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Lethal and sublethal effects of the pirimicarb-based formulation Aficida® on *Boana pulchella* (Duméril and Bibron, 1841) tadpoles (Anura, Hylidae)

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ABSTRACT

Acute lethal and sublethal toxicity of the pirimicarb-based commercial formulation Aficida® were evaluated on *Boana pulchella* tadpoles. Whereas mortality was used as end point for lethality, frequency of micronuclei and other nuclear abnormalities as well as alterations in the frequency of erythroblasts in circulating blood as biomarkers for genotoxicity and cytotoxicity, respectively. Swimming, growth, developmental and morphological abnormalities were also employed as sublethal end points. Results show that the species is within the 13th percentile of the distribution of acute sensitivity of species to pirimicarb for aquatic vertebrates. Results revealed values of 23.78 and 101.45 mg/L pirimicarb as $LC50_{96\ h}$ for GS25 and GS36 tadpoles, respectively. The most evident effects were related with the swimming activity with NOEC and LOEC values within the 0.005–0.39 mg/L pirimicarb concentration range. Aficida® induced DNA damage at the chromosomal level by increasing micronuclei frequency and other nuclear abnormalities, *i.e.*, lobbed and notched nuclei and binucleated cells. Cellular cytotoxicity was found after Aficida® treatment. The presence of abdominal oedemas in exposed organisms and thus flotation response of organisms could be proposed as a new sensitive exposure parameter. The multiple end point assessment approach used allowed a complete understanding the multi level of effects occurring by exposure to pirimicarb, at least in *B. pulchella*.

1. Introduction

Worldwide, many ecosystems around the world are being constantly challenged due to growing human and industrial pressure exerted upon them. The use of various biomarkers in local, easily available species can be of use to evaluate the response of the biota to such environmental pollutants (Larramendy, 2017a, 2017b). Several biological parameters mirror the interactions between toxic agents and biotic matrices. These are powerful tools that can be applied to monitor the quality of the environment. Their responses may reveal general deleterious effects to the organism in general, pinpointing alterations at cellular, biochemical and molecular level, as well as higher levels of organization (USEPA, 1975, 2002). In this sense, anthropogenic activities are continuously introducing extensive amounts of pesticides into the environment regardless of their persistence, bioaccumulation and toxicity. Furthermore, pesticides are able not only to affect target organisms, but concomitantly exert side effects on nontarget organisms (www.epa.gov/pesticides). Accordingly, the use of pesticides requires predictive, rapid and practical techniques for toxicity assessment, especially those concerning to their lethal and sublethal effects, including genotoxic and cytotoxic properties (OECD, 1997).

Pirimicarb (2-dimethylamino-5, 6-dimethylpyrimidin-4-yl dimethylcarbamate) is a selective carbamate acaricide-insecticide mostly used for aphid control in crops, including cereal, horticultural, fruit and ornamental plants, among others (CASAFE, 2015). Pirimicarb acts by contact, ingestion and inhalation (WHO-FAO, 2009) and its mode of action is inhibiting cholinesterase activity (Sultatos, 2008). According the USEPA (2005), it has been classified within the group of compounds with possible carcinogenic potential in humans (Class II-III). Overall, pirimicarb has been classified as a moderately hazardous compound (Class II) by the WHO (2009).

Available information indicates the existence of more than 23 pirimicarb-based insecticide formulations registered worldwide (http://environmentalchemistry.com/yogi/chemicals/cn/Pirimicarb.html). Pirimicarb is considered as a moderately persistent insecticide employed on integrated pest management practices because is the most

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selective aphid insecticide reported so far (Xiao et al., 2015). Several pirimicarb formulations have been found to induce adverse effects on microbial communities from freshwater sediments (Widenfalk et al., 2004, 2008), microcrustaceans (Andersen et al., 2006; Mansour and Hassan, 1993; Syberg et al., 2008) as well as insects (Magnin et al., 1988). For *Daphnia magna,* pirimicarb is a very highly toxic compound with a EC50 48 h of 16 mg/L (Kusk, 1996). However, the insecticide is considered as slightly toxic to several fish and amphibians species with LC50 values of 55–410 mg/L and 40 mg/L, respectively (https://cfpub.epa.gov/ecotox/report.cfm?type=short). For terrestrial vertebrates, pirimicarb is considered as a highly to moderately toxic compound in *Anas platyrhynchos* with an oral LD 50 of 17.2 mg/kg (Tomlin, 1994). Similarly, pirimicarb is considered moderately toxic to mammals (USEPA, 1974).

While pirimicarb was first introduced to agriculture in 1970 as a sole compound for aphid control, little information on its occurrence in the environmental compartment is available. The freshwater estimation environmental concentration (EEC) from runoff following ground application provides a value of 1.14 ppb while for aerial application, the EEC from drift and runoff is 1.26 ppb (USEPA, 1974). Pirimicarb together carbaryl and metalaxyl were the most frequently detected compounds when base flow conditions and wet weather events were analyzed in the southern Ontario surface water samplings (Struger et al., 2016). Furthermore, pirimicarb was detected within the range of 0.02–4.00 μg/L in surface water near agricultural fields as well as in rainfall samples at 0.08–0.42 μg/L concentrations (Charizopoulos and Mourkidou, 1999).

Few studies analyzing the genotoxic and cytotoxic effects exerted by both pirimicarb and pirimicarb-containing commercial formulations have been performed (IARC, 1976; WHO, 2004). Pirimicarb is being generally documented as nongenotoxic to bacteria, yeast, fungi, and in mammalian cells (USEPA, 1974). However, positive response was observed in mouse lymphoma cells (WHO, 2004) and in the *Drosophila melanogaster* w/w^+ eye mosaic system (Aguirrezabalaga et al., 1994). Pirimicarb did not induce chromosomal alterations in rat bone marrow cells (Anderson et al., 1980) and in *in vitro* human lymphocytes (USEPA, 1974). However, increased frequency of chromosomal aberrations in peripheral lymphocytes from occupational exposed workers (Pilinskaia, 1982), and chromosomal aberrations, sister chromatid exchange frequencies, DNA single-strand breaks, altered cell-cycle progression and mitodepressive effect in mammalian cells *in vitro* (Soloneski and Larramendy, 2010; Ündeger and Basaran, 2005; Valencia-Quintana et al., 2016). For aquatic vertebrates, including amphibians and fish, available information is rare. We have previously demonstrated that both the pirimicarb-based formulations Aficida® and Patton Flow® induced micronucleus (MN), DNA-single strand breaks and cytotoxicity in blood cells of *Rhinella arenarum* tadpoles and in the Neotropical fish *Cnesterodon decemmaculatus* exposed under laboratory conditions (Vera-Candioti et al., 2010a, 2010b, 2013, 2015).

Amphibians have certain characteristics rendering them useful indicator species for measuring the effects of changes in the environment (Hayes, 2010). However, in recent decades, amphibian populations have been reported to suffer significant decline worldwide (Hayes, 2010), a phenomenon in most cases attributed to pollution from agricultural areas with the use of pesticides (Mann et al., 2009). Negative effects against wild anuran populations exerted by emerging pollutants, including agrochemicals, have been reported (Davidson et al., 2007; Relyea, 2009; Sparling and Fellers, 2009). Furthermore, a correlation between the use of agrochemicals and the decline of amphibian populations has been well documented (Beebee, 2005; Jones et al., 2009). The effects of pesticides are particularly detrimental to amphibian species because of their aquatic life cycle, sensitive skin and unprotected eggs, among other factors (Bradford et al., 2011; Brühl et al., 2011; Sparling and Fellers, 2009; van Meter et al., in press).

The Montevideo tree frog *Boana pulchella* (Duméril and Bibron, 1841) (formerly named *Hypsiboas pulchellus*) (Anura) is an arboreal species in the family Hylidae. This species has an extensive distribution in the Neotropical America and is an abundant species in the Pampasic region of Argentina (Cei, 1980). Its natural habitats are subtropical or tropical dry lowland grasslands, subtropical or tropical seasonally wet or flooded lowland grasslands, intermittent freshwater lakes and marshes and pasturelands (Kwet et al., 2004). This species lays its eggs in masses attached to the submerged stems of the aquatic plants, and it is easy to handle and acclimatize to laboratory conditions (Lajmanovich et al., 2005; Pérez-Iglesias et al., 2014, 2015, 2016, 2017; Ruiz de Arcaute et al., 2014a).

The aim of the present study is to characterize the acute toxicity of the 50% pirimicarb-based insecticide Aficida® on *B. pulchella* tadpoles exposed under laboratory conditions employing a static acute experimental method. Mortality was used as end point for lethality whereas the frequency of MNs and other nuclear abnormalities as well as alterations in the frequency of erythroblasts (ERBs) among circulating blood cells were employed as biomarkers for genotoxicity and cytotoxicity, respectively. Furthermore, behavioural, growth, development and morphological abnormalities were also estimated as sublethal end points. This commercial formulation represents not only the major pirimicarb-based insecticide used in Argentina but also on a worldwide scale for cereal production and gardening aphid selective control (CASAFE, 2015).

2. Materials and methods

2.1. Chemicals

Pirimicarb [(2-dimethylamino-5,6-dimethylpyrimidin-4-yl) *N,N*-dimethylcarbamate; CAS 23103-98-2; trade name Aficida®; 50% pirimicarb, excipients q.s.] was purchased from Syngenta Agro S.A. (Buenos Aires, Argentina). Cyclophosphamide (CP, CAS 6055-19-2) and $K_2Cr_2O_7$ [Cr(VI)] (CAS 7778-50-9) were obtained from Sigma Chemical Co. (St. Louis, MO, USA) and Merck KGaA (Darmstadt, Germany), respectively. All other chemicals and solvents of analytical grade were purchased from Sigma Chemical Co.

2.2. Chemical analysis

Concentration levels of pirimicarb in test solutions were analyzed by QV Chem Laboratory (La Plata, Buenos Aires, Argentina) by high-performance liquid chromatography (Agilent 1100) with UV diode array at a wavelength detection of 230 nm, and separation was done in a 15 cm C_{18} column (4.6 mm inner diameter), using acetonitrile in 10 mM KH_2PO_4 pH 4 buffer with a 60:40 ratio, at 0.8 ml/min, as mobile phase. Stability of the compound along the time of exposure was identified by comparison of chromatographic peaks corresponding to solutions from the initial time and 24 h thereafter. Detection limit for pirimicarb was 0.2 mg/L.

2.3. Test organisms

All tadpoles employed in this study were collected from a permanent and unpolluted pond free from pluvial runoff from agricultural areas located in the vicinity of La Plata city (35°010′S, 57°510′W, Buenos Aires Province, Argentina), at a late cleavage stage (GS) 9 according to Gosner (1960)'s classification. Hatches were transported to the laboratory and then acclimatized to 16/8 light/dark cycles in aquaria with dechlorinated tap water with artificial aeration. 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/N746).

2.4. Experimental design

Tadpoles were maintained in aquaria at a density of 10 individuals/L. The physical and chemical parameters of the water were as follows: 25.0 ± 1 °C; pH 7.5 ± 0.1; dissolved oxygen, 6.3 ± 0.3 mg/L; conductivity, 99 ± 8.5 μS/cm; hardness, 143 ± 23.5 mg $CaCO_3$/L. Boiled lettuce as a food source was supplied twice a week.

Experimental procedures were done when individuals reached, according to Gosner´s classification (1960) GS25 (premetamorphic stage) and GS36 (range, 35–37, prometamorphic stage) following the recommendations proposed elsewhere (ASTM, 2007; Pérez-Iglesias et al., 2015; USEPA, 2002). Two acute toxicity assays were carried out. One of them employed GS25 tadpoles to estimate Aficida®-induced lethal and sublethal effects including behavioural, growth, developmental as well as morphological alterations. The second assay, employing GS36 tadpoles, was conducted to evaluate not only those lethal and sublethal effects included in the first experiment but also other sublethal effects such as Aficida®-induced genotoxicity by means of the induction of MNs and other erythrocytic nuclear abnormalities as well as cytotoxicity by alterations in the frequency of erythrocytes/erythroblasts.

Acute toxicity assays were carried out following standardized methods proposed by USEPA (1975, 2002) and ASTM (2007) with minor modifications reported previously (Natale et al., 2006; Nikoloff et al., 2014; Perez-Iglesias et al., 2014, 2015, Ruiz de Arcaute et al., 2014a; Vera-Candioti et al., 2010a). For each experimental point, experiments were performed using 10 tadpoles were maintained in 1 L glass container and exposed to different concentrations of pirimicarb for 96 h. To determine the concentrations of pirimicarb employed in the acute toxicity tests, preliminary experiments were performed. Whereas GS25 tadpoles were exposed to six different concentrations of Aficida® (0.005, 0.04, 0.39, 3.90, 39.02, and 195.08 mg/L pirimicarb), GS36 tadpoles were exposed to 19.51, 39.02, 58.52, 78.03, and 93.54 mg/L pirimicarb. Whereas the negative control group consisted of ten organisms kept in dechlorinated tap water, the positive control group consisted of five tadpoles treated with 23 mg/L $Cr_{(VI)}$ as reported previously (Nikoloff et al., 2014; Pérez-Iglesias et al., 2015; Vera-Candioti et al., 2010a).

For cytotoxic and genotoxic analyses, experiments were performed using five GS36 tadpoles for each experimental point, maintained in a 500 ml glass container and exposed to three different concentrations of Aficida® (19.51, 39.02, and 58.52 mg/L pirimicarb). Negative (dechlorinated tap water) and positive controls (40 mg CP/L) were conducted and run simultaneously with treatments for Aficida®-exposed tadpoles.

All test solutions were prepared immediately before use and replaced every 24 h. Aficida® was first dissolved in dechlorinated tap water at a concentration of 1 g/L to obtain a stable stock solution, and then diluted in test water at the concentrations previously specified (USEPA, 1975). Experiments were run simultaneously and performed in quadruplicate or triplicate for each experimental point for acute toxicity and cytogenetic experiments, respectively. Tadpoles were not fed throughout the experiment.

2.5. Lethal end-points

Mortality was evaluated by visual observation every 24 h. Individuals were considered dead when no movement was detected after gently prodding the tadpoles with a glass rod compared to control larvae. Dead individuals were taken at each observation time as well as all tadpoles alive at the end of the experiment were labeled and fixed in 10% v/v formaldehyde for further evaluation of growth and morphological abnormalities.

2.6. Sublethal end-points

2.6.1. Behavioural changes

Behavioural changes were evaluated in those specimens employed for acute toxicity tests (Section 2.5.). Changes were registered every 24 h after gently swirling the water five times with a glass rod and observing for 1 min the swimming activity and behavior of each organism. Irregular swimming (IS) and immobility (IM) were categorized according to Brunelli et al. (2009). In addition, a behavioural change relative to the buoyancy of exposed-tadpoles, referred as floatation (FT), was defined for floating tadpoles in upside down position that were not able to maintain normal submerged swimming, and registered. The prevalence of a type of abnormality was calculated by dividing the number of larvae with the particular behavioural abnormality by the total number of individuals examined.

2.6.2. Growth and development

Growth and development abnormalities were evaluated in those specimens employed for acute toxicity tests (Section 2.5.). Growth was assessed by measuring body length with a digital caliper of 0.01 mm after 96 h of Aficida®-exposure according to McDiarmid and Altig (1999). Developmental stages were registered under a Wild Heerbrugg M8 binocular stereoscope microscope and determined according to the GS categories proposed elsewhere (Gosner, 1960).

2.6.3. Morphological abnormalities

Morphological abnormalities were evaluated in those specimens employed for acute toxicity tests (Section 2.5.). Morphological abnormalities were registered under binocular stereoscope microscope (Section 2.6.2.) and classified according to the categories proposed elsewhere (Bantle et al., 1998) with minor modifications. Briefly, among them body abnormalities (BAB), tail abnormalities (TAB; *e.g.*, simple minor axial abnormalities, dorsal flexure of the tail), gut abnormalities (GAB; *e.g.*, decrease in keratodont numbers, gut reduction) and presence of several abdominal oedemas (SAO). The prevalence of a type of abnormality was calculated as indicated in Section 2.6.1.

2.6.4. Micronuclei and other erythrocytic nuclear abnormalities

MN assay was performed on peripheral circulating blood erythrocytes according to the procedure described previously (Nikoloff et al., 2014; Perez-Iglesias et al., 2014, 2015; Ruiz de Arcaute et al., 2014a; Vera-Candioti et al., 2010a). Experiments were performed using five GS36 tadpoles for each experimental, maintained in a 500 ml glass container and exposed to 19.51, 39.02, and 58.52 mg/L pirimicarb. Negative (dechlorinated tap water; Section 2.4) and positive controls (40 mg CP/L) were conducted and run simultaneously with treatments for Aficida®-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 criteria (ASIH, 2004). At the end of each experiment, tadpoles were anaesthetized 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% (v/v) cold methanol (4 °C) for 20 min, and then stained with 5% Giemsa solution for 12 min. Slides were coded and blind-scored by one researcher at 1000× magnification. Data are expressed as the total number of MNs per 1000 cells, as suggested previously (Vera-Candioti et al., 2010a). MN frequency was determined following the examination criteria reported in detail previously (Nikoloff et al., 2014; Perez-Iglesias et al., 2014, 2015; Vera-Candioti et al., 2010a).

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 cells (BNs), whereas cells with one nucleus presenting a relatively small evagination of the nuclear membrane that contained euchromatin were classified as blebbed nuclei (BLs). Nuclei with evaginations of the nuclear membrane larger than those of the BLs, which could have several lobes, were considered lobed nuclei (LBs). Finally, nuclei with vacuoles and appreciable depth into a nucleus without containing nuclear material were recorded as notched nuclei (NTs).

2.6.5. Cytotoxicity assay

ERB frequencies were blind determined by one researcher at 1000× magnification by analyzing a total of 1000 red blood cells from each tadpole in those slides employed for MNs analysis. Results are expressed as the number of ERBs per 1000 cells as suggested elsewhere (Vera-Candioti et al., 2010a).

2.7. Statistical analyses

Mortality and swimming data were analyzed using the Probit Analysis, according to the Finney (1971)'s method. Concentration-response (C–R) curves at 96 h were estimated with their 95% confidence limits. Regression (a and b) and correlation (r) coefficients were calculated for each C–R curves. The LC50/EC50 values and the 95% confidence interval were calculated from C–R curves. The same toxicological parameters and statistical assumptions were corroborated using the Probit Analysis statistical software, version 1.5 (http://www.epa.gov/nerleerd/stat2.htm#tsk). Tests of significance of the regressions and correlations coefficients were performed following Zar (1999). The proportion of affected individuals per test chamber (n = 10) at 96 h was calculated for lethal and sublethal end-points assayed by a one-way ANOVA with Dunnett's test (Zar, 1999) in order to determine significant differences with the control group and estimate NOEC y LOEC (for proportions data angular transformation was applied). The ANOVA assumptions were corroborated with Barlett test for homogeneity of variances and $\chi 2$ test for normality. Finally, the relationships between different end points (mortality, swimming, growth, development, frequency of morphological abnormalities, frequency of erythrocytes/erythroblasts, MNs and nuclear abnormalities) were evaluated with a correlation matrix using a Pearson product moment correlation coefficient and a principal components analysis using concentration as a grouping variable. The level of significance chosen was 0.05, unless indicated otherwise.

3. Results

3.1. Chemical analysis

HPLC results of chemical analyses showed no significant changes ($p > 0.05$) in the concentration of the toxicant in treatments during the 24 h interval replacements of the testing solutions (concentration range, 97 ± 5% recovery). Concentrations assessed throughout the study represent the nominal concentrations of active ingredient present within the pirimicarb-based formulation Aficida®.

3.2. Lethal end points

Probit analyses of mortality data from experiments employing GS25 tadpoles yielded values of 23.78 mg/L pirimicarb (confidence limits 95%: 16.20–33.77) for the $LC50_{96\ h}$ value. ANOVA analyses showed significant differences between the proportions of dead individuals between treatments ($p < 0.05$). Dunnett's test allowed calculation of the acute NOEC and LOEC values in mg/L pirimicarb of 3.90 ($p > 0.05$) and 39.02 ($p < 0.01$), respectively (Table 1).

Probit analysis of mortality data from the in which GS36 tadpoles were employed allowed determination of the following toxicity values in mg/L pirimicarb: $LC50_{96\ h}$ = 101.45 [Graphic method] (a = −55.99; b = 25.25). ANOVA analysis showed significant differences between the proportions of dead individuals between treatments ($p < 0.05$). Dunnett's test allowed calculation of the acute NOEC and LOEC values in mg/L pirimicarb of 78.03 and 97.54 ($p < 0.05$), respectively (Table 1).

Table 1
Mortality induction by pirimicarb-based insecticide Aficida® on *Boana pulchella* tadpoles for 96 h.

Cleavage stage	LC50[a]	95% CI[a,b]	NOEC[a]	LOEC[a]
GS25	23.78	16.20–33.77	3.9	39.02
GS36	101.45[c]	–	78.03	97.54

[a] mg/L.
[b] 95% confidence interval.
[c] Calculated by linear interpolation method when the Probit was not applicable.

3.3. Behavioural, growth and development changes

The behavioural swimming categories considered in the study, namely IS and IM, were independently analyzed in accordance to the experimental design. However, due their concomitant progressive effects (*i.e.*, IM individuals previously experienced IS conditions), both categories were merged and considered as a sole category designated as swimming effects (SE).

The EC50 values from Probit analysis for GS25 tadpoles were: $EC50_{48\ h}$ = 63.05 (confidence limits 95%: 59.23–66.68) and $EC50_{96\ h}$ = 15.61 [Graphic method] (a = 3.248; b = 0.397). ANOVA analyses showed significant differences between the proportions of affected individuals showing SE between all treatments ($p < 0.05$). Dunnett's test allowed calculation of the acute NOEC and LOEC values in mg/L pirimicarb of 0.005 ($p > 0.05$) and 0.04 ($p < 0.005$) as well as 0.04 ($p > 0.05$) and 0.39 ($p < 0.001$) for treatments lasting 48 h and 96 h, respectively.

The EC50 values from Probit analysis for GS36 tadpoles for SE did not show a dose-response relationship with exposure time, though ANOVA showed significant differences between the proportions of affected individuals for all treatment ($p < 0.05$). Dunnett's test allowed calculation of the acute NOEC and LOEC values in mg/L pirimicarb of 39.02 and 58.52 ($p < 0.05$) for 48 and 96 h, respectively.

Results from Probit analysis for FT were $EC50_{96\ h}$ = 55.21 (confidence limits 95%: 50.88 – 59.34). The ANOVA analyses showed significant differences between the proportions of affected individuals for all treatment ($p < 0.001$). Dunnett's test allowed calculation of the acute NOEC and LOEC values in mg/L pirimicarb of 39.02 ($p > 0.05$) and 58.52 ($p < 0.001$), respectively.

An ANOVA analysis for GS25 tadpoles showed significant differences in body length between Aficida®-exposed tadpoles and control group ($p < 0.05$). The NOEC and LOEC values were 3.90 mg/L pirimicarb ($p > 0.05$) and 39.02 mg/L pirimicarb ($p < 0.05$), respectively. On the other hand, results for GS36-exposed tadpoles did not reveal significant differences in body length between Aficida®-exposed individuals and controls ($p > 0.05$). Furthermore, a Kruskal-Wallis test performed on the development grade induced on both GS25 and GS36 Aficida®-exposed tadpoles did not showed significant differences between exposed and non-exposed individuals ($p > 0.05$).

3.4. Morphological abnormalities

Abnormalities were recorded within the 3.90–97.54 and 58.52–97.54 mg/L pirimicarb concentration-ranges for STG25 and STG36, respectively. ANOVA results showed significant differences in the occurrence of abnormalities between Aficida®-treated (Fig. 1d-i) and non-treated tadpoles (Fig. 1a–c) regardless of the exposure period

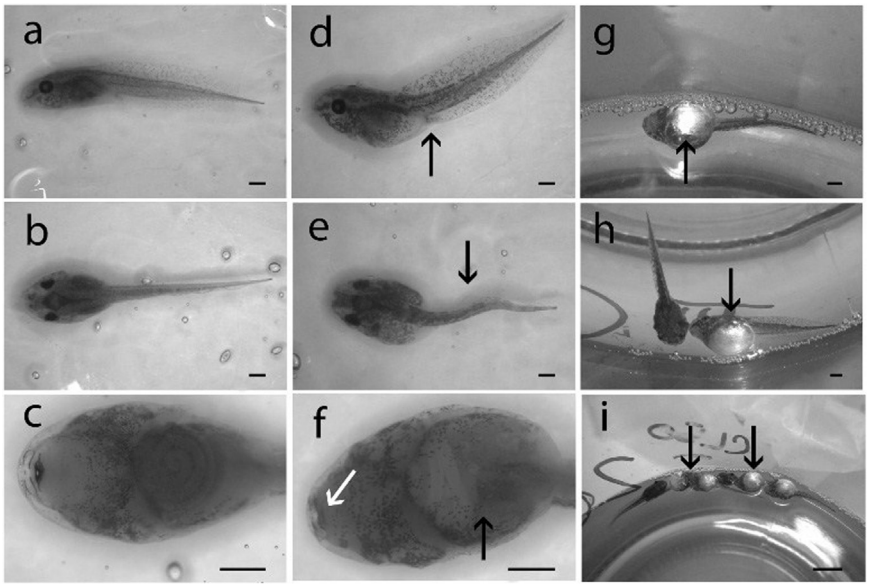

Fig. 1. Morphological abnormalities observed in *Boana pulchella* (Anura, Hylidae) tadpoles exposed to Aficida®. Lateral (a), dorsal (b) and ventral (c) views of negative control individuals; exposed tadpoles showing dorsal (d) and axial (e) flexure of the tail, loss of keratodonts (white arrow) and gut reduction (black arrow) (f); presence of several abdominal oedemas associated with floatation (g-i); the arrow in (g) indicates rupture of the peritoneum. Bars represent 1.5 mm (a-h) and 10 mm (i).

($p < 0.001$). The most conspicuous type of abnormalities observed in GS25-exposed tadpoles was the development of SAO ($p < 0.001$) associated with a reduction of the gut development ($p < 0.001$) with concentrations higher than 3.90 mg/L pirimicarb. Similarly, in GS25-exposed tadpoles, BAB ($p < 0.001$) was observed within the 3.90 – 97.54 mg/L pirimicarb concentration-range. Furthermore, when GS36 tadpoles were analyzed, the most conspicuous abnormalities observed were also SAO, with rupture of the peritoneum in treatments within 58.52–97.54 mg/L pirimicarb concentration-range (Fig. 1f–i). In all cases, SAO was always preceded by the swimming change described as FT. Consequently, individuals exhibiting FT also showed SAO after 96 h of exposure (Fig. 1g-i). Similarly, in GS36-exposed tadpoles, BAB ($p < 0.001$) was observed within the 58.50–97.54 mg/L pirimicarb whereas GAB ($p < 0.001$) was recorded within the 78.03–97.54 mg/L pirimicarb concentration-ranges. On the other hand, no significant alterations on TAB were observed in those GS25 and GS36 Aficida®-exposed tadpoles in relation to control specimens ($p > 0.05$) (Fig. 1d-e).

3.5. Micronuclei and other erythrocytic nuclear abnormalities

Table 2 shows the results of the analysis of Aficida®-induced MNs in peripheral blood erythrocytes of *B. pulchella* tadpoles. An increased frequency of MNs was observed in tadpoles exposed to CP (positive control) at 96 h ($p < 0.001$) but not in those treatments lasting for 48 h ($p > 0.05$). ANOVA analysis demonstrated an increase in MN frequencies in Aficida®-treated tadpoles during both 48 h ($p < 0.001$) and 96 h ($p < 0.001$). In tadpoles exposed for 48 h, a significant increase in the frequency of MNs was found in 19.51 mg/L pirimicarb-treated individuals compared to negative controls ($p < 0.01$). On the other hand, no increased MN frequency was observed in tadpoles exposed to 39.02 ($p > 0.05$) and 58.52 mg/L pirimicarb ($p > 0.05$). However, a regression analysis demonstrated that the frequency of MNs decreased as a dependent function of the Aficida® concentration ($r = 0.58$; $p < 0.001$). After 96 h exposure, the frequency of MNs was only enhanced in tadpoles exposed to 58.52 mg/L pirimicarb compared to negative controls ($p < 0.001$). On the other hand, no increased MN frequency was observed in tadpoles exposed to 19.51 ($p > 0.05$) and 39.02 mg/L pirimicarb ($p > 0.05$). Overall, a regression analysis demonstrated that the induction of MNs at 96 h of exposure increased as a dependent function of the Aficida® concentration ($r = -0.65$, $p < 0.001$) (Table 2).

When the other nuclear abnormalities were analyzed, significant increased frequencies were observed for treatments lasting 48 and 96 h. In tadpoles exposed for 48 h, only an increase in the frequency of LBs was observed with 19.51 mg/L of pirimicarb ($p < 0.01$). On the other hand, insecticide treatments, regardless of concentration, did not modify the frequencies of NTs, BNs, and BLs in regard to control values ($p > 0.05$) (Table 2). In treatments lasting 96 h, an increased frequency of NTs was observed in Aficida®-treated tadpoles with concentrations of 19.51, 39.02 and 58.52 mg/L pirimicarb ($p < 0.01$). Additionally, only the treatment of 58.52 mg/L pirimicarb for 96 h showed an increased frequency of BNs ($p < 0.05$). Finally, no enhanced frequencies of LBs and BLs were observed in 96 h-exposed tadpoles, regardless of the insecticide concentration ($p > 0.05$) (Table 2).

3.6. Erythroblast frequencies

The results of the analysis of the proportion of circulating ERBs in the blood of those tadpoles exposed for 48 h and 96 h are shown in Table 2. A significant increase in the frequency of ERBs was observed in the CP-exposed (positive control) tadpoles in regard to negative control values when the analysis was performed at 48 h of treatment ($p < 0.05$). On the other hand, after 96 h of exposure, no alteration in the ERBs counting was observed in those positive control tadpoles in regard to negative controls ($p > 0.05$) (Table 2). In treatments lasting 48 h, an increased frequency of ERBs was observed in Aficida®-treated tadpoles with 19.51 ($p < 0.01$) and 58.52 mg/L pirimicarb ($p < 0.001$). Finally, when treatments lasted 96 h, only 58.52 mg/L pirimicarb induced an increase in ERB frequencies ($p < 0.001$) (Table 2). Overall, a significant relationship between Aficida® concentration and increase in ERB frequencies was observed after 96 h of Aficida® exposure ($r = 0.61$; $p < 0.001$). Furthermore, a trend to a similar relationship was observed in those tadpoles after 48 h of exposure although not reaching statistical significance ($r = -0.21$; $p > 0.05$) (Table 2).

3.7. Relationships between lethal and sublethal end points

The correlation matrix of the principal component analysis from all estimated end points after 96 h exposure of *B. pulchella* tadpoles exposed to sublethal concentrations of Aficida® is presented in Table 3. The correlation matrix highlights the existence of different associations between several pairs of tested end points. Among the major significant associations between pairs of variables ($p < 0.05$) can be included, SAO with SE ($r = 0.99$), FT ($r = 0.97$) and with GAB ($r = 0.92$) as well as FT with BAB ($r = 0.96$) GAB ($r = 0.92$) and with SE ($r = 0.95$). MOR was highly associated with TAB ($r = 1.00$), and to a lesser extend with GAB

Table 2
Frequencies (‰) of MNs, other nuclear abnormalities, and erythroblasts among peripheral blood cells from *Boana pulchella* tadpoles exposed to the pirimicarb-based insecticide Aficida*.

Exposure time (h)	Concentration (mg/L)	No. of animals analyzed	No. of cells analyzed	MNs[a,b]	Other nuclear abnormalities[a,b]				ERBs[c]
					NTs	LBs	BNs	BLs	
48	Control	9	8990	6.01 ± 1.44	6.80 ± 1.59	0.00 ± 0.00	0.11 ± 0.11	1.89 ± 0.75	1.12 ± 1.12
	Positive control[d]	15	14,874	5.04 ± 1.19	8.82 ± 1.81	1.08 ± 0.40	0.13 ± 0.09	3.29 ± 0.72	8.53 ± 2.02*
	19.51	11	10,867	19.77 ± 4.17**	13.24 ± 2.98	2.29 ± 0.81**	0.18 ± 0.12	2.48 ± 0.68	12.40 ± 4.04**
	39.02	15	14,935	9.15 ± 1.67	8.15 ± 1.89	0.80 ± 0.37	0.00 ± 0.00	0.67 ± 0.32	4.43 ± 2.32
	58.52	12	11,777	4.50 ± 1.18	5.84 ± 1.68	0.59 ± 0.34	0.08 ± 0.08	0.42 ± 0.34	19.42 ± 6.84***
96	Control	11	10,993	8.55 ± 1.21	4.91 ± 0.71	0.09 ± 0.09	0.18 ± 0.12	4.37 ± 2.18	0.64 ± 0.43
	Positive control[d]	13	12,948	32.18 ± 5.57***	12.7 ± 2.05**	1.16 ± 0.45	1.16 ± 0.42	8.87 ± 1.94	4.09 ± 2.42
	19.51	15	14,949	6.81 ± 1.70	11.1 ± 1.74**	0.27 ± 0.27	0.00 ± 0.00	3.07 ± 0.57	3.42 ± 0.97
	39.02	8	7948	14.09 ± 3.05	11.95 ± 2.65**	0.63 ± 0.38	0.88 ± 0.35	2.39 ± 1.07	6.58 ± 2.35
	58.52	9	8719	23.87 ± 3.97***	11.74 ± 1.51**	0.46 ± 0.35	1.38 ± 0.71*	4.56 ± 1.69	33.15 ± 11.16***

*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; significant differences with respect to control values.
[a] Results are expressed as mean number of abnormalities/1000 cells ± SE.
[b] MNs, micronucleus; ERBs, erythroblasts; NTs, notched nuclei; LBs, lobed nuclei; BNs, binucleated nuclei; BLs, blebbed nuclei.
[c] Results are expressed as mean number of erythroblasts/1000 blood cells ± SE.
[d] Cyclophosphamide (40 mg/L) was used as positive control.

(r = 0.79), DEV (r = 0.65), FT (r = 0.60) as well as with BAB (r = 0.60). Finally, it should be noticed that SE correlated with DEV (r = 0.63) and DEV with variables such as SAO (r = 0.69), MAB (r = 0.67), TAB (r = 0.65), GAB (r = 0.60), and BAB (r = 0.59) (Table 3). Table 3 also demonstrates that among sublethal end points for evaluation of genotoxicity, MNs correlated with the several behavioural parameters such as SE (r = 0.79), FT (r = 0.76), and consequently with SAO (r = 0.73) and BAB (r = 0.69). Similarly, BNs presented high correlation with FT (r = 0.81), GAB (r = 0.76), BAB (r = 0.71), and with SAO (r = 0.70). Other associations with a lower significance correlation coefficient are depicted in Table 3.

The analysis of the main factors biplot (Fig. 2) allows separation of the highest concentrations data (over 58.52 mg/L pirimicarb) for all end points analyzed. Fig. 2 highlights that the separation of effects at concentrations of 58.52 mg/L pirimicarb is explained in a greater degree (52.88%) by several genotoxic end points such as BNs, ERBs and MNs toward negative values, as well as the contribution of behavioural effects (FT and SE) and presence of several other abnormalities (*e.g.*, SAO, BAB and GAB) toward positive values of F1.

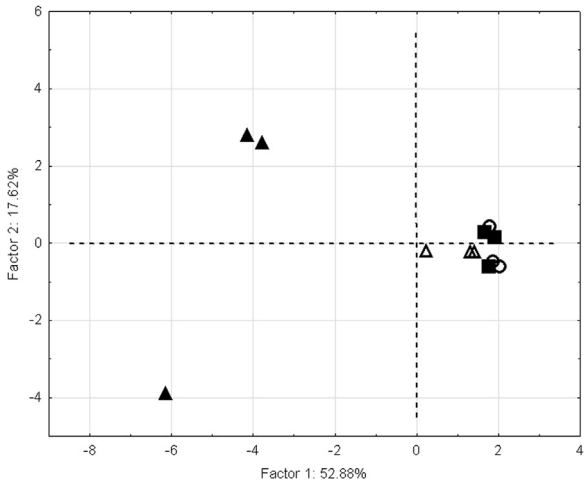

Fig. 2. Principal components analysis. The biplot reflects the distribution of results of the different end points evaluated as a function of the exposure concentrations on the principal plane (F1 x F2). White circles, negative control group; black squares, 19.51 mg/L pirimicarb-; empty triangles, 39.02 mg/L pirimicarb-; black triangles, 58.52 mg/L pirimicarb-exposed tadpoles.

Table 3
Correlation matrix between all tested end points analyzed on *Boana pulchella* tadpoles exposed to the pirimicarb-based insecticide Aficida*[a,b].

	CONC	MOR	FT	SE	GRO	DEV	TAB	MAB	BAB	GAB	SAO	MNs	NTs	LBs	BNs	BLs	ERBs
CONC																	
MOR	0.40																
FT	**0.81**	**0.60**															
SE	**0.77**	0.42	**0.95**														
GRO	0.39	0.26	0.41	0.50													
DEV	0.52	**0.65**	**0.71**	**0.63**	**0.59**												
TAB	0.40	**1.00**	**0.60**	0.42	0.26	**0.65**											
MAB	0.40	0.09	0.46	0.57	**0.63**	**0.67**	0.09										
BAB	**0.76**	**0.60**	**0.96**	**0.96**	0.40	**0.59**	**0.60**	0.34									
GAB	**0.70**	**0.79**	**0.92**	**0.85**	0.34	**0.60**	**0.79**	0.16	**0.96**								
SAO	**0.77**	0.55	**0.97**	**0.99**	0.50	**0.69**	0.55	0.51	**0.98**	**0.92**							
MNs	**0.73**	0.02	**0.76**	**0.79**	0.22	0.34	0.02	0.53	**0.69**	0.52	**0.73**						
NTs	**0.64**	−0.11	0.39	0.34	0.01	0.20	0.11	0.37	0.25	0.14	0.30	0.54					
LBs	0.31	**0.61**	0.33	0.05	0.25	0.37	**0.61**	0.20	0.19	0.35	0.15	0.07	0.29				
BNs	**0.78**	**0.68**	**0.81**	**0.64**	0.20	**0.61**	**0.68**	0.19	**0.71**	**0.76**	**0.70**	**0.63**	0.29	0.56			
BLs	0.02	0.17	0.24	0.28	0.05	0.17	0.17	0.07	0.25	0.15	0.23	0.41	0.20	0.01	0.07		
ERBs	**0.71**	0.24	**0.75**	**0.82**	0.16	0.13	0.24	0.12	**0.85**	**0.76**	**0.80**	**0.72**	0.30	0.01	0.50	0.35	

[a] , Values in table correspond to Pearson´s coefficients; in bold, significant differences in relation to negative controls, $p < 0.05$.
[b] , Abbreviations: CONC, concentration; MOR, mortality; FT, Floatation; SE, Swimming effects; GRO, Growth; DEV, Development; TAB, Tail abnormalities; MAB, Mouth abnormalities; BAB, Body abnormalities; GAB, Gut abnormalities; SAO, Several abdominal oedemas; MNs, Micronuclei; NTs, Notched nuclei; LBs, Lobbed nuclei; BNs, Binucleated erythrocytes; BLs, Blebbed nuclei; ERBs, Erythroblasts.

4. Discussion

In the present report, the acute lethal (mortality) and sublethal effects at the individual level (swimming, growth, developmental and morphological abnormalities), as well as the induction of MNs, other nuclear abnormalities and alterations circulating blood cells as biomarkers for genotoxicity and cytotoxicity were evaluated on *B. pulchella* tadpoles exposed to Aficida® using acute standardized tests under laboratory conditions.

Based on LC50 data for aquatic species reported for pirimicarb (http://cfpub.epa.gov/ecotox/), a bimodal distribution of sensitivities plot (n = 19), two major groups of organisms with distinctive range values (95% confidence limits do not overlap), can be observed. The group of the most sensitive species includes crustaceans (*Daphnia magna*, *D. pulex, Ceriodaphnia quadrangula*, *Asellus aquaticus*, *Cyclops spp*, *Gammarus pulex*) and hexapods (*Chironomus riparius, Culex quinquefasciatus*), with values between 0.0014 and 8.5 mg/L of pirimicarb, with a mean and confidence limit of 1.53 [0.00–3.91]. The second and more tolerant group is represent by aquatic vertebrates such as fish and amphibians (*Oncorhynchus mykiss, B. pulchella, Bufo japonicus formosus*, *Lepomis macrochirus, Cyprinodon variegatus*, *Rhinella arenarum*, *Cnesterodon decemmaculatus*, *Poecilia reticulata* and *Cyprinus carpio*) with a range between 29 and 410 mg/L of pirimicarb, and a mean and confidence limits of 131.80 [35.021–228.58] (https://cfpub.epa.gov/ecotox/report.cfm?type = short). Expressing the obtained toxicity end point values as mg pirimicarb/L we can situate *B. pulchella* within the 52th percentile when taking into account data on all aquatic organisms, showing relative moderate sensitivity of the species to the pesticide. However, whether we only take aquatic vertebrates, *B. pulchella* could be situated within the 13th percentile. The calculation of the Chemical Hazard Index (CHI) according to Birge et al. (2000) and employing the pirimicarb $LC50_{96\ h}$ values reported for *B. pulchella* (GS25) and *O. mykiss* (http://cfpub.epa.gov/ecotox/), provides a value of CHI = 1.05. Thus, *B. pulchella* could possess a very similar sensitivity as the mostly used reference organism in aquatic ecotoxicology, *O. mykiss*.

Respect to the differential response to pirimicarb according the $LC50_{96\ h}$ values obtained in the current study in relation with the stage of development of *B. pulchella* tadpoles, GS25 are 4.26 times more sensitive than GS36. A similar difference has been previously reported for *R. arenarum* by Brodeur et al. (2009). These authors suggested that larval sensitivity is inversely proportional to its developmental stage and consequently to its size. Our observations are in total accordance with this assumption. Regarding the acute lethal effects of the studied pirimicarb-based formulation on *B. pulchella*, the chemical could be ranked from moderate to low concern according to the scoring used by the Office of Pollution Prevention and Toxics of the USEPA (2001) for GS25 and GS36, respectively. Information on concentrations of pirimicarb in environmental compartments will be necessary to achieve a complete exposure profiles to be included in future risk assessments studies.

The comparison of our results with those reported by Vera-Candioti et al. (2010a) for the common South American toad *R. arenarum* reveals that the sensitivity of *B. pulchella* GS25 tadpoles when exposed to Aficida® is 3.7 times higher than that observed for *R. arenarum* tadpoles at the same stage. Contrarily, the *R. arenarum* GS36 tadpoles are 1.73 more sensitive than *B. pulchella* at the same developmental stage. Taking into account that experiments were performed using the same experimental conditions, these observations demonstrate a significantly and opposite response between both species, pinpointing a clear inter specific variability in sensitivity when exposed to pirimicarb. Thus, the consequences of such difference in sensitivity could became more relevant considering that they are sympatric species, inhabiting the same ecosystems, and exposed to the same local selection pressure. Several reports agree with this observation for other anuran species (Egea-Serrano et al., 2009; Kawecki and Ebert, 2004; Marquis and Miaud, 2008; Marquis et al., 2009).

The assessment of the response of sublethal end points shows that the most evident effects were related to the swimming activity. This end point allowed assessing differences in sensitivity between stages at low concentrations of the toxicant (0.04–0.39 mg/L pirimicarb). No further differences in sensitivity were detected at higher concentrations. Pirimicarb-exposed GS36 individuals exhibited a conspicuous and unequivocal effect on behavior, *i.e.*, FT, clearly specific for Aficida®-exposed larvae. No such alteration has been previously observed in the species when were exposed to several other toxicants (Agostini et al., 2013; Natale et al., 2006; Pérez-Iglesias et al., 2015). It could be also suggested that the evolution of the FT would lead to the conspicuous abnormality denominated SAO, leading to tissue laceration (*e.g.*, the peritoneum in association with the digestive system) which was observed to be correlated with other abnormalities such as GAB and BAB, and finally death of the exposed tadpoles. However, in order to be able to consider FT as a new parameter to test toxicity exposure, further studies employing a battery of distinct pesticides on the same species and/or using different anuran species exposed to the same pesticide are required. Finally, whether this panorama could happens in natural conditions, floating individuals would not be able to continue normal development, being, then, easily subjected to predation or inability to adapt to environmental stressors as previously suggested (Junges et al., 2010).

The induction of different behavioural and morphological alterations observed in our study has been also reported to be induced by other pirimicarb-based formulations. Honrubia et al. (1993) reported the induction of structural changes of gills, liver, gall-bladder, heart, and notochord of *P. perezi* tadpoles exposed to ZZ-Aphox formulation. Additionally, Alvarez et al. (1995) reported skeletal malformations induced by the same formulated product during the larval development of the same species.

The analysis of growth and development of the GS25 larvae showed an inhibition of growth but in the growth rate when tadpoles were exposed to 3.90 mg/L of pirimicarb. In agreement, Johansson et al. (2006) observed an increase in mortality concomitantly with a decrease in three endpoints evaluated, namely body length, tail length and dry weight in *R. temporaria* tadpoles exposed to a concentration of pirimicarb up to 16 mg/L during 72 h.

The evaluation of the frequency of MNs and other nuclear abnormalities in circulating blood cells represents a widely used method for the detection of damage at chromosomal level exerted by different emerging pollutants, including insecticides (Cavaş and Ergene-Gözükara, 2003; Nikoloff et al., 2014; Pérez-Iglesias et al., 2015; Ruiz de Arcaute et al., 2014a). Available information on the genotoxic property(ies) exerted by pirimicarb is scarce. So far, previous reports demonstrated the ability of pirimicarb-based formulated products to jeopardize aquatic species. We employed *R. arenarum* tadpoles and the Neotropical fish *Cnesterodon decemmaculatus* as experimental models to demonstrate the induction of MNs after exposure to the Aficida®- and Patton Flow®-pirimicarb-based insecticides (Vera-Candioti et al., 2010a, 2010b, 2015). Accordingly, our current results represent the first evidence of the acute genotoxic effects exerted by the pirimicarb-based formulation Aficida® on another anuran species such as *B. pulchella* exposed under laboratory conditions. We observed that the lowest concentration of Aficida® used (19.51 mg/L pirimicarb) was able to induce DNA damage leading to MN formation when tadpoles were exposed for 48 h. Furthermore, an enhanced MN frequency was noticed when 58.52 mg/L pirimicarb were assayed after 96 h of treatment but not for lower concentrations (*i.e.,* 19.51 and 39.02 mg/L pirimicarb). So far, we do not have any explanation for this particular finding. However, a plausible possibility could be related to the induction of a selective cell lost by insecticide-induced cell death of the most damaged cells, persisting only in circulating blood those not severely damaged cells. This observation could be also associated with a process of detoxification and renewal of damaged cells in hematopoietic organs as previously stated (Kaur and Dua, 2016; Mohanty et al., 2011; Pérez-

Iglesias et al., 2016). Finally, the inability of the most severely damaged cells to enter mitosis and/or a selective delay in the cell-cycle progression of the most severely damaged cells retarding the presence of micronucleated erythrocytes in circulation could not be ruled out. In agreement with the latter, an enhanced frequency of circulating erythroblasts was observed for all but one experimental point. When other chromatin instabilities were included in the study, the induction of NTs but not others nuclear abnormalities was significantly enhanced in Aficida®-treated tadpoles groups exposed for 96 h but not 48 h. Previous reports demonstrated that this morphological nuclear abnormalities as well as other nuclear abnormalities can be considered as genetic damage indicators (Cavaş and Ergene-Gözükara, 2003; Ruiz de Arcaute et al., 2014a, 2014b). Moreover, although the mechanism(s) responsible for such abnormalities have not been fully described, nuclear abnormalities have been used by some authors as a signal of cytogenetic damage in freshwater species (Cavaş and Ergene-Gözükara, 2003; Metcalfe, 1988).

5. Conclusions

The widespread distribution of *B. pulchella* and conspicuous presence in the habitats, reproduction along the year associated to heavy rainfall, easiness of maintenance under laboratory conditions, number of eggs per lay, larvae size, and the chance of maintaining both winter and spring larvae (long and short life cycles, respectively), make the species a good candidate for ecotoxicological studies at different levels of response. Our current results agree well with this concept. Thus, the species should be considered a good reference organism in environmental control programs of the region.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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