Ecotoxicology and Environmental Safety 148 (2018) 1–10

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

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

Are the damaging effects induced by the imazethapyr formulation Pivot® H in *Boana pulchella* (Anura) reversible upon ceasing exposure?

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ARTICLE INFO

Keywords:
Imazethapyr
Pivot® H
Recovery capabilities
Comet assay
Micronuclei
Morphological abnormalities

ABSTRACT

In the present study, the damage recovery capabilities of *Boana pulchella* tadpoles after acute exposure (96 h) to 0.39 mg/L concentration of the imazethapyr (IMZT)-based herbicide formulation Pivot® H (25% IMZT LC_{50} value) were assessed during a period of 7 to −21 days. To appraise the recovery capabilities, frequency of micronuclei (MNs), other nuclear abnormalities and DNA single-strand breaks evaluated by single cell gel electrophoresis assay on circulating blood cells were employed as endpoints for genotoxicity. Growth, development, body mass, and morphological abnormalities were also employed as individual endpoints in the recovery assay. Results demonstrated that IMZT induced sublethal effects at both the individual (i.e., loss of keratodonts) and cytogenetic levels (e.g., increase of MN frequency, other nuclear abnormalities and DNA single-strand breaks). At 11 days of the exposure phase, tadpoles recovered their basal levels of frequency of MNs, other nuclear abnormalities, and comets. However, loss of keratodonts, observed at the end of the exposure period, was present up to 21 days thereafter. Finally, axial abnormalities and delay in development stage were observed only during the postexposure phase in IMZT-exposed tadpoles at 18 and 25 days, respectively and were observed until the end of the experiment. This is the first evidence of use the comet assay as cytogenetic biomarker of genotoxicity in evaluating the recovery capabilities of amphibians in general and also those of *B. pulchella* after exposure to IMZT.

1. Introduction

In the last decades, the expansion of agricultural activities together with the exhaustive use of agrochemicals has led to the widespread presence of pesticides in every area of the environment and thus represents one of the major anthropogenic factors leading to increased contaminants and environmental stressors in aquatic ecosystems (Cooper, 1993; Schwarzenbach et al., 2006). Among these agrochemicals are included herbicides, known to exert changes in the biochemical and metabolic parameters of wild nontarget species that inhabit aquatic systems (Köhler and Triebskorn, 2013; Salbego et al., 2010). It is well known that while a part of the amount of herbicides employed in agronomical practices reach the target, the remainder flows into the ground, through air or water, contaminating several microhabitats, including ponds and wetlands in the proximity of crops where amphibians live and breed, then affecting all amphibian life stages (Greulich and Pflugmacher, 2003; Harris et al., 1998; Rohr and Crumrine, 2005). The accumulation of such environmental stressors in aquatic environments has been proposed as a causative agent of amphibian decline, because they can have adverse effects on anuran health (Beebee and Griffiths, 2005; Blaustein and Wake, 1990; Houlahan et al., 2000; Kiesecker et al., 2001). However, it is imperative to highlight that due to the seasonality of crops concomitantly with the subsequent periodic/seasonal application of these agrochemicals, their input into aquatic systems become then, typically intermittent. Accordingly, nontarget species exposure under these types of situations to these types of environmental stressors can be short (time scale of days) and followed by periods of permanence in noncontaminated areas. Moreover, there is little information concerning the reversibility or irreversibility of biochemical, physiological, or genetic effects in aquatic organisms after the decrease or disappearance of contamination (Guilherme et al., 2014). Assessing the responses generated by pesticide exposure and the persistence of disturbances after contamination ends would provide a better understanding of environmental stressor depuration and

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http://dx.doi.org/10.1016/j.ecoenv.2017.10.009
Received 14 July 2017; Received in revised form 27 September 2017; Accepted 3 October 2017

recovery processes in aquatic organisms. In addition, the dynamics of pesticide-induced toxicity is an important step to improve the knowledge of the actual magnitude of risk posed by these compounds to aquatic biota (Guilherme et al., 2014; Mouchet et al., 2015). Summarizing, it has been recommended that to achieve a complete analysis of pesticide impact, the recovery mechanisms immediately following chemical exposure and in the period after exposure should be also considered (Bernabò et al., 2013).

Several authors have studied the recovery capabilities of aquatic biota after exposure to environmental stressors, including fish (Bearr et al., 2010; Corcoran et al., 2014; de Menezes et al., 2011; Dû-Lacoste et al., 2013; Ferrari et al., 2004; Guilherme et al., 2014; Guzmán-Guillén et al., 2014; Michel and Vincent-Hubert, 2015; Mohanty et al., 2011; Stankevičiūtė et al., 2016; Wessel et al., 2010) and amphibians (Bernabò et al., 2013; Bulaeva et al., 2015; Ferrari et al., 2004; García-Muñóz et al., 2009; Lajmanovich et al., 2009; Marques et al., 2014; Mouchet et al., 2015; Vogiatzis and Loumbourdis, 1997). In particular, studies on anurans tadpoles have focused on assessing recovery capabilities after exposure to environmental stressors at ecotoxicological, individual (e.g., behavior, growth, and morphological abnormalities) (Bernabò et al., 2013; Bulaeva et al., 2015; García-Muñóz et al., 2009), biochemical (e.g., enzymatic responses) (Ferrari et al., 2004; Lajmanovich et al., 2009; Vogiatzis and Loumbourdis, 1997), immune/histological (Bernabò et al., 2013), teratogenic (Svartz et al., 2012), and cytotoxic and genotoxic levels (Bulaeva et al., 2015; Mouchet et al., 2015). Nevertheless, information about these recovery capabilities on Neotropical species is scarce (Ferrari et al., 2004; Lajmanovich et al., 2009; Svartz et al., 2012), and there is no record about recovery capabilities after induction of genetic damage in Neotropical anurans reported so far.

Imidazolinones represent a group of agrochemicals widely used in more than 200 countries worldwide as selective pre- or postemergence herbicides that effectively controls a broad spectrum of weed species. This class of herbicides currently consists of six commercially available enantiomers and their methyl derivatives, namely, imazapic, imazapyr, imazethapyr (IMZT), imazamox, imazaquin, and imazamethabenz-methyl. Imidazolinone herbicides inhibit the action of the acetohydroxyacid synthase, also called acetolactate synthase (Lin et al., 2007). These agrochemicals are among the most popular herbicides because while they have proved to be potent and highly selective for plants, they are considered overall as nontoxic for vertebrates like mammals and fishes (PPDB, 2014).

IMZT [5-ethyl-2-(4-isopropyl-4-methyl-5-oxo-4,5-dihydroimidazol-1H-2-yl) nicotinic acid] is employed to control grasses and broad-leaved weeds in a variety of crop and noncrop areas (MacBean, 2012). The herbicide has a low sorption coefficient, though it possess high solubility in water (1400 mg/L), and thus has a high affinity with water (Johnson et al., 2000; Senseman, 2007). Furthermore, the California Department of Pesticide Regulation (www.pesticideinfo.org) has ranked this herbicide as a probable ground water contaminant. According to the U.S. Environmental Protection Agency (U.S. EPA), IMZT has been classify as a chemical with slight toxicity (Class III) (USEPA, 1989), whereas it has been considered as unlike hazardous chemical by the World Health Organization (www.pesticideinfo.org). The European Union has concluded that IMZT is an unsafe compound for the environment and it has been associated with human irritant effects on the eyes and skin, respiratory tract irritation (PPDB, 2014).

Overall, toxic, genotoxic, and cytotoxic studies of IMZT are limited and contradictory. Very little is known about IMZT-induced toxicity in nontarget organisms. Low levels of toxicity have been reported for the algae *Raphidocelis subcapitata* and aquatic invertebrates such as *Daphnia magna* after 72 and 48 h of exposure, respectively. Contrarily, high acute toxicity was observed in *Lemna gibba* (Magdaleno et al., 2015; PPDB, 2014; Reimche et al., 2015). Among terrestrial invertebrates, honey bees and earth worms have been reported to have particularly high sensitivity and low sensitivity to IMZT, respectively (PPDB, 2014). Toxicity exerted by IMZT has been found not to be acutely toxic for fish including *Ictalurus punctatus*, *Oncorhynchus mykiss* and *Lepomis macrochirus* with reported LC_{50} 96 h values of 240, 344, and 423 mg/L IMZT, respectively (Kegley et al., 2014; PPDB, 2014). Nevertheless, Moraes et al. (2011) demonstrated in hepatic tissues of *Cyprinus carpio* alterations in oxidative stress parameters after 0.0148 mg/L exposure to both the active ingredient IMZT and to the IMZT-based commercial herbicide formulation Only®. In addition, inhibition of acetylcholinesterase in the Mozambique tilapia *Oreochromis mossambicus* was reported after exposure to commercial formulations of IMZT (Pasha, 2013; Pasha and Singh, 2005). Finally, when IMZT was administered to Sprague Dawley rats by oral route or by dermal exposure to New Zealand White rabbits, low or moderate acute toxicity was reported after 96 h of treatment (USEPA, 2002a).

In analyses of cyto- and genotoxic effects, IMZT has generally been reported to be nonmutagenic in the *Salmonella T*yphimurium and *Escherichia coli* reversion assay both in the presence and absence of S9 metabolic fraction (Magdaleno et al., 2015). When the SMART in *Drosophila melanogaster* and the CHO/HGPRT assays were employed, negative results were observed, regardless of the presence of S9 mix (Fragiorge et al., 2008; USEPA, 2002a). Whereas chromosome alterations were not detected in bone marrow cells from IMZT-treated rats, both negative and positive results have been found to be induced in CHO cells with and without metabolic activation, respectively (USEPA, 1989). Growth inhibition was reported after exposure to a IMZT commercial formulation Verosil® in the green micro alga *Pseudokirchneriella subcapitata* (Magdaleno et al., 2015). IMZT altered nontarget communities of rotifers, cladocers, copepods and chironomids (Marchiori et al., 2012; Reimche et al., 2015). In addition, IMZT induced both cytotoxicity and chromosomal anomalies in meristematic root cells of *Triticum durum* and *Vicia faba* (El-Nahas, 2000; Rad et al., 2011). Cytotoxicity estimated by both root growth and mitotic index inhibition was reported in *Allium cepa* root tip cells after treatment with pure IMZT (Liman et al., 2015). Similarly, Magdaleno et al. (2015) observed that the IMZT-based formulation Verosil® induced cytotoxicity by arresting cells at prophase in meristematic tissues. Furthermore, toxicity of IMZT using the root elongation assay, chromosomal aberrations and DNA single-strand breaks evaluated by the single cell gel electrophoresis. This bioassay, also called the comet assay, is one of the most widely used methods to detect the genotoxic capability of xenobiotics both in vivo and in vitro since it is simple, fast, specific, and sensitive. The methodology in its alkaline or neutral version, detects a variety of DNA lesions at the single-cell level, including single-DNA strand breaks as well as alkali-labile lesions and double-strand breaks, respectively (Azqueta et al., 2014; Collins et al., 2014). Applying this methodology, it has been also demonstrated that IMZT induced DNA damage in *Lactuca sativa* after 120 h of exposure (Liman et al., 2015). Recently, an increased frequency of MNs was reported in *A. cepa* after exposure to an IMZT-based preparation (Magdaleno et al., 2015). Qian et al. (2015) demonstrated that phytotoxicity following IMZT-treatment was due to inhibition of the biosynthesis of branched chain amino acids (BCAAs) valine, leucine, and isoleucine affecting, then, the pattern of growth on roots, shoots, and leaves of *Arabidopsis thaliana.* Recently, we reported that an acute in vivo exposure to the herbicide jeopardized anuran Montevideo tree frog *Boana pulchella* tadpoles (Pérez-Iglesias et al., 2015). We found in the species an increase in the frequency of MNs, other nuclear abnormalities as well as the induction of primary DNA lesions after an acute IMZT-based herbicide commercial formulation Pivot® H exposure (Pérez-Iglesias et al., 2015). Recently, using the same biotic matrix, we were able to demonstrate that the herbicide induces DNA oxidative lesions at purine but pyrimidines bases which may be at least partially responsible for IMZT-induced genotoxicity (Pérez-Iglesias et al., 2017).

However, no approach to analyze whether *B. pulchella* tadpoles possess the capacity to recover the acute IMZT-inflicted morphological and cytogenetic damage has been included in none of our previous

studies. Accordingly, the present study we aimed to appraise the recovery capabilities of *B. pulchella* tadpoles after an acute exposure to the IMZT-based herbicide formulation Pivot® H (10.59% IMZT). MN frequency, other nuclear abnormalities and DNA single-strand breaks estimated by the SCGE assay on circulating blood cells were employed as endpoints for genotoxicity after a recovery period of 7–21 days from the end of the herbicide exposure. Furthermore, growth, development, body mass, and morphological abnormalities were also employed as sublethal individual endpoints in the recovery assay.

2. Material and methods

2.1. Chemicals

Pivot® H (10.59% IMZT; CAS081335-77-5) was purchased from BASF Argentina S.A. (Buenos Aires, Argentina). Cyclophosphamide (CP; CAS 6055-19-2) as well as all other chemicals and solvents of analytical grade were purchased from Sigma Chemical Co. (St. Louis, MO).

2.2. Quality control

Concentration levels of IMZT in the test solutions were analyzed by QV Chem Laboratory (La Plata, Buenos Aires, Argentina) according to U.S. Geological Survey Report 01-4134 (Furlong et al., 2011). Analyte levels were estimated by high performance liquid chromatography. Active ingredient samples from test solutions (0.39 mg/L) correspond to values obtained immediately after preparation (0 h) and 24 h thereafter. The detection limit for IMZT was 0.5 μg/L.

2.3. Test organisms

B. pulchella (Duméril and Bibron, 1841), formerly named *Hypsiboas pulchellus*, is an anural arboreal species in the family Hylidae with extensive distribution in Neotropical America, representing an abundant species in the Pampasic region of Argentina (For review, see Larramendy and Soloneski (2017), and references therein). This species is easy to handle and acclimate to laboratory conditions.

2.4. Experimental design

Tadpoles used for this study were obtained from a temporary and unpolluted pond away from agricultural areas, approximately 20 km away of La Plata City (35°10′ S, 57° 51′ W; Buenos Aires Province, Argentina). Pond water characteristics were as follow: pH 7.5 (7 − 8); conductivity, 195 (140 − 285) μS/cm; suspended matter, 30 (8 − 51) mg/L; hardness, 90 (63 − 137) mg/L $CaCO_3$; NO_2^-, 11 (6 − 14) μg/L; NO_3^-, 107 (33 − 180) μg/L; NH_4^+, 25 (5 − 73) μg/L; soluble reactive phosphorus, 604 (492 − 704) μg/L. No pesticides were detected in this area (Solis et al., 2016). Larvae were collected at late cleavage stage (GS) 9 according to Gosner's (1960) classification from 10 different hatches. From each hatch, 10–25% of the egg masses was collected and placed in plastic bags with 1 L of water of the collected natural environment with an equivalent volume of air for transportation to the laboratory according to recommendation of Heyer et al. (1994). Hatches were acclimatized in the laboratory to 16/8 h light/dark cycles in dechlorinated tap water with artificial aeration aquaria at 25 °C following recommendations of Cei (1980) and Kehr (1989). The physical and chemical parameters of the water were as follows: temperature, 25.0 ± 1 °C; pH 8.0 ± 0.1; dissolved oxygen, 6.3 ± 0.3 mg/L; conductivity, 663 ± 15.0 μS/cm; hardness, 181 ± 35.0 mg/L $CaCO_3$. Fish food (TetraMin®, Tetra Werke, Germany) as a food source was supplied twice a week until the beginning of the experimental procedures. 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 of the National University of La Plata (code 11/N619). Once tadpoles reached GS36 (range, 35 to − 37) according to Gosner (1960), acute toxicity tests were conducted to estimate sublethal effects including growth, developmental, body mass, and morphological effects and induction of MNs, other erythrocyte nuclear abnormalities, and DNA single-strand breaks. Individuals were randomly deposited in standard glass tanks with 40 L of capacity according to the experimental design (see Section 2.4.1 for details). Briefly, the experimental protocol consisted in two consecutive experimental phases. The first phase involved an acute and steady exposure to IMZT for 4 days, and the second phase was a postexposure period in IMZT-free water tanks lasting 21 days. Each treatment group consisted of three replicates.

2.4.1. Exposure phase

Acute toxicity tests were performed following assays proposed by the USEPA (2002b) and ASTM (2007), with minor modifications reported previously by Pérez-Iglesias et al. (2015). For each experimental point, experiments were performed using 50 tadpoles maintained in a 40 L glass tank. Experiments were performed in triplicate and run simultaneously for each experimental point. Tadpoles were exposed to 0.39 mg/L IMZT, a concentration equivalent to 25% of the IMZT LC_{50} value reported previously (Pérez-Iglesias et al., 2015). This concentration was selected since it represents the lowest IMZT concentration able to cause a significant increase in sublethal effects, including morphological abnormalities, MNs, and DNA damage (Pérez-Iglesias et al., 2015). Whereas a negative control group consisted of 50 specimens kept in dechlorinated tap water, a positive control group consisted of 50 tadpoles exposed to 40 mg/L cyclophosphamide. As reported previously, cyclophosphamide represents a widely clastogenic compound used as positive control, including anuran species (Krishna et al., 1995; Nikoloff et al., 2014; Pérez-Iglesias et al., 2015; Vera-Candioti et al., 2010). Both control groups were run simultaneously with IMZT-exposed tadpoles. All test solutions were prepared immediately before use and replaced every 24 h. Tadpoles were not fed throughout the exposure period of 4 days following the recommendations reported elsewhere (Pérez-Iglesias et al., 2014, 2015; USEPA, 2002b). Experiments were performed accepting the criteria that the density of tadpoles cannot not exceed 10 individuals/L in order to obtain an optimum growth and to achieve the lowest possible stress (Kehr, 1987, 1989) and do not exceed 1 gr body mass/L according to the recommendations of USEPA (2002b). Mortality was recorded daily.

2.4.2. Postexposure phase

This phase was performed following the procedures described by Mouchet et al. (2015) and Bernabò et al. (2013) with minor modifications. Accordingly, genetic recovery capabilities were evaluated by MN and SCGE assays as suggested by Mouchet et al. (2015), whereas individual recovery capabilities, evaluated by growth, developmental, body mass, and morphological abnormalities analyses, were analyzed following the procedures of Bernabò et al. (2013). At the end of the IMZT exposure period (Section 2.4.1), tadpoles were moved into a 40 L glass tank containing IMZT-free clean water (dechlorinated tap water) with continuous gentle aeration. Tadpoles were fed every 2 days (Section 2.4) and maintained for up to 21 days after the end of the exposure phase, when nearly 80% of tadpoles within the control group had reached GS42, starting the metamorphosis mechanism and thus indicating the end of the assay. During the assay, 30 tadpoles were sacrificed every 7 days from each experimental group to evaluate all sublethal endpoints. Out of the 30 larvae, 15 were employed for analyses of growth, development, body mass and morphological abnormalities whereas the remaining 15 tadpoles were employed for cytogenetic endpoints. Briefly, from the beginning of assay, tadpoles were sacrificed at day 4, the end of the acute exposure phase, and at days 11, 18, and 25 (during the postexposure phase) following the experimental design proposed by Mouchet et al. (2015). The total volume of IMZT-free water from each tank was renewed every 3 days, and water quality parameters (pH, dissolved oxygen, conductivity, and hardness) were

monitored before renewal.

2.5. Sublethal endpoints

2.5.1. Growth, development, and body mass

Growth was assessed at day 4 (end of the exposure phase) and at days 11, 18, and 25 from the beginning of the experiment during the postexposure phase by measuring body length according to McDiarmid and Altig (1999) with a digital caliper of 0.01 mm. Developmental stage was ranked according to Gosner (1960). The wet mass of tadpoles was weighed up to the nearest 0.001 mg.

2.5.2. Morphological abnormalities

Abnormalities were assessed at the end of the exposure phase and during the postexposure phase as indicated in Section 2.5.1. Tadpoles were analyzed under a Wild Heerbrugg M8 binocular stereoscope microscope and morphological characteristics were determined according to Bantle et al. (1998), with minor modifications. Among them, the presence of oedema, axial abnormalities (lateral flexure of tail), decrease in keratodont numbers including total or partial losses of keratodonts' rows, and gut abnormalities were included following previous recommendations (Pérez-Iglesias et al., 2015). The prevalence of a type of abnormality was calculated by dividing the number of larvae with the particular abnormality by the number of individuals examined.

2.5.3. Micronuclei and other erythrocytic nuclear abnormalities

The MN assay was performed on mature peripheral circulating erythrocytes according to the procedure described previously (Vera-Candioti et al., 2010). Tadpoles were euthanized following the criteria proposed by the American Society of Ichthyologists and Herpetologists (ASIH, 2004). The frequency of MNs was determined at the end of the exposure phase and during the postexposure phase as indicated in Section 2.5.1. The sacrifices of negative (dechlorinated tap water, see Section 2.4) and positive (40 mg/L CP) controls were conducted similarly with treatments for IMZT-exposed tadpoles. At the end of harvesting time, 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 on clean slides, air dried, fixed with 100% (v/v) cold methanol (4 °C) for 20 min, and then stained with 5% Giemsa solution for 12 min. Slides were coded and MN frequency blind scored from 1000 erythrocytes per tadpole from each experiment at 1000× magnification. Data are expressed as a total number of MNs per 1000 cells following the examination criteria reported previously (Vera-Candioti et al., 2010).

Other erythrocytic nuclear abnormalities than MNs, i.e., binucleated cells (BNs), blebbed (BLs), lobed (LBs) and notched nuclei (NTs) were blind scored from 1000 erythrocytes per tadpole from each experiment at 1000× magnification. Examination criteria followed those established previously for the species (Pérez-Iglesias et al., 2015).

2.5.4. Single cell gel electrophoresis assay

Specimens employed for the MN assay (see Section 2.5.3) were also used for the SCGE assay. The SCGE assay was performed following the alkaline procedure described elsewhere (Vera-Candioti et al., 2013). Blood samples were diluted with 1 ml phosphate-buffered saline, centrifuged (2000 rpm, 9 min), and resuspended in a final volume of 50 µl of phosphate-buffered saline. An aliquot of 30 µl of the diluted samples was mixed with 70 µl of 0.5% low-melting-point agarose and was then layered on a slide precoated with 100 µl of 0.5% normal-melting-point agarose. The slide was covered with a coverslip and placed at 4 °C for 10 min. After solidification, the coverslip was removed, and the slide was covered with a third layer of 50 µl of 0.5% low-melting-point agarose. After solidification, the coverslip was removed, and slides were further processed according to the procedure described in detail elsewhere (Pérez-Iglesias et al., 2015, 2017). Slides were examined under an Olympus BX50 fluorescence photomicroscope equipped with an appropriate filter combination. The extent of DNA damage was quantified by the length of DNA migration, which was visually determined in 100 randomly selected and nonoverlapping cells (Azqueta et al., 2011; Collins, 2004; Collins et al., 1995). DNA damage was classified in five classes (0–I, undamaged; II, minimum damage; III, medium damage; IV, maximum damage) as suggested previously (Çavaş 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 experimental point 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} represent the total number of nucleoids scored.

2.6. Statistical analysis

The proportion of individuals affected per test chamber (n = 15) was calculated for sublethal endpoints (morphological abnormalities, MNs, BNs, BLs, LBs, NTs, and damaged cell frequencies) and values were angular transformed. Data were checked for normality using the Shapiro-Wilk test, and for homogeneity of variances using Bartlett's test. One-way analysis of variance (ANOVA) was applied to analyze overall differences among time and treatments (controls, IMZT-exposed, and post-IMZT-exposed groups) with respect to tadpoles exhibiting sublethal effects. If statistically significant differences were found, Tukey's HSD tests were performed as *post hoc* multiple comparison tests. In cases that did not meet the assumptions of normality, a Kruskal–Wallis or Mann-Whitney tests was performed. Moreover, *t*-tests of mean difference were performed for comparisons in chemical analyses. The level of significance chosen was $\alpha = 0.05$ for all tests, unless indicated otherwise.

3. Results

3.1. Chemicals analyses

Results of chemical analyses by HPLC revealed IMZT concentrations of 0.407 ± 0.20 and 0.388 ± 0.01 mg/L for 0 and 24 h, respectively. Overall, the analysis showed no significant changes ($P > 0.05$) in the concentration of the toxicant in treatments during the 24 h interval renewals of the testing solutions during the exposure phase of the experiments (concentration range, 98 ± 5% recovery). Concentrations assessed throughout the study represent the nominal concentrations of active ingredient present within the IMZT-based formulation Pivot® H.

3.2. Growth, development, and body mass

A *t*-test analysis revealed that during the exposure phase of the experiments, results showed no alterations in body growth or body mass between IMZT-based formulation Pivot® H-exposed tadpoles and negative control groups ($P > 0.05$). Similar results were also observed during the postexposure phase ($P > 0.05$). On the other hand, whereas no differences in the developmental rate between exposed and non-exposed tadpoles were observed during the exposure phase ($P > 0.05$), a significant decrease in the parameter was observed during the post-exposure phase ($P = 0.023$, $F = 3.82$, $df = 21$). Results demonstrated that at the end of the postexposure phase (day 25), 47% of the IMZT-exposed tadpoles had reached GS42, whereas in the negative control group, such values reached as high as 76% of individuals ($P < 0.05$) (Fig. 1).

3.3. Morphological abnormalities

Examples of morphological abnormalities observed in IMZT-treated tadpoles are presented in Fig. 2 whereas the prevalence of the abnormalities is depicted in Fig. 3. A *t*-test demonstrated loss of

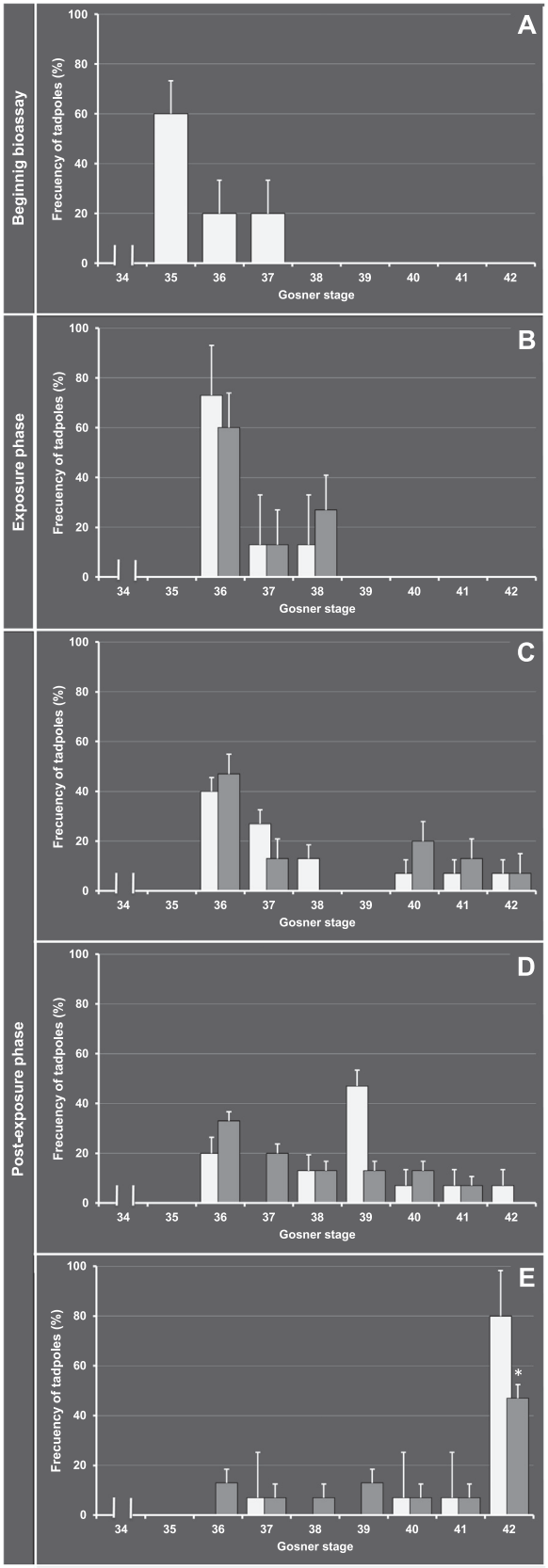

Fig. 1. Imazethapyr-based commercial herbicide formulation Pivot® H-induced alterations in the developmental rate of *Boana pulchella* (Anura, Hylidae) tadpoles exposed to 0.39 mg/L Pivot® H for 4 days (exposure phase) and submitted to a period in herbicide-free water for 7, 14, or 21 days (postexposure phase). Results are presented as percentages of pooled data from three independent experiments. Experiments for negative controls (untreated tadpoles, white bars) and Pivot® H-exposed tadpoles (gray bars) were conducted and run simultaneously. *, $P < 0.05$, significant differences with respect to control values after *t*-test analysis.

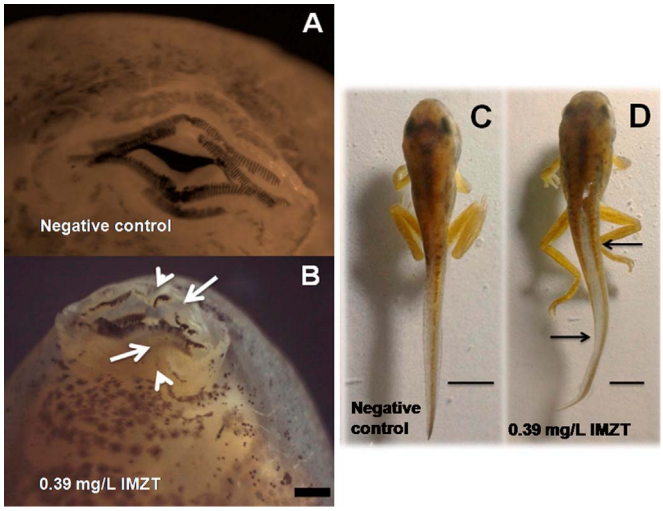

Fig. 2. Imazethapyr-based commercial herbicide formulation Pivot® H-induced oral (B) and axial (D) abnormalities in *Boana pulchella* (Anura, Hylidae) tadpoles exposed to 0.39 mg/L Pivot® H for 4 days (exposure phase) and submitted to a period in herbicide-free water for 7, 14, or 21 days (postexposure phase). (A) A GS36 negative control tadpole showing normal rows of keratodonts. (B) Exposed GS36 tadpole submitted to a period in herbicide-free water for 7 days (day 11, postexposure phase) showing partial loss of the upper first row, partial loss of the lower first row, and total loss of the upper last and lower last rows of keratodonts. Arrows indicate partial loss and arrowheads depict total loss of rows of keratodonts. (C) Dorsal view of GS42 negative control tadpole showing no axial malformations. (D) Dorsal view of exposed GS42 tadpole submitted to a period in herbicide-free water for 21 days (postexposure phase) showing lateral flexure of tail (arrows). Bars represent 0.5 mm (A, B) or 5 mm (C, D).

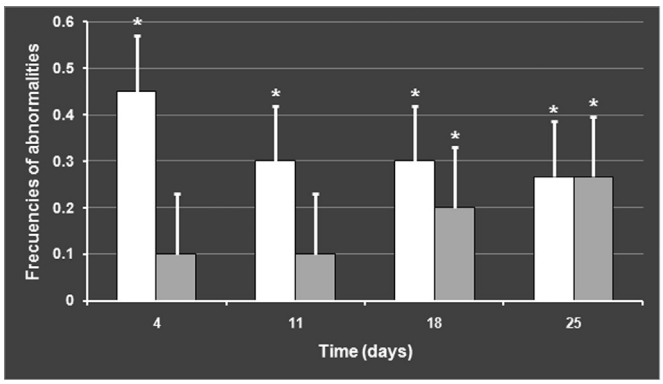

Fig. 3. Imazethapyr-based commercial herbicide formulation Pivot® H-induced morphologic abnormalities in *Boana pulchella* (Anura, Hylidae) tadpoles exposed to 0.39 mg/L Pivot® H for 4 days (exposure phase) and submitted to a period in herbicide-free water for 7, 14, or 21 days (postexposure phase). Results are presented as frequencies of pooled data from three independent experiments run simultaneously. Bars are showing frequencies of oral (white bars) and axial (gray bars) abnormalities in Pivot® H-exposed tadpoles during recovery assay.

keratodonts in at the end of the exposure phase relative to negative control values ($P = 0.019$, *t*-value = 3.16, df = 6) as well as at 11 ($P = 0.017$, *t*-value = 3.87, df = 4), 18 ($P = 0.017$, *t*-value = 3.87, df = 4) and 25 days during the postexposure phase ($P = 0.039$, *t*-value = 2.35, df = 4) (Fig. 2A, B). Besides, axial abnormalities (lateral flexure of tail) were observed in tadpoles when the analyses were performed at 18 ($P = 0.039$, *t*-value = 2.35, df = 4) and 25 days after the initial treatment ($P = 0.017$, *t*-value = 3.87, df = 4) (Fig. 2C, D).

Table 1
Frequencies (%) of MNs and other nuclear abnormalities in peripheral blood erythrocytes from *Boana pulchella* tadpoles exposed to 0.39 mg/L to the imazethapyr-based herbicide formulation Pivot® H for 4 days (Exposure phase) and submitted to a period in herbicide-free water for 7, 14, and 21 days (Postexposure phase).

Exposure time (d)	Treatment	No. of animals analyzed	No. of cells analyzed	MNs[a]	Other Nuclear Abnormalities[a]			
					NTs	LNs	BNs	BLs
Exposure phase								
0	Control	13	13405	4.95 ± 1.03	0.53 ± 0.23	1.66 ± 0.38	0.89 ± 0.28	2.46 ± 0.35
4	Control	14	14421	4.55 ± 1.09	0.63 ± 0.24	0.42 ± 0.17	1.35 ± 0.38	2.77 ± 0.37
	Pivot® H	15	15169	7.60 ± 0.98**	1.44 ± 0.36*	1.10 ± 0.25	1.93 ± 0.23	5.74 ± 0.84**
	CP[b]	15	15556	11.34 ± 2.18**	0.65 ± 0.25	2.12 ± 0.39**	2.26 ± 0.53	6.02 ± 1.00**
Postexposure phase								
11	Control	14	14302	6.00 ± 1.27	0.53 ± 0.19	0.47 ± 0.19	2.24 ± 0.65	5.96 ± 0.99
	Pivot® H	15	15753	5.11 ± 1.13	0.89 ± 0.29	0.26 ± 0.11	1.07 ± 0.37	5.13 ± 0.99
	CP	15	15649	21.35 ± 2.51***	1.54 ± 0.59	0.90 ± 0.27	4.40 ± 1.35	6.07 ± 0.97
18	Control	13	12633	5.03 ± 1.23	0.95 ± 0.24	0.83 ± 0.29	1.94 ± 0.52	4.28 ± 1.03
	Pivot® H	15	15204	4.81 ± 1.20	0.32 ± 0.15	0.65 ± 0.18	1.09 ± 0.34	3.52 ± 0.59
	CP	13	13437	5.68 ± 0.96	0.67 ± 0.26	0.60 ± 0.18	1.51 ± 0.56	2.99 ± 0.70
25	Control	15	15068	4.65 ± 1.82	1.03 ± 0.38	0.51 ± 0.19	1.18 ± 0.35	4.86 ± 0.87
	Pivot® H	15	15539	3.76 ± 0.78	0.77 ± 0.27	0.33 ± 0.16	1.22 ± 0.29	5.72 ± 1.01
	CP	9	9329	4.94 ± 1.21	0.68 ± 0.45	0.43 ± 0.23	1.17 ± 0.31	3.64 ± 0.98

Results are expressed as mean number of abnormalities/1000 cells ± SE.
significant differences with respect to control after one-way ANOVA.
[a] MN micronucleus, NT notched nucleus, LN lobed nucleus, BN binucleated nucleus, BL blebbed nucleus.
[b] Cyclophosphamide (CP, 40 mg/L), was used as positive control.
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

3.4. Micronuclei and other erythrocytic nuclear abnormalities

Results of MNs and other nuclear abnormalities induced in peripheral blood erythrocytes of *B. pulchella* tadpoles are summarized in Table 1. ANOVA analysis demonstrated that at the end of the exposure phase, an increased frequency of MNs was observed in CP-exposed tadpoles (positive control) ($P = 0.009$, F = 3.40, df = 69). When other nuclear abnormalities were analyzed, an increase in the frequency of LNs ($P = 0.026$, H = 11.05, N = 74) and BLs ($P = 0.025$, F = 3.0, df = 39) but not NTs or BNs was also found ($P > 0.05$). Tadpoles treated with IMZT for 4 days showed a significant increase in MN frequency relative to negative controls ($P = 0.004$, F = 6.30, df = 41; Fig. 4A). When other nuclear abnormalities were analyzed, an increase in the frequency of both NTs ($P = 0.016$, F = 1.24, df = 23; Fig. 4B) and BLs ($P = 0.0002$, F = 1.10, df = 24; Fig. 4C), but not LNs or BNs ($P > 0.05$), was observed (Table 1).

During the postexposure phase, CP-exposed tadpoles showed a significant increase of MNs compared to their negative control group only in those specimens analyzed at day 11 ($P = 0.0000$, F = 24.12, df = 2), and not those analyzed at days 18 and 25 of the experiment ($P > 0.05$). In addition, no significant differences in the frequencies of other nuclear abnormalities between CP-treated and negative controls were observed ($P > 0.05$). Results also demonstrated that IMZT-treated specimens did not show significant differences either in the frequency of MNs or in other nuclear abnormalities with regard to the negative control group, regardless of the postexposure lapse ($P > 0.05$) (Table 1).

3.5. DNA damage

Table 2 presents the results of the SCGE assay obtained in peripheral blood erythrocytes of *B. pulchella* tadpoles after exposure and during the postexposure phase, whereas the mean frequencies of the different nucleoid categories are depicted in Fig. 5 for each sampling time.

ANOVA analysis revealed that at the end of the exposure phase, CP treatment (positive control) induced an enhancement of the GDI as well as the frequency of damaged cells compared to the negative control group ($P = 0.0002$, F = 10.84, df = 2) (Table 2). Such increase was due to an increase in the frequency of type III nucleoids ($P = 0.0003$, F = 9.73, df = 2) and a concomitant decrease of type 0–I nucleoids ($P = 0.0004$, F = 9.58, df = 2) (Fig. 5C). In tadpoles exposed to IMZT, a significant increase in the GDI ($P = 0.003$, F = 1.39, df = 26) and in the frequency of damaged cells was observed relative to negative controls ($P = 0.004$, F = 1.27, df = 26) (Table 2, Fig. 5B). Furthermore, the increase was caused by an increase in the frequency of type II, III, and IV nucleoids ($P = 0.03$, F = 1.44, df = 26; $P = 0.003$, F = 1.13, df = 26; $P = 0.021$, F = 3.83, df = 26, respectively) and a concomitant decrease of type 0–I nucleoids ($P = 0.004$, F = 1.27, df = 26) (Fig. 5B).

During the postexposure phase, CP treatment (positive control) did not modify the GDI ($P > 0.05$) or the frequency of damaged cells ($P > 0.05$) compared to the negative control group, regardless of the sampling time of the CP-exposed tadpoles (Table 2, Fig. 5C). In

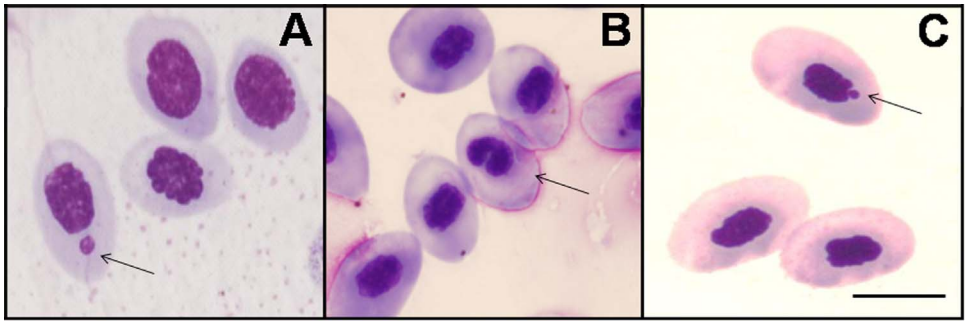

Fig. 4. Imazethapyr-based commercial herbicide formulation Pivot® H-induced nuclear abnormalities in *Boana pulchella* (Anura, Hylidae) tadpoles exposed to 0.39 mg/L Pivot® H for 4 days (exposure phase). Blood smears showing an erythrocyte with a micronucleus (A, arrow), an erythrocyte depicting a notched nucleus (B, arrow) and an erythrocyte with a blebbed nucleus (C, arrow). Cells were stained with 5% Giemsa and viewed at 1000× magnification. Bar represents 10 μm.

Table 2
Analysis of DNA damage measured by comet assay in *Boana pulchella* tadpoles blood cells exposed to 0.39 mg/L to the imazethapyr-based herbicide formulation Pivot® H for 4 days (Exposure phase) and submitted to a period in herbicide-free water for 7, 14, and 21 days (Postexposure phase).

Exposure time (d)	Treatment	No. of animals analyzed	No. of nucleoids analyzed	% of damaged nucleoids (II + III + IV)	GDI[a] ± SE
Exposure phase					
0	Control	15	1620	30.93	1.15 ± 0.10
4	Control	15	1614	32.28	1.22 ± 0.06
	Pivot® H	13	1362	50.44**	1.56 ± 0.08**
	CP[b]	15	1633	53.77***	1.65 ± 0.07***
Postexposure phase					
11	Control	14	1200	33.83	1.27 ± 0.06
	Pivot® H	13	1341	32.21	1.26 ± 0.07
	CP[b]	15	1676	38.31	1.33 ± 0.08
18	Control	12	1100	37.64	1.19 ± 0.05
	Pivot® H	13	1423	34.01	1.15 ± 0.02
	CP[b]	13	1522	34.63	1.24 ± 0.05
25	Control	13	1400	29.71	1.10 ± 0.07
	Pivot® H	13	1515	33.93	1.23 ± 0.06
	CP[b]	10	1000	28.43	1.19 ± 0.05

Significant differences with respect to control after one-way ANOVA.
[a] GDI, Genetic damage index.
[b] Cyclophosphamide (CP, 40 mg/L) was used as positive control.
** $P < 0.01$.
*** $P < 0.001$.

postexposure phase, no significant differences were observed in the GDI ($P > 0.05$) or in the frequency of damaged cells ($P > 0.05$) between the negative control group and IMZT-treated tadpoles regardless of the sampling time (Table 2, Fig. 5B).

4. Discussion

We recently employed *B. pulchella* as a target species to evaluate the acute sublethal effects of the herbicide IMZT-based commercial formulation Pivot® H (Pérez-Iglesias et al., 2015, 2017). We demonstrated that an increase in MN frequency, other nuclear abnormalities and primary DNA single-strand breaks were induced after IMZT exposure in *B. pulchella* tadpoles (Pérez-Iglesias et al., 2015, 2017). In this study, and in total agreement with our previous reports, tadpoles exposed to IMZT presented an increase of MN, NT, and BL frequencies and primary DNA lesions at the end of the exposure phase. However, in the post-exposure phase, we demonstrated that *B. pulchella* tadpoles recovered their basal levels of MNs, NTs, BLs, and comets frequencies after the first 7 days of the postexposure phase. Our results are consistent with responses found, among others, in the African clawed frog *Xenopus laevis* tadpoles, a well-known conventional anuran experimental model (Morse et al., 1996; Mouchet et al., 2015). *X. laevis* tadpoles, when exposed to environmental stressors such as cadmium (Mouchet et al., 2015) or to the polycyclic aromatic hydrocarbon benzo[a]pyrene (Morse et al., 1996), returns to their basal levels of genetic damage during the postexposure phase after treatment cessation. Similar observations have been also observed in other aquatic invertebrate and vertebrate species. Among them can be included, the zebra mussel *Dreissena polymorpha* after exposure to benzo[a]pyrene and cadmium (Michel and Vincent-Hubert, 2015); the flatfish *Scophthalmus maximus* and the sole *Solea solea* exposed to polycyclic aromatic hydrocarbons (Dû-Lacoste et al., 2013; Wessel et al., 2010); the fathead minnow *Pimephales promelas* when exposed to brominated flame-retardant mixtures (Bearr et al., 2010); the European eel *Anguilla anguilla* exposed to glyphosate- or triclopyr-based herbicides (Guilherme et al., 2014; Marques et al., 2014); the common carp *Cyprinus carpio* after exposure to clotrimazole (Corcoran et al., 2014); the rainbow trout *Oncorhynchus*

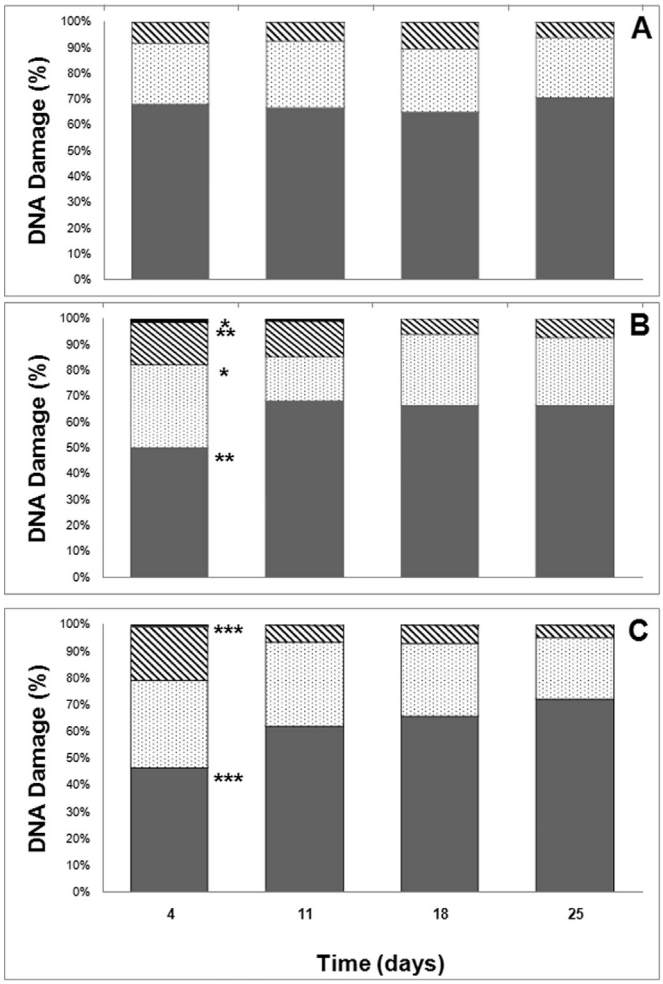

Fig. 5. Imazethapyr-based commercial herbicide formulation Pivot® H-induced DNA damage measured by the single cell gel electrophoresis assay in peripheral blood cells from *Boana pulchella* (Anura, Hylidae) tadpoles exposed for 4 days (exposure phase) and submitted to a period in herbicide-free water for 7, 14, or 21 days (post-exposure phase) (B). The frequencies of undamaged (type 0-I, dark gray bar sections), type II (dotted bar sections), type III (stripped bar sections), and type IV nucleoids (black bar sections) were determined by analyzing 100 nucleoids from each tadpole. Results are presented as percentages of pooled data from three independent experiments. Negative (A; untreated tadpoles) and positive controls (C; 40 mg/L cyclophosphamide-treated tadpoles) were conducted and run simultaneously with 0.39 mg/L Pivot® H exposure treatments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, significant differences with respect to control values after one-way ANOVA.

mykiss treated with heavy metals (Stankevičiūtė et al., 2016); the rohu carp *Labeo rohita* after an exposure to phorate, an organophosphate insecticide/acaricide (Mohanty et al., 2011); and the Nile tilapia *Oreochromis niloticus* after cyanotoxin exposure (Guzmán-Guillén et al., 2014). Finally, it has been also observed that Wistar rats recover their basal levels of genotoxic damage after exposure to ethyl-carbamates (Prado-Ochoa et al., 2016).

Previous studies have shown that the time to recover a basal level of genetic damage can vary greatly depending upon the species. Periods as short as 72 h for fish (Mohanty et al., 2011), of 14 days for amphibians (Mouchet et al., 2015), and up to 21 days for rats (Prado-Ochoa et al., 2016) have been reported. According to Mohanty et al. (2011), the explanation for this recovery process is associated with a selective loss of damaged cells and/or the onset of the DNA repair process. In this sense, several authors have suggested that the rapid recovery from the genetic damage induced by pesticides is related to the onset of DNA repair systems by DNA repair enzymes through the process of base excision repair and/or nucleotide excision repair, acting in both aquatic

invertebrates and vertebrates (Guilherme et al., 2014; Michel and Vincent-Hubert, 2015; Mohanty et al., 2011; Mouchet et al., 2015). However, as mentioned previously, the genetic recovery could be also associated with a process of detoxification and renewal of damaged cells in hematopoietic organs (Kaur and Dua, 2016; Mohanty et al., 2011; Pérez-Iglesias et al., 2016).

In agreement with these explanations, our current results clearly demonstrated a lack of cells with IMZT-induced damage carrying MNs or other nuclear abnormalities after the first 7 days of the postexposure phase of the experiment. Similarly, at this sampling time, no variations in the frequencies of different nucleoid types, regardless of the degree of damage in exposed tadpoles compared to nonexposed organisms were registered. On the other hand, at this sampling time (7 days in IMZT-free water), but not in further samplings, a prevalence of damaged erythrocytes with higher MN frequency was observed in CP-exposed tadpoles (positive control).

Although also speculative, another plausible explanation for our findings in *B. pulchella* can be envisioned. Years ago, Mohanty et al. (2011) suggested that the earlier stage of apoptosis may contribute to the production of highly damaged comets (nucleoid types III and IV) during the initial periods of pesticide exposure followed by the elimination of dead cells by apoptosis concomitantly with the lengthening of the recovery time. Our observations could not accept or rule out this possibility. No estimation of the frequency of apoptotic cells was included in our study. Thus, whether the frequency of nucleoids with a high grade of damage is the result of IMZT-induced DNA damage or could be the sum of IMZT-induced DNA damage and an enhancement in the frequency of earlier stage apoptotic cells is still an open question.

Focusing on the effects at the individual level registered in the postexposure phase of the experiment, we found tadpoles retaining those morphological abnormalities (i.e., loss of keratodonts) observed at the end the exposure period up to the end of the experiment, regardless of the sampling time. However, new individual alterations such as delay in development stage and axial abnormalities were only observed during the postexposure phase in previously IMZT-exposed tadpoles and kept 14 days in IMZT-free water. Supporting these results, several authors have mentioned that the presence of sublethal effects at the individual level observed only in the postexposure phase could be due to a persistence of the contaminant by pesticide bioaccumulation in tadpoles' tissues (Bernabò et al., 2013; Vogiatzis and Loumbourdis, 1997). Although speculative, the absence of significant alterations in development stage during the exposure phase we observed could be related to the use of a low concentration of the xenobiotic during a short exposure time as previously suggested by Bulaeva et al. (2015) in tadpoles of the wood frog *Lithobates sylvaticus* exposed to sodium perchlorate. However, it has also been reported that unless tadpoles are transferred into pesticide-free water after an acute exposure to a contaminant, they could retain such abnormalities, and also the development stage could be affected up to the metamorphic climax (Bernabò et al., 2013; Bulaeva et al., 2015). Our results showed that tadpoles do not recover from sublethal effects at the individual level (e.g., morphological abnormalities and delay in development stage) after acute exposure occurs. Last, but not least, it is important to highlight that the sublethal effects induced by environmental stressors at the individual level may have greater consequences at the population level. It has been suggested that pollutant-induced morphological abnormalities could lead to difficulty in escaping from predators, and thus the expenditure of more energy resources in the mere acquisition of food or the impossibility of foraging, which would affect tadpole growth and development and consequently have adverse effects on tadpoles' fitness and possibly on amphibian populations within an agroecosystem (Bach et al., 2016; Bernabò et al., 2013; Pérez-Iglesias et al., 2015). Furthermore, in agreement with our observations, Xu and Huang (2017) recently analyzed the effects of α-cypermethrin enantiomers on the growth and biochemical parameters of and bioaccumulation in *Rana nigromaculata* tadpoles. They observed delayed growth in tadpoles exposed to sublethal concentrations of S-(1R, 3R)-cypermethrin, highlighting that the inhibition of larval growth should be considered as a sensitive and valid bioindicator of developmental toxicity. Mouchet et al. (2015) suggested that the development stage preceding the metamorphosis mechanism is a very important process allowing for rapid recovery after cytogenetic damage due to high levels of metabolic activity occurring during this stage of the life cycle. This is worth considering for tadpoles employed as biotic matrices in ecotoxicological studies. Our data support this concept since exposed *B. pulchella* tadpoles recovered their basal levels of the biomarkers employed for genotoxicity during the first week after injury.

We suggest that the experimental design used here should be considered in future investigations in which the toxic and genotoxic recovery capabilities of amphibians are evaluated. This bioassay contemplates not only a period that completely covers the detoxification stage, but also the development stage of the whole larval cycle. However, further studies that linking bioaccumulation of contaminants in tissues and posterior depuration, the activity of the enzymes involved in depuration and repair, and erythrocateresis processes involved in these responses are required to improve our understanding of the recovery capacities of aquatic vertebrates like amphibians and the effects of environmental stressors at higher ecological levels.

The IMZT treatment in this study assays only one concentration of 0.39 mg/L. However, it constitutes a relative high end of the threshold value of 14 μg/L IMZT found in the surface water of the Azul River basin from Buenos Aires Province (Argentina) (Peluso et al., 2008), even considering the recommended application concentrations of 800 mg/L reported for Argentina (Bindraban et al., 2009; CASAFE, 2013). Furthermore, years ago it has been demonstrated that approximately only a 3% of the IMZT applied field concentration reaches the aquatic surrounding environment by runoff after continuous and intermittent irrigation (Dias Martini et al., 2013). It is worth to mention that the IMZT concentrations found in Argentinean crop production is nearly 7.6 times higher than the highest concentrations reported for surface water in United States (Mattice et al., 2012) or even 51.8 and 40.0 times higher than that reported for Brazilian drinking and surface waters, respectively (Souza Caldas et al., 2011). Accordingly, the concentration IMZT employed in the current study would be expected to be almost improbable in the environment, perhaps been only observed when specific event occurred, e.g., direct application, drainage into ditches or accidental discharge. Thus, the possibility that amphibian populations and even occupationally exposed workers could be accidentally to this agrochemical at this range of concentrations could not be ruled out. Nevertheless, our results pinpoint that the methodologies employed in the current study can be used as a sensitive and informative biosensors for estimating the recovery capacity of the jeopardizing effects inflicted by xenobiotics in aquatic vertebrates, at least *B. pulchella* tadpoles.

To the best of our knowledge, this study represents the first evidence of the use the comet assay for cytogenetic biomarkers of genotoxicity in evaluating the recovery capabilities of amphibians in general and specifically those of the Neotropical hylid *B. pulchella* after exposure to an environmental pollutant such as the herbicide IMZT.

Further studies are required to achieve a better understanding of the responses generated by exposure to this herbicide, to improve the knowledge on the persistence of disturbances after contamination ends, and to provide better tools for environmental management decisions when depuration processes apply after contamination events. Although we have been able to demonstrate that recovery of the DNA damage occurs in tadpoles exposed to IMZT under laboratory conditions, a total and different scenario most probably occurs in environmental natural conditions. Under natural conditions, tadpoles are not exposed only to one xenobiotic but to a mixture of several of contaminants to a longer period than that as short as 4 days like the employed in our experimental design. Accordingly, it could be pinpointed that despite the reversibility of some of the IMZT-induced deleterious effects, some

morphological and functional damage which in a realistic scenario are still added to other countless environmental stressors.

Acknowledgements

This study was supported by grants from the National University of La Plata (Grants 11/N746 and 11/N817), the National Council for Scientific and Technological Research (CONICET, PIP Number 0344), and the National Agency of Scientific and Technological Promotion (PICT 2015 no. 3059) from Argentina.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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