors, *e.g.*, overexploitation, diseases, habitat loss and/or modification, introduced species, and climate change, can also contribute to their decline (Mann et al., 2009). Negative effects against wild anuran populations, among others, exerted by emerging pollutants, including agrochemicals, have been reported (Davidson et al., 2007; Relyea, 2009; Sparling and Fellers, 2009). Although environmental pollution might interfere with normal amphibian growth, development, and susceptibility to disease, the induction of genetic injury into DNA after chronic exposure to agrochemicals is perhaps the most relevant biological effect. Furthermore, a correlation between the use of agrochemicals and the decline of amphibian populations has been reported (Beebee, 2005; Jones et al., 2009). The effects of pesticides, including insecticides as well as herbicides, are particularly detrimental to amphibian species. Several factors contribute to this, *e.g.*, their aquatic habitat, unprotected eggs, and sensitive, highly permeable skin, which is involved in gas, water, and electrolyte exchange with the environment (Blaustein et al., 1994; Bradford et al., 2011; Brühl et al., 2011; Sparling and Fellers, 2009).

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 genotoxicity and cytotoxicity have been employed on aquatic organisms, including amphibians. Among them, analysis of micronucleus (MN) frequency and the induction of DNA single-strand breaks by the single cell gel electrophoresis (SCGE) assay are the most frequently used and recommended end points for detecting cytogenetic and DNA damage in circulating nucleated erythrocytes, respectively (Hartmann et al., 2003; Lajmanovich et al., 2013; Maselli et al., 2010; Mouchet et al., 2007; Nikoloff et al., 2014; Tice et al., 2000; Vera Candioti et al., 2010).

Hypsiboas pulchellus, the Montevideo tree frog, also called the common tree frog, is an arboreal anuran species in the family Hylidae. The species was recently reported as threatened by agricultural water pollution (specifically pesticide runoff) in the central inner part of Argentina (Junges et al., 2012). Previous studies have stressed that tadpoles of this species can be considered a suitable *in vivo* model for detecting lethal and sublethal effects induced by several emerging pollutants, including agrochemicals. Among them, the oxidizing agent potassium dichromate (Natale et al., 2006); the chemotherapeutic cyclophosphamide (Lajmanovich et al., 2005); the insecticides fenitrothion (Junges et al., 2010), cypermethrin (Agostini et al., 2010), and endosulfan (Agostini et al., 2013; Lajmanovich et al., 2005); as well as the herbicide glufosinate ammonium (Peltzer et al., 2013) can be included.

The aim of the present study is to characterize the acute toxicity of the IMI-based insecticide formulation Glacoxan Imida (35 percent IMI) on *H. pulchellus* tadpoles exposed under laboratory conditions. The study was performed employing lethal and several sublethal short-term end points for genotoxicity, namely, MN and SCGE bioassays.

2. Materials and methods

2.1. Chemicals

IMI (CAS 138261-41-3; recommended application field ratio up to 700 g a.i./ha (CASAFE, 2011)) commercial grade trade formulation Glacoxan Imida (35 percent IMI) was purchased from Punch Química S.A., Argentina. Cyclophosphamide (CP; CAS 6055-19-2) and dimethyl sulfoxide (DMSO; CAS 67-68-5) were purchased from Sigma Chemical Co. (St. Louis, MO), and $K_2Cr_2O_7$ [$Cr_{(VI)}$] (CAS 7778-50-9) was obtained from Merck KGaA (Darmstadt, Germany). All other chemicals and solvents of analytical grade were purchased from Sigma Chemical Co.

2.2. Quality control

Determination of the concentration levels of IMI in the test solutions was done by QV Chem Laboratory (La Plata, Buenos Aires, Argentina) according to U.S. Geological Survey Report 01-4134 (Furlong et al., 2011). IMI levels were analyzed by high performance liquid chromatography using an ultraviolet detector. Active ingredient samples from test solutions (10.0 and 100.0 mg/L) correspond to values obtained immediately after preparation (0 h) and 24 h thereafter. The detection limit for IMI was 0.5 mg/L. Concentrations assessed throughout the study represent the nominal concentration of active ingredient in the IMI-based Glacoxan Imida formulation.

2.3. Test organisms

H. pulchellus tadpoles were selected as test organisms. This species has an extensive distribution in Neotropical America, including Argentina, Brazil, Paraguay, and Uruguay, and is a very widespread and abundant species in the Pampasic region of Argentina (Cei, 1980). Its natural habitats are subtropical or tropical dry lowland grassland, subtropical or tropical seasonally wet or flooded lowland grassland, intermittent freshwater lakes and marshes, and pastureland (Junges et al., 2012; Kwet et al., 2004). This species is easy to handle and acclimate to laboratory conditions (Lajmanovich et al., 2005; Natale et al., 2006). Egg masses used for this study were collected from a temporary and unpolluted pond free from pluvial runoff from agricultural areas, in the vicinity of La Plata City (35°10'S, 57°51'W; Buenos Aires Province, Argentina), at the late cleavage stage, stage nine according to Gosner's (1960) classification. Hatches were transported to the laboratory and then acclimatized to 16/8 h light/dark cycles in aquaria at 25 °C with dechlorinated tap water with artificial aeration. Physical and chemical parameters of the water were as follows: temperature, 25.0 ± 1 °C; pH 7.5 ± 0.1; dissolved oxygen, 6.3 ± 0.3 mg/L; ammonium (NH_4^+) < 0.2 mg/L; conductivity, 994 ± 8.5 μS/cm; hardness, 143 ± 23.5 mg $CaCO_3$/L. Boiled lettuce as a food source was supplied twice a week until the beginning of the experimental procedures. Experiments were performed on tadpoles at stage 36 (range, 35–37) according to the classification proposed by Gosner (1960). Hatches were collected with the permission of the Flora and Fauna Direction from the Buenos Aires Province (Buenos Aires, Argentina) (code 22500-22339/13) and the Ethical Committee from the National University of La Plata (code 11/N699).

2.4. Determination of LC_{50}

Experiments for toxicity assessment were performed on tadpoles at Gosner stage 36 following standardized methods proposed by the U.S. EPA (1975, 2002) and ASTM (2007) with minor modifications reported previously for native species (Nikoloff et al., 2014; Vera Candioti et al., 2010). Experiments were performed using five tadpoles for each experimental point, maintained in a 500 ml glass container, and exposed to six different concentrations of IMI (25.0, 37.5, 50.0, 75.0, 100.0, and 124.5 mg/L) during 96 h. Whereas the negative control group consisted of five organisms kept in dechlorinated tap water (see Section 2.3.), the positive control group consisted of five tadpoles treated with 23 mg/L $Cr_{(VI)}$, as reported previously (Nikoloff et al., 2014; Vera Candioti et al., 2010). Controls were conducted and run simultaneously with treatments for IMI-exposed tadpoles. All test solutions were prepared immediately before use and replaced every 24 h. Tadpoles were not fed

throughout the experiment. Experiments were performed in quadruplicate and run simultaneously for each experimental point.

2.5. Sublethal end points

2.5.1. Micronuclei and other erythrocytic nuclear abnormalities

MN assay was performed on peripheral circulating blood erythrocytes according to the procedure described previously (Vera Candioti et al., 2010). Experiments were performed using five tadpoles for each experimental point at Gosner stage 36, maintained in a 500 ml glass container and exposed to three different concentrations of Glacoxan Imida equivalent to 25 percent, 50 percent, and 75 percent of the corresponding $LC_{50\ 96\ h}$ value. To achieve these concentrations, tadpoles were exposed to 12.5, 25.0, and 37.5 mg/L IMI, respectively (see Section 2.4). Negative (dechlorinated tap water; see Section 2.4) and positive controls (40 mg/L cyclophosphamide, CP) were conducted and run simultaneously with treatments for IMI-exposed tadpoles. All test solutions were prepared immediately before each experiment. The frequency of MNs was determined in peripheral mature erythrocytes at 48 and 96 h after initial treatment. Experiments were performed in triplicate and run simultaneously for each experimental point. Tadpoles were killed according to American Society of Ichthyologists and Herpetologists (ASIH, 2004) criteria. At the end of each experiment, tadpoles were anesthetized by immersion in ice water, and blood samples were obtained by sectioning behind the operculum. Peripheral blood smears were performed for each animal onto clean slides, air dried, fixed with 100 percent (v/v) cold methanol (4 °C) for 20 min, and then stained with five percent Giemsa solution for 12 min. Slides were coded and blind-scored by one researcher at 1,000 × magnification. Data are expressed as the total number of MNs per 1000 cells, as suggested previously (Vera Candioti et al., 2010). MN frequency was determined following the examination criteria reported previously (Fenech, 2007; Vera Candioti et al., 2010). Briefly, the criteria employed in identifying MNs were as follows: a diameter smaller than 1/3 of that of the main nuclei, nonrefractibility, the same staining intensity as or staining intensity lighter than that of the main nuclei, no connection or link with the main nuclei, no overlapping with the main nuclei, MN boundary distinguishable from the main nuclei boundary, and no more than four MNs associated with the nuclei.

Other erythrocytic nuclear abnormalities were blind-scored from 1000 erythrocytes per experimental point from each experiment at 1000 × magnification. Examination criteria followed those established previously (Cavaş and Ergene-Gözükara, 2003; Strunjak-Perovic et al., 2009). Briefly, cells with two nuclei were considered binucleated (BN), whereas cells with one nucleus presenting a relatively small evagination of the nuclear membrane that contained euchromatin were classified as blebbed nuclei (BL). Nuclei with evaginations of the nuclear membrane larger than those of the BLs, which could have several lobes, were considered lobed nuclei (LB). Finally, nuclei with vacuoles and appreciable depth into a nucleus without containing nuclear material were recorded as notched nuclei (NT).

2.5.2. Single cell gel electrophoresis assay

Tadpoles exposed for MN assay were also employed for SCGE assay (see Section 2.5.1). Negative (dechlorinated tap water; see Section 2.4) and positive controls (40 mg/L CP) were conducted and run simultaneously with treatments for IMI-exposed tadpoles. The SCGE assay was performed following the alkaline procedure described by Singh (1996) with minor modifications reported elsewhere (Nikoloff et al., 2014; Vera-Candioti et al., 2013). 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 non-overlapping cells. DNA damage was classified in four classes (0-I, undamaged; II, minimum damage; III, medium damage; IV, maximum damage), as suggested previously (Cavaş and Könen, 2007). Data are expressed as the mean number of damaged cells (sum of Classes II, III, and IV) and the mean comet score for each treatment group. The genetic damage index (GDI) was calculated for each test compound following Pitarque et al. (1999) using the formula GDI=[I (I)+2 (II)+3 (III)+4 (IV)/N (0-IV)], where 0-IV represents the nucleoid type, and N_0–N_{IV} represents the total number of nucleoid scored.

2.6. Statistical analysis

Mortality data were analyzed using the U.S. EPA Probit Analysis, version 1.5, statistical software (http://www.epa.gov/nerleerd/stat2.htm) and based on Finney's (1971) method. Data of MNs, BNs, BLs, LBs, NTs, and SCGE were analyzed by one-way ANOVA with Dunnett's test (Zar, 1999) to determine significant differences from the control group. ANOVA assumptions were corroborated with Barlett's test for homogeneity of variances and a χ^2 test for normality. The relationships between concentration and GDI and MN data were evaluated by simple linear regression and correlation analyses. Concentration–response (C–R) curves at 96 h were estimated with their 95 percent confidence limits. Regression coefficients (r) were calculated for each C–R curve. Tests of significance of the regressions coefficients were performed following Zar (1999). The level of significance was 0.05 for all tests, unless indicated otherwise.

3. Results

Results obtained from the t-test between chemical analyses showed no significant changes ($P>0.05$) in the concentration of the pure analyte in treatments after the 24 h interval renewals of the testing solutions (concentration range, 98 ± 5 percent recovery).

3.1. Mortality

Probit analysis of the mortality data allowed determination of the LC_{50} values of IMI after 24, 48, 72, and 96 h of exposure. Results revealed the following mean values of $LC_{50\ 24\ h}$=69.412 mg/L (range, 62.872–75.522), $LC_{50\ 48\ h}$=58.225 mg/L (range, 26.494–127.876), $LC_{50\ 72\ h}$=56.772 mg/L (range, 27.535–116.963), and $LC_{50\ 96\ h}$=52.622 mg/L (range, 48.470–58.185).

3.2. MN and other erythrocytic nuclear abnormality frequencies

Table 1 shows the results of the analysis of IMI-induced MNs as well as other nuclear abnormalities in peripheral blood erythrocytes of *H. pulchellus* tadpoles. An increased frequency of MNs was observed in tadpoles exposed to CP (positive control) for 96 h ($P<0.05$), but not in those in treatments lasting 48 h ($P>0.05$).

Table 1
Frequencies (‰) of MN and other nuclear abnormalities in peripheral blood erythrocytes of *Hypsiboas pulchellus* tadpoles exposed to the imidacloprid-based insecticide Glacoxan Imida[a].

Exposure time (h)	Concentration (mg/L)	No. of animals analyzed	No. of cells analyzed	MN[b]	Other nuclear abnormalities[b]			
					NT	LB	BN	BL
48	Control	15	15,059	4.20 ± 0.68	3.40 ± 0.95	0.20 ± 0.14	0.33 ± 0.12	4.67 ± 0.99
	CP[c]	16	16,150	4.15 ± 0.81	4.06 ± 0.98	0.38 ± 0.15	0.63 ± 0.15	5.44 ± 0.92
	12.5	15	15,097	6.11 ± 1.06	4.60 ± 1.87	0.27 ± 0.15	0.13 ± 0.09	5.87 ± 1.45
	25.0	15	14,646	6.75 ± 1.32	6.80 ± 1.36	0.20 ± 0.14	0.47 ± 0.19	7.00 ± 1.33
	37.5	15	15,086	6.15 ± 1.34	3.67 ± 1.24	0.07 ± 0.06	0.47 ± 0.29	4.87 ± 1.13
96	Control	15	15,213	5.46 ± 0.78	3.67 ± 0.76	0.60 ± 0.23	1.13 ± 0.27	6.20 ± 1.07
	CP[c]	18	18,157	10.14 ± 1.69*	8.33 ± 2.66	0.67 ± 0.45	0.89 ± 0.16	9.06 ± 1.42
	12.5	15	15,067	6.18 ± 0.95	3.40 ± 0.88	0.07 ± 0.06	0.67 ± 0.23	3.87 ± 0.79
	25.0	15	15,126	10.06 ± 1.52*	6.80 ± 1.67	1.00 ± 0.28	1.33 ± 0.37	9.47 ± 0.81
	37.5	15	15,169	5.40 ± 1.30	3.53 ± 0.90	0.13 ± 0.09	0.53 ± 0.25	3.93 ± 1.13

[a] Results are expressed as mean number of abnormalities/1000 cells ± SE.
[b] MN, micronucleus; NT, notched nuclei; LB, lobed nuclei; BN, binucleated nuclei; BL blebbed nuclei.
[c] Ciclophosphamide (CP, 40 mg/L) was used as positive control.
* $P<0.05$; significant differences with respect to control values.

Table 2
Analysis of DNA damage measured by comet assay in *Hypsiboas pulchellus* tadpoles cells exposed to the imidacloprid-based insecticide Glacoxan Imida.

Chemicals	Concentration (mg/L)	Exposure time (h)	No. of animals analyzed	No. of cells analyzed	Percent of damaged cells (II+III+IV)	GDI ± SE[a]
Control		48	15	1561	33.06	1.13 ± 0.19
		96	15	1589	29.76	1.19 ± 0.14
Glacoxan Imida	12.5	48	16	1538	46.16	1.82 ± 0.13**
		96	15	1345	39.55	1.53 ± 0.14
	25.0	48	15	1448	56.15*	1.83 ± 0.17**
		96	16	1495	56.39**	1.71 ± 0.13
	37.5	48	15	1324	60.12**	1.91 ± 0.22**
		96	10	757	61.55**	1.96 ± 0.20*
CP[b]	40.00	48	14	1425	79.93***	2.81 ± 0.22***
		96	15	1536	74.94***	2.42 ± 0.24***

[a] GDI: genetic damage index.
[b] Cyclophosphamide (CP, 40 mg/L) was used as positive control.
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$; significant differences with respect to control values.

Results revealed that the frequency of MNs in tadpoles exposed to IMI for 48 h did not differ from that in the negative controls, regardless of the concentration assayed within the 12.5–37.5 mg/L IMI concentration range ($P > 0.05$). On the other hand, tadpoles exposed to 25.0 mg/L IMI for 96 h showed a significant increase in MN frequency relative to negative controls ($P > 0.05$). When the frequencies of other nuclear abnormalities were analyzed, IMI treatments, regardless of both concentration and exposure time, did not show modified frequencies compared to negative control values ($P > 0.05$) (Table 1).

3.3. DNA damage

The results of the SCGE assay obtained in peripheral blood erythrocytes of *H. pulchellus* tadpoles exposed for 48 and 96 h to IMI are presented in Table 2, and mean frequencies of cells from each damage grade are depicted in Fig. 1. CP treatment (positive control) induced an enhancement of the GDI as well as the frequency of damaged cells compared to negative controls in specimens exposed for either 48 or 96 h ($P < 0.001$) (Table 2, Fig. 1). In tadpoles exposed to IMI, a significant increase of the GDI was observed at 48 h of treatment for all tested concentrations ($P < 0.01$) (Table 2). In tadpoles exposed for 48 h, an increased frequency of type IV nucleoids within the 12.5–37.5 mg/L concentration range was observed ($0.05 > P < 0.01$). In addition, an enhancement of the frequency of type III nucleoids was observed when tadpoles were exposed to a 37.5 mg/L concentration ($P < 0.05$) (Fig. 1A). When the analysis was performed in tadpoles exposed for 96 h, an increased GDI value was found only for tadpoles exposed to 37.5 mg/L IMI ($P < 0.05$) (Table 2). Furthermore, such increase was due to an increase in the frequency of type II nucleoids ($P < 0.01$) and a concomitant decrease of type 0-I nucleoids ($P < 0.01$) (Fig. 1B). Overall, a regression analysis demonstrated that whereas the GDI did not vary as a function of the IMI concentration in tadpoles treated for 48 h ($r = 0.16$, $P > 0.05$), a significant dose-dependent increase in the GDI was observed in tadpoles exposed for 96 h ($r = 0.33$, $P < 0.05$).

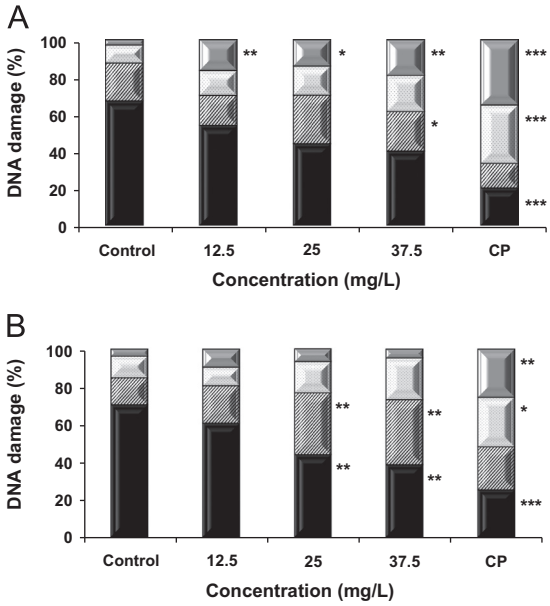

Fig. 1. Glacoxan Imida-induced DNA damage measured by comet assay in circulating blood cells from *Hypsiboas pulchellus* (Anura, Hylidae) tadpoles exposed for 48 h (A) and 96 h (B). The frequencies of undamaged (type 0-I nucleoids; black bar sections), type II (stripped bar sections), type III (dotted bar sections), and type IV nucleoids (gray bar sections) were determined by analyzing 100 nucleoids from each tadpole. Results are presented as percentages of pooled data from three independent experiments. Negative (untreated tadpoles) and positive controls (CP, 40 mg/L cyclophosphamide-treated tadpoles) were conducted and run simultaneously with treatments for Glacoxan Imida-exposed tadpoles. *$P < 0.05$; **$P < 0.01$;***$P < 0.001$ (significant differences with respect to control values).

4. Discussion

In the present report, the acute lethal toxicity and sublethal effects of the IMI-based formulation Glacoxan Imida were evaluated on *H. pulchellus* (Anura, Hylidae) tadpoles exposed under laboratory conditions. Regarding the acute lethal effects of the studied formulation on the species, the chemical could be ranked, according the scoring proposed by the Office of Pollution Prevention and Toxics of the U.S. EPA (Smrchek et al., 1993; Wagner et al., 1995), as a compound with moderate ecotoxicity concern for *H. pulchellus* tadpoles. However, it can be classified as a harmful compound for aquatic organisms (Category III) following either the European Union directives (Mazzatorta et al., 2002) or the classification criteria proposed by the United Nations (UN, 2011). Despite this fact, the insecticide presents identifiable sublethal effects at concentrations nearly fourfold lower than those corresponding to the lethal end point, which are associated with the appearance of genotoxic effects. The results demonstrate that IMI is not a potent DNA-damaging agent at the chromosomal level since the insecticide increased the frequency of MNs in peripheral erythrocytes of *H. pulchellus* only when exposed for 96 h to

25.0 mg/L. Furthermore, the insecticide failed to induce other nuclear abnormalities, *i.e.*, binucleated cells or blebbed, lobed or notched nuclei. On the other hand, we observed that acute exposure to all concentrations of the IMI-based formulation increased the frequency of primary DNA lesions estimated by alkaline SCGE. Additionally, our data revealed that the SCGE assay was more sensitive than the MN test in detecting early DNA damage when the same IMI concentrations were employed for tadpole exposure.

The variability in pesticide-induced toxicity to different amphibian species is a phenomenon known worldwide (Jones et al., 2009; Relyea, 2009; Vera Candioti et al., 2010). To the best of our knowledge, acute lethality data of IMI have been reported previously for two amphibian species. Years ago, Feng et al. (2004) reported $LC_{50\ 96\ h}$ values of 82.0 and 129.0 mg/L for *R. limnocharis* and *P. nigromaculatus* premetamorphic tadpoles exposed to the active ingredient under laboratory conditions, respectively. However, no indication of the developmental growth states of the tadpoles was given (Feng et al., 2004). Accordingly, our current observations indicate that *H. pulchellus* could be considered the most sensitive anuran species to IMI reported so far.

Our results revealed an IMI concentration of 52.622 mg/L (range, 48.470–58.185) as the $LC_{50\ 96\ h}$ value for *H. pulchellus* tadpoles. Previous studies have demonstrated that the tested hylid frog tadpoles can be considered adequate reference organisms in toxicity risk assessment studies of different xenobiotics, including pesticides. Among these results, the following reported $LC_{50\ 96\ h}$ maximum values can be included: 29.60 mg/L for $Cr_{(VI)}$ (Natale et al., 2000, 2006), 0.47 mg/L for the insecticide cypermethrin or 0.175 mg/L for the cypermethrin-based commercial formulation Sherpa® (Agostini et al., 2010), and 0.00013 mg/L for an endosulfan-based insecticide formulation (Agostini et al., 2009). Accordingly, it seems evident that this anuran species is nearly 405,000, 301, 112, and two times less sensitive to IMI than to endosulfan, Sherpa®, cypermethrin, and $Cr_{(VI)}$ or glufosinate ammonium, respectively. In other words, IMI is the least toxic emerging pollutant reported so far for *H. pulchellus* tadpoles. Furthermore, it could be valid to suggest that the species seems to be more sensitive to pesticides belonging to the organochlorine and pyrethroid chemical groups than to the neonicotinoid group.

It should be mentioned that in our study, an IMI-based insecticide containing only 35 percent of the active ingredient within the formulation was assayed, an aspect that should be further considered. It is well known that in agriculture, pesticides are usually applied in their formulated forms, where the active ingredient is combined with organic solvents and emulsifying and wetting agents, which affect the pesticide penetration and performance (WHO, 1990). The additives may synergize or antagonize the toxicity of the active ingredient. Although additive compounds frequently make up part of a commercial pesticide formulation, they are not usually included in any discussion of the effects on living organisms, and their adverse effects may exceed those of the active ingredient. Although pesticides are developed through very strict regulation processes to function with reasonable certainty and minimal impact on human health and the environment, serious concerns have been raised about health risks resulting from occupational exposure and from residues in food and drinking water (WHO, 1990). Several investigations have demonstrated that the additive compounds present in pesticide commercial formulations have the ability to induce toxicity and cellular damage by themselves, separate from the active ingredient (Belden et al., 2010; Brühl et al., 2013; Lin and Garry, 2000; Mann and Bidwell, 1999; Molinari et al., 2013; Nikoloff et al., in press, 2012; Rayburn et al., 2005; Soloneski and Larramendy, 2010; Zeljezic et al., 2006). Hence, risk assessment must also consider additional toxic effects caused by the excipient(s). Unfortunately, the identities of the additive compounds present in the commercial formulation Glacoxan Imida were not made available to us by the manufacturers. It should be mentioned that according to our Argentinean administration, the excipients present in any agrochemical are not required to be listed on the agrochemical data sheet and can be kept as a "trade secret". Years ago, the U.S. EPA (1982) claimed that the acute toxicity of a technical active ingredient can differ significantly from that of the end-use formulation containing that active ingredient. Our results are in total agreement with this concept and pinpoint the necessity of further studies on *H. pulchellus* tadpoles employing the active ingredient IMI as test compound to reveal whether the high sensitivity of the *H. pulchellus* we observed is specific to the species or results from the presence of xenobiotic(s) within the formulated technical formulation Glacoxan Imida assayed in our study.

The MN analysis is employed worldwide as genotoxic bioassay to detect small chromosomal fragments, *i.e.*, acentric fragments and chromatid fragments, induced by clastogens or vagrant chromosomes produced by aneugens (Fenech, 2007; OECD, 2007). Previous reports demonstrated the induction of MNs in circulating erythrocytes from amphibian tadpoles as a consequence of pesticide treatments (Bouhafs et al., 2009; Li et al., 2010; Nikoloff et al., 2014; Vera Candioti et al., 2010). Furthermore, employing *H. pulchellus* as an experimental model, Lajmanovich et al. (2005) demonstrated the induction of MNs after exposure to the insecticide endosulfan. In our current study, only the concentration of 25.0 mg/L of IMI employed was able to induce DNA damage leading to MN formation when tadpoles were exposed for 96 h but not when treatments lasted for 48 h. So far, we do not have any explanation for this particular observation. However, a plausible possibility could be related to the presence of toxic coformulants in the commercial preparation of Glacoxan Imida that may pose genotoxic risk and have cytotoxic properties as well, preventing the most severe cells from completing one cell-cycle division by the harvesting time, and thus not inducing an increase of MNs. Furthermore, the possibility of induction of a selective cell loss by insecticide-induced cell death of the most damaged cells after treatment, leaving only a reduced proportion of cells capable of reaching the M1 status nearly after 96 h of exposure, could not be ruled out. Whether the latter is true or not, the present results could confirm the importance of studying complete agrochemical formulations in toxicity screenings because the excipient(s) may have toxicological properties completely different from those of the active ingredients alone, and their impacts may be quite different, as demonstrated previously in different biotic matrices (Cox and Surgan, 2006; Nikoloff et al., in press, 2014; Soloneski and Larramendy, 2010).

The SCGE test has become extensively valuable as a biomarker in amphibians to monitor contaminated areas (*in situ* assay) (Burlibasa and Gavrila, 2011; Maselli et al., 2010) as well as for screening xenobiotics after direct or indirect exposure (*in vivo* assay) (Knakievicz et al., 2008; Mouchet et al., 2007; Nikoloff et al., 2014). We observed that, regardless of the length of treatment, an IMI concentration of 12.5 mg/L was unable to increase the frequency of damaged nucleoids in tadpoles treated for 48 and 96 h. However, acute exposure to IMI concentrations higher than 25.0 mg/L, regardless of the exposure period, increased the frequency of primary DNA lesions estimated by alkaline SCGE, a result opposite that of the MN test. One possible explanation for this observation could be related to the different cellular status of the target cells that are included for analysis for each end point. Whereas DNA damage is estimated by SCGE in resting cells, MNs are determined on proliferating cells with lesions that have lasted for at least one mitotic cell cycle and that probably retain their repair properties, as suggested elsewhere (He et al., 2000). Finally,

it should be mentioned that our results demonstrated that whereas the GDI did not vary as a function of the IMI concentration in tadpoles treated for 48 h, a significant dose-dependent increase in the GDI was observed in tadpoles exposed for 96 h. Similar observations have been previously reported for coelomocytes of *Eisenia fetida* earthworms (Zang et al., 2000) and erythrocytes of *P. nigromaculatus* tadpoles (Feng et al., 2004) exposed to IMI.

Although reports in which the frequency of agrochemical-induced DNA single-strand breaks has been used as a bioassay for evaluating genetic damage induced in amphibians are well documented worldwide (Mouchet et al., 2007), data available for native Argentinean amphibians are scarce. To the best of our knowledge, induction of primary DNA damage on tadpoles of *Rhinella arenarum* as the target has been reported previously for only two flurochloridone-based commercial formulations, Twin Pack Gold® and Rainbow® (Nikoloff et al., 2014). Accordingly, our current results represent the first evidence of the acute genotoxic effects exerted by the IMI-based commercial insecticide formulation Glacoxan Imida on tadpoles under laboratory conditions. Furthermore, no other SCGE study has been previously reported employing *H. pulchellus* as the test organism. Finally, our findings support the view that the SCGE assay is a highly sensitive method for the detection of DNA damage induced by environmental pollutants.

Although the *in vivo* IMI treatments in this study covered a wide range of concentrations, the concentration range represents a relatively high end of the threshold value of 0.1 μg/L IMI allowed in ground, surface, and drinking water (RIVM, 2008), even considering the tolerances for residues of IMI and its metabolites on food/feed additives ranging from 20 μg/L in eggs to 3 mg/L in hops (http://extoxnet.orst.edu/) or that the recommended application rates to foliage or soil range from approximately 50–320 g a.i./ha (PMRA, 2005), or as high as 700 g a.i./ha reported for Argentina (CASAFE, 2011). Thus, the concentrations of IMI employed in this investigation would be expected to be almost improbable in the environment, perhaps observed only when specific events occurred (*e.g.*, direct application, drainage ditches, or accidental discharge). Although, we cannot rule out that amphibian populations, and also occupationally exposed human workers, could be exposed accidentally to these agrochemicals at this range of concentrations.

Acknowledgments

This study was supported by grants from the National University of La Plata (Grants 11/N619 and 11/N699) and the National Council for Scientific and Technological Research (CONICET, PIP N 0106 and PIP N 0344) from Argentina.

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