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Research article

Multi-biomarker approach to evaluate the toxicity of chlorpyrifos (active ingredient and a commercial formulation) on different stages of *Biomphalaria straminea*

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ABSTRACT

Biomphalaria straminea is a freshwater gastropod native to South America and used in toxicological assessments. Our aim was to estimate 48 h-LC_{50} and sub-chronic effects after the exposure to low concentrations of chlorpyrifos as commercial formulation (CF) and active ingredient (AI) on *B. straminea* adult, embryos and juveniles. Concentrations between 1 and 5000 μg L^{-1} were chosen for acute exposures and 0.1 and 1 μg L^{-1} for the sub-chronic one. After 14 days biochemical parameters, viability and sub-populations of hemocytes, reproductive parameters, embryotoxicity and offspring' survival were studied. Egg masses laid between day 12 and 14 were separated to continue the exposure and the embryos were examined daily. Offspring' survival and morphological changes were registered for 14 days after hatching. 48 h-LC_{50}, NOEC and LOEC were similar between CF and AI, however the CF caused more sub-lethal effects. CF but not the AI decreased carboxylesterases, catalase and the proportion of hyalinocytes with respect to the total hemocytes, and increased superoxide dismutase and the % of granulocytes with pseudopods. Also CF caused embryotoxicity probably due to the increase of embryos' membrane permeability. Acetylcholinesterase, superoxide dismutase, hemocytes sub-populations, the time and rate of hatching and juveniles' survival were the most sensitive biomarkers. We emphasize the importance of the assessment of a battery of biomarkers as a useful tool for toxicity studies including reproduction parameters and immunological responses. Also, we highlight the relevance of incorporating the evaluation of formulations in order to not underestimate the effects of pesticides on the environment.

1. Introduction

Bivalves are considered useful bioindicator organisms for environmental biomonitoring and toxicity assessments due to their ability to bioaccumulate chemical contaminants in their soft tissues through both dermal and dietary routes and their low metabolic capacity of xenobiotics (Cortez et al., 2018). *Biomphalaria* sp. (Phylum: Mollusca, Class: Gasteropoda, Subclass: Pulmonata, Order: Basommatophora, Family: Planorbidae) can replace higher invertebrates and vertebrates' species in ecotoxicological experiments due to the actual bioethics criteria (Ibrahim and Sayed, 2019). Furthermore, early developmental stages involve less-invasive procedures, so they are recommended by the ethics principles of replacement, reduction, and refinement. *Biomphalaria* spp. is an excellent model to assess oviposition, embryotoxicity and hatching due to its great reproductive potential, rapid embryonic development and growth and early sexual maturation (Caixeta et al., 2022). These

Abbreviations: AChE, acetylcholinesterase; AcSCh, acetylthiocholine iodide; AI, active ingredient; BSA, bovine serum albumin; CAT, catalase; CDNB, 2,4-dinitrochlorobenzene; CEs, carboxylesterases; CF, commercial formulation; ChE, cholinesterase; DTNB, 5,5-dithio-2-bis-nitrobenzoate; EROS, reactive oxygen species; GR, glutathione reductase; GSH, glutathione; GSSG, glutathione disulfide; GST, glutathione S-transferase; NADPH, nicotinamide adenine dinucleotide phosphate; NBT, nitroblue tetrazolium; OP, organophosphate insecticide; p-NPA, *p*-nitrophenyl acetate; p-NPB, *p*-nitrophenyl butyrate; SC, solvent control; SOD, superoxide dismutase; WC, water control.

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gastropods are simultaneous hermaphrodites that lay their embryonated eggs in egg masses (gelatinous capsules). The embryos develop inside the eggs and after a few days juveniles hatch from them (Cossi et al., 2018). *B. straminea* is a freshwater pulmonate gastropod native to South America but rapidly expanded to other locations (Paraense, 2001; Pointier et al., 2005; Rumi et al., 2008; Habib et al., 2018). This species has been previously used in acute and sub-chronic ecotoxicological assays using environmental samples and pesticides (Cossi et al., 2018, 2020; Paredes et al., 2022; Bianco et al., 2023).

Pesticides are used worldwide, mainly in the agricultural field, and can produce toxic effects on species that are not their target, representing a threat to aquatic and terrestrial ecosystems (Schäfer et al., 2011). In addition, they can have important consequences on human health generating neurological, cytotoxic and genotoxic effects, among others. They are applied as commercial formulations (CF) which contain the active ingredient (AI) plus excipients. CFs frequently result more toxic than the AI alone (Nagy et al., 2020; Rozman et al., 2010) due to the toxicological properties per se of the excipients, by increasing the bioavailability of the AI or due to additive or synergic effects of the mix of compounds.

Within pesticides, organophosphate insecticides (OPs) are neurotoxic agents, which upon entering organisms, act through irreversible inhibition of the enzyme acetylcholinesterase (AChE) which is responsible for hydrolyzing the neurotransmitter acetylcholine. When this enzyme is inhibited, acetylcholine accumulates and leads to overstimulation of cholinergic receptors being able to cause neurotoxicity and even death (Galloway and Handy, 2003).

Taking into account their main mechanism of action, AChE activity is the most used biomarker for OPs exposure and effect. However, in recent years the combined use of AChE and carboxylesterases (CEs) is recommended as a more suitable strategy to assess OPs toxicity (Cossi et al., 2018). CEs comprise a family of isoenzymes that were included in the B-esterases group like cholinesterases (ChEs), which are esterases irreversibly inhibited by OPs (Aldridge, 1953). CEs were generally more sensitive to OPs than ChEs in invertebrates (Wheelock et al., 2008; Otero and Kristoff, 2016) being an alternative binding target for OPs and therefore protecting organisms from neurotoxic effects (Kristoff et al., 2012). In addition, CEs participate in the detoxification of xenobiotics by hydrolyzing carboxyl ester bonds present in some of them.

The detoxifying enzyme glutathione S-transferase (GST), as well as antioxidants, are frequently used as additional biomarkers, taking into account that OPs cause oxidative stress modifying antioxidant levels or activities and/or increasing the reactive oxygen species (EROS) in several invertebrate species (Kristoff et al., 2008; Lavarías and Garcia, 2015; Chatterjee et al., 2021). GST catalyzes the conjugation of electrophilic compounds with reduced glutathione (GSH) increasing the solubility of xenobiotics and their elimination. GSH is a soluble non-enzymatic antioxidant that plays a central role in maintaining cellular redox status and protecting cells from oxidative injury (Dickinson and Forman, 2002). GSH is oxidized by glutathione peroxidase to glutathione disulfide (GSSG) while glutathione reductase (GR) catalyzes the reduction of GSSG to GSH, maintaining the proper GSH concentrations in cells. Superoxide dismutase (SOD) and catalase (CAT) are considered the first defense lines against EROS. SOD converts superoxide anion to hydrogen peroxide (H_2O_2) while CAT catalyzes the decomposition of H_2O_2 to water.

The study of other biomarkers directly related to species integrity and survival, such as effects on the immune system and reproduction, has a high ecological relevance.

Hemocytes are the key components of the innate immune systems for cell-mediated immune responses of recognition, phagocytosis and cytotoxic reactions in invertebrates (Herbert et al., 2018; Rodriguez et al., 2023). Common effects of contaminants on the immune system of mollusks involve the decrease in hemocytes viability and changes in the proportion of their subpopulations (Ibrahim and Hussein, 2022). Due to the great heterogeneity of morphology, structure and functions of hemocytes among different mollusks species and between the same species at different development stages, environmental conditions and experimental procedures, the classification of the hemocytes is until today a controversial topic (Rebelo et al., 2013; Weng et al., 2022). Based on the morphological and cytochemical features of cell size and granularity, two basic morphotypes of hemocytes are categorized in the hemolymph for many bivalve species: granulocytes and hyalinocytes. Granulocytes contain a variety of membrane limited granules (or vesicles) in the cytosol and generally possess a larger size, a low nucleus/cytoplasm ratio, often featuring thin pseudopodia at the external hyaline area. In contrast, hyalinocytes are round shaped cells with small size and show the features of undifferentiated cells with a high nucleus/cytoplasm ratio, containing none or very few cytoplasmic granules. Granulocytes play a dominant role in cellular immunity being also the main cells involved in phagocytosis, while hyalinocytes are less active in phagocytosis and are mainly involved in early inflammatory responses such as wound healing and agglutination (Weng et al., 2022; Russo and Lagadic, 2004).

Sub-chronic exposures allow the assessment of long-term effects such as reproduction and offspring' alterations, which are one of the most harmful effects on the environment. Regarding reproductive biomarkers, the number of egg masses and embryonated eggs per egg mass, embryos viability, hatching success and time of hatching are the main parameters studied in oviparous invertebrates (Caixeta et al., 2022). Decrease of embryos' hatching is one of the most toxic responses observed in *Biomphalaria* spp. exposed to inorganic/organic pollutants and environmental water samples (Agrelo et al., 2019; Kristoff et al., 2011; Tallarico et al., 2014; Oliveira-Filho et al., 2016; Paredes et al., 2022). The use of embryos and juveniles in toxicity tests is a suitable strategy.

This work aims to study the toxicity of chlorpyrifos AI alone and the toxicity of a commercial formulation, including environmental concentrations, on different stages of *B. straminea,* improving the knowledge about pesticides toxicity and biomarkers' sensitivity. CF toxicity could be caused by the AI or the AI plus excipients. AChE, detoxifying enzymes, antioxidants, hemocytes' parameters and effects on reproduction were evaluated.

2. Materials and methods

2.1. Snails

B. straminea snails were reared in our laboratory in aerated glass aquaria with passively dechlorinated tap water (pH: 7.66 ± 0.09, conductivity: 205.75 ± 1.26 μS/cm, dissolved oxygen: 8.5 ± 0.1 mg L^{-1} and 95.5 ± 4.7 % oxygen saturation), under controlled conditions of temperature (22 ± 1 °C) and artificial photoperiod (12:12 h - Light:Dark). Physicochemical parameters were measured using a Hanna HI 9811–5 multiparameter and a Hanna HI 9145 portable oximeter. Snails were fed *ad livitum* with *Lactuca sativa* var. *capitata L.* (butterhead lettuce). The use of *B. straminea* was approved by the Hygiene and Safety Service, Faculty of Exact and Natural Sciences, University of Buenos Aires (Resol. 1722/2003; 1401/2018).

2.2. Chemicals

Acetylthiocholine iodide (AcSCh), *p*-nitrophenyl acetate (p-NPA), *p*-nitrophenyl butyrate (p-NPB), 5,5-dithio-2-bis-nitrobenzoate (DTNB), 2,4-dinitrochlorobenzene (CDNB), GSH, GSSG, nicotinamide adenine dinucleotide phosphate (NADPH), bovine serum albumin (BSA), nitroblue tetrazolium (NBT), methionine and were purchased from Sigma–Aldrich. All other chemicals used were also of analytical reagent grade.

The CF used was CLORP-F® 48 %, FALCROP S.A., Argentina. The concentration of chlorpyrifos in the formulation was 48 g 100 mL^{-1} solvents and emulsifiers. Solvents and emulsifiers are not declared in the formulation, so it is not possible to use an excipients-control group in the

bioassays. CLORP-F was diluted in distilled water. The concentration of chlorpyrifos in the CF stock solution was 480 mg of chlorpyrifos L^{-1}. All CF concentrations refer to the concentration of chlorpyrifos in the solutions.

Chlorpyrifos AI PESTANAL® (O,O-diethyl O-3,5,6-trichloro-2-pyridyl phosphorothioate, CAS: 2921-88-2, 98 % pure) was purchased from Sigma–Aldrich. It was dissolved in acetone considering its low water solubility (Herbert et al., 2021) reaching a concentration of 5000 mg L^{-1} (stock solution).

Working concentrations were prepared by diluting the stock solutions in dechlorinated tap water.

2.3. Bioassays

One week before the bioassays, adult snails of similar weights (0.081 ± 0.008 g) were separated in 1 L glass containers for acclimation. All exposures were conducted in glass containers under the same breeding conditions of temperature and artificial photoperiod. Snails were fed during sub-chronic exposures but not during the acute ones.

The percentage of acetone in the AI working solutions were lower than the ones recommended for general aquatic toxicity (0.01 %) and for reproduction studies (0.002 %; Hutchinson et al., 2006).

Dechlorinated tap water control (WC) and acetone controls (SC) were included in the bioassays.

2.3.1. Acute exposures

In order to estimate 48 h-LC_{50} (concentration that causes lethality in the 50 % of the total exposed organisms), lethality NOEC (No Observed Effects Concentration) and LOEC (Lowest Observed Effects Concentration), nine concentrations of chlorpyrifos AI were used: 1, 10, 100, 500, 1000, 1500, 2000, 4000 and 5000 μg L^{-1} and eight concentrations of chlorpyrifos CF: 1, 10, 100, 500, 1000, 1500, 2000 and 4000 μg L^{-1}. Two bioassays were performed using in the first one 40 snails per AI concentration and per solvent control (0.001 % and 0.005 % acetone) (Table 1). Chlorpyrifos solutions >100 mg L^{-1} contain a percentage of acetone of 0.005. In the second bioassay, 20 snails were used for WC and 20 snails for each CF concentration.

Table 1
Acute toxicity test of chlorpyrifos on *Biomphalaria straminea* snails.

Treatment	Chlorpyrifos concentration (μg L^{-1})	Number of total snails	Number of dead snails	Lethality (%)
Water control	–	20	0	0
Commercial formulation	1		0	0
	10		0	0
	100		2	10
	500		2	10
	1000		6	30
	1500		11	55
	2000		16	80
	4000		17	85
Solvent control 1	–	40	0	0
Solvent control 2	–		0	0
Active ingredient	1		0	0
	10		0	0
	100		2	5
	500		4	10
	1000		10	24
	1500		14	35
	2000		22	55
	4000		28	70
	5000		33	83

Number of dead snails and % of lethality after 48 h exposure to dechlorinated tap water control (water control), solvent control 1 (0.001 % acetone), solvent control 2 (0.005 % acetone) and different concentrations of chlorpyrifos as a commercial formulation and the active ingredient.

Snails were considered dead when there was no response to mechanical stimuli checked under stereoscopic microscope (Nikon SMZ645) or when shells were empty (Cossi et al., 2018).

48 h-LC_{50} was calculated following the method of probit analysis. To determine NOEC and LOEC of snails' lethality, the results of each treatment were contrasted with the corresponding control (WC for CF and SC for AI) (Green et al., 2018).

2.3.2. Sub-chronic exposures

One bioassay was performed using eight glass containers (250 mL) holding 6 snails each per treatment (WC, SC 0.001 % acetone, 0.1 μg L^{-1} and 1 μg L^{-1} of chlorpyrifos AI, 0.1 μg L^{-1} and 1 μg L^{-1} of chlorpyrifos CF). All solutions were renewed every 48 h to avoid pesticide degradation (chlorpyrifos disappearance time 50 [DT_{50}] hydrolysis: 16 days at 25 °C, pH 9; chlorpyrifos DT_{50} photolysis: 29.6 days at 25 °C, pH 7; Herbert et al., 2021) and to maintain the dissolved oxygen stable during the bioassay.

Every 48 h mortality and the number of laid egg masses per treatment were registered.

After 14 days exposure, 5 snails of the same container were homogenized together due to their small size ($N = 8$ homogenates per site) and the remaining organism was used for hemolymph extraction. Homogenates were performed according to Cossi et al. (2018), centrifuged at 11,000×*g* for 15 min at 4 °C and supernatants were used for biochemical determinations.

2.4. Biochemical parameters

Protein content was measured as described by Lowry et al. (1951), using a calibration curve with BSA, and results were expressed as $mg_{protein}\ g_{tissue}^{-1}$.

AChE activity was measured using100 mM phosphate buffer, pH 8.0, 0.2 mM DTNB and 0.75 mM AcSCh as substrate as previously described (Bianco et al., 2014). Activity was expressed as μmol of 2-nitro-5-thiobenzoate anion produced per min per $mg_{protein}$.

CEs activities were determined using p-NPA and p-NPB as substrates in 100 mM phosphate buffer pH 8.0, containing 5 % of acetone (Bianco et al., 2014). Activities were expressed as μmol of p-nitrophenol produced per min per $mg_{protein}$.

GST activity was measured according to Habig et al. (1974), using 100 mM phosphate buffer pH 6.5, 1 mM GSH and 1 mM CDNB. The formation of the CDNB-GSH conjugate was monitored for 1 min at 340 nm and the activity was expressed as μmol per min per $mg_{protein}$.

For CAT activity determination, the decomposition of H_2O_2 was monitored for 1 min at 240 nm according to Cossi et al. (2018). Activity was expressed as μmol of H_2O_2 degraded per min per $mg_{protein}$.

SOD activity was measured by the method of Beauchamp and Fridovich (1971), based on the inhibition of the photochemical reduction of NBT. The reaction medium (final volume of 3 mL) consisted of 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 0.1 mM EDTA, 75 μM NBT, 20 μM riboflavin and 5, 10 and 15 μL of the supernatants. After light exposure (10 min), the absorbance at 560 nm was monitored. A negative control (without exposure to light) and a control without the supernatant fractions were used. The activity was expressed as units per $mg_{protein}$. 1 Unit of SOD is defined as the amount of supernatant necessary to produce 50 % inhibition of NBT reduction.

GR activity was measured using 143 mM phosphate buffer pH 7.5, 0.13 mM NADPH and 10 mM GSSG. NADPH consumption was monitored for 1 min at 340 nm (Venturino et al., 2001) and the activity was expressed as μmol of NADPH consumed per min per $mg_{protein}$.

GSH content was measured as described in Bianco et al. (2013) in deproteinized supernatants using 6 mM DTNB. Absorbance was measured at 412 nm after 30 min at room temperature. GSH content was determined using a calibration curve with GSH standard and expressed as μmoles g_{tissue}^{-1}.

2.5. Immunological parameters

2.5.1. Hemolymph extraction

Hemolymph samples were obtained on ice by direct puncture of each snail to evaluate the type, proportion and viability of circulating hemocytes. Snails were cleaned with absorbent paper and excess fluids were removed. Then, a small cut was made in the anterior ventral region of the shell using surgical scissors under a magnifying glass and approximately 20 μL of hemolymph per snail ($N = 4–6$) were extracted.

2.5.2. Cell count and determination of cell viability by exclusion with trypan blue

The samples were diluted in Trypan Blue solution (0.4 %) in a 1:1 ratio and an aliquot was placed in the Neubauer chamber. After incubating for 10 min in a humid chamber at room temperature, the number of adherent hemocytes was counted under an optical microscope at 400× magnification, and they were differentiated between granulocytes, granulocytes with pseudopods, and hyalinocytes. Viability was also evaluated through the Trypan Blue exclusion method. Only cells with intact membranes can effectively exclude the dye, thus dead cells with compromised membranes stain blue (Strober, 2015).

2.6. Reproductive parameters

Egg masses laid between day 12 and 14 of adult exposure were examined under a stereoscopic microscope and broken masses were dismissed. Remaining egg masses ($N = 7–10$ per treatment) were carefully removed from the containers, placed individually in plaques of 6-well plates (10 mL) and continued to be exposed under the same controlled conditions. Solutions were renewed every 48 h. Total egg masses were examined daily under a stereoscopic microscope. The number of eggs and embryonated eggs per mass, morphological abnormalities, arrested eggs and the time of hatching were registered.

The percentage of hatching was calculated on the total number of embryonated eggs per treatment.

2.7. Offspring' survival

Morphological abnormalities and survival were evaluated daily using a stereoscopic microscope. Malformations were classified in four categories by Oliveira-Filho et al. (2010): hydropic, shell, cephalic (eyes and tentacles) and unspecified malformations.

Offspring' survival was calculated as the percentage of juveniles that survive 14 days after hatching on the total number of hatched juveniles.

2.8. Statistical analysis

48 h-LC_{50} was obtained using the probit analysis method, with a trust interval of 95 %. NOEC and LOEC were calculated by contrasting concentrations with their respective control by Fisher-test. Assumptions of normality (Kolmogorov-Smirnov test) and homogeneity of variances (Levene's test) were verified. Differences of biomarkers and hemocytes parameters were tested using a one-way ANOVA and Tukey-Kramer Multiple Comparisons Test. When assumptions were not met, differences were analyzed by Kruskal-Wallis test and Dunn's Multiple Comparisons test. The number of egg masses laid and the percentage of hatching and offspring' survival were analyzed using Chi-squared test. Data was analyzed comparing the CF treatments with respect to Water Control and AI treatments with respect to Solvent Control (acetone).

Statistical tests were performed using GraphPad InStat 3.0 and GraphPad Prism 8. Level of significance was 0.05 for all tests.

No statistically differences between both controls were observed ($p > 0.05$).

3. Results

3.1. Acute lethality

Lethality was not registered in snails of water or solvent controls. The number of dead snails pert treatment are shown in Table 1. The percentage of dead snails with each chlorpyrifos concentration was used to estimate 48 h-LC_{50} (Table 1). Chlorpyrifos CF and AI 48 h-LC_{50} were 1340 μg L^{-1} (1018–1700 μg L^{-1}) and 1690 μg L^{-1} (1590–1800 μg L^{-1}) and respectively.

Chlorpyrifos NOEC of snails' lethality was 500 μg L^{-1} and LOEC 1000 μg L^{-1} for both AI and CF (Fisher Test, NOEC $p > 0.05$, LOEC $p < 0.05$).

3.2. Sub-chronic exposures

3.2.1. Lethality

Low percentages of lethality were observed in snails exposed to all the treatments (2–10 %) which were not statistically significant between groups (Kruskal-Wallis Test, $p > 0.05$).

3.2.2. Biochemical parameters

Total protein content did not vary significantly among treatments (ANOVA, $p > 0.05$) being the mean ± SD of all treatments 1.83 ± 0.15 mg g_{tissue}^{-1}.

AChE activity measured in organisms exposed to 1 μg L^{-1} chlorpyrifos CF was significantly inhibited with respect to WC (48 %) and to 0.1 μg L^{-1} of CF (47 %) (ANOVA, $p < 0.05$, Fig. 1.A). The AI caused inhibition at both concentrations of 0.1 and 1 μg L^{-1} with respect to SC (44 and 62 % respectively) (ANOVA, $p < 0.05$, Fig. 1.B).

When CE activity was measured with p-NPA as substrate, the higher concentration of chlorpyrifos CF caused a significant inhibition (62 %) with respect to WC (ANOVA, $p < 0.05$, Fig. 2.A) while no statistical differences between snails exposed to AI with respect to SC were observed (ANOVA, $p > 0.05$, Fig. 4.B).

In CEs activity measured with p-NPB, no statistical differences between chlorpyrifos CF and WC and between AI concentrations and SC were observed (ANOVA, $p > 0.05$, Fig. 2.C,D).

CAT activity was significantly inhibited (75 %) by the higher concentration of chlorpyrifos CF with respect to WC (ANOVA, $p < 0.05$, Fig. 3.A). Despite the fact that Fig. 3.B shows a decrease in CAT activity with both AI concentrations, there were no statistical differences between both concentrations of AI and with respect to SC (ANOVA, $p > 0.05$).

In the case of SOD, the activity increased in organisms exposed to 0.1 μg L^{-1} chlorpyrifos CF with respect to WC group and to the snails exposed to 1 μg L^{-1} (ANOVA, $p < 0.05$, Fig. 3.C). On the contrary no statistically differences between snails exposed to the AI with respect to the SC (ANOVA, $p > 0.05$, Fig. 3.D).

GST (0.216 ± 0.023 μmol min^{-1} $mg_{protein}^{-1}$) and GR (0.1 ± 0.015 μmol min^{-1} $mg_{protein}^{-1}$) activities plus GSH content (0.336 ± 0.071 μmol g_{tissue}^{-1}) did not vary between treatments (main ± SD of all treatments, ANOVA, $p > 0.05$).

3.2.3. Hemocytes

When the viability of circulating hemocytes was evaluated by the Trypan blue exclusion test 2–6 % of dead hemocytes were observed. No statistically differences between groups were observed (ANOVA, $p > 0.05$).

In control groups, the proportion of granulocytes and hyalinocytes with respect to the total hemocytes were 49 and 51 % in WC and 48 and 52 %, respectively.

It was found a significant increase in the percentage of granulocytes in organisms exposed to both concentrations of chlorpyrifos CF and AI with respect to the controls (ANOVA, $p < 0.05$, Fig. 4.A,B). In addition, the results observed with the high concentration of AI with respect to the

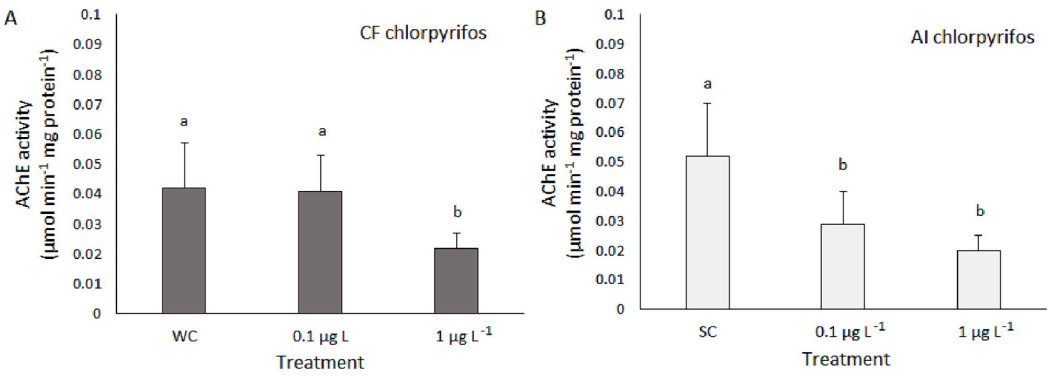

Fig. 1. Acetylcholinesterase (AChE) activity of *Biomphalaria straminea* after 14 days of exposure to a commercial formulation (CF) and the active ingredient (AI) of chlorpyrifos, dechlorinated tap water (WC) and solvent control (0.001 % acetone, SC). (A) WC group and two concentrations of chlorpyrifos in the CF: 0.1 and 1 µg L^{-1}. (B) SC group and two concentrations of AI: 0.1 and 1 µg L^{-1}. Results represent mean ± SD. Different letters indicate statistical differences between treatments ($p < 0.05$).

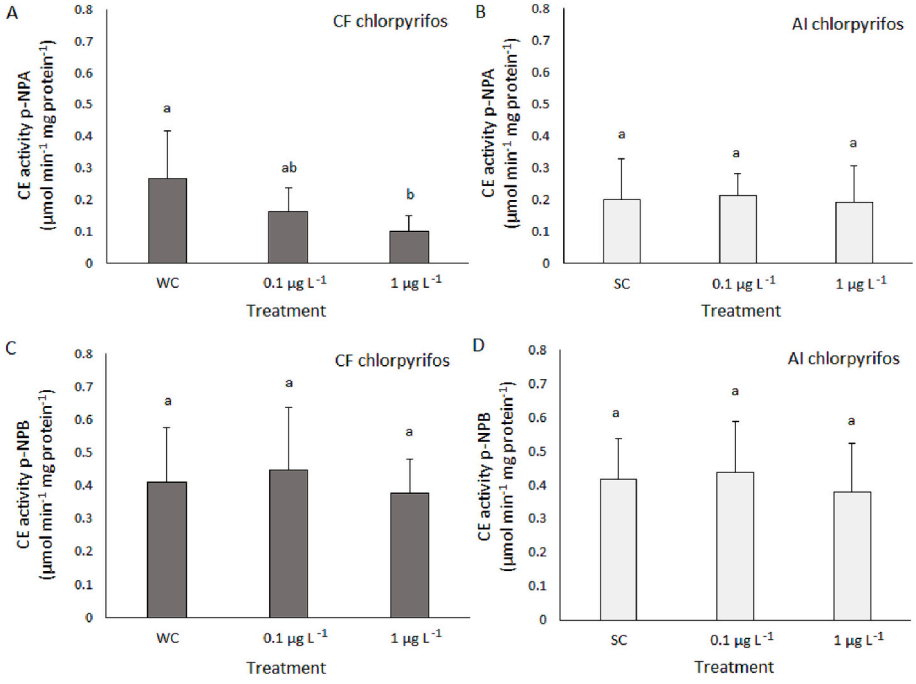

Fig. 2. Carboxylesterase (CE) activity of *Biomphalaria straminea* using *p*-nitrophenylacetate (p-NPA) and *p*-nitrophenylbutyrate (p-NPB) as substrates after 14 days of exposure to two concentrations (0.1 and 1 µg L^{-1}) of chlorpyrifos in a commercial formulation (CF) and chlorpyrifos active ingredient (AI), dechlorinated tap water (WC) and solvent control (0.001 % acetone, SC). (A) CES in WC and CF groups with p-NPA, (B) CES in SC and AI groups with p-NPA, (C) CES in WC and CF groups with p-NPB, (D) CES in SC and AI groups with p-NPB. Results represent mean ± SD. Different letters indicate statistical differences ($p < 0.05$).

lower one were statistically significant (ANOVA, $p < 0.05$, Fig. 4.B).

There was also a significant decrease in the percentage of hyalinocytes in organisms exposed to 0.1 and 1 µg L^{-1} of CF and in snails exposed to the higher concentration of AI with respect to the controls (ANOVA, $p < 0.05$, Fig. 4.C,D).

When analyzing the percentage of granulocytes with pseudopods with respect to the total granulocytes, only a significant increase was observed in the snails exposed to CF chlorpyrifos 1 µg L^{-1} with respect to the WC group (ANOVA, $p < 0.05$, Fig. 4.E,F).

3.2.4. Egg masses

The total number of egg masses laid by the adult snails throughout the 14 days of exposure did not vary significantly between treatments. All egg masses showed a similar number of eggs per mass, which were 99–100 % embryonated (ANOVA, $p > 0.05$, Table 2).

No morphological abnormalities in the eggs or egg masses were observed in any treatment.

3.2.5. Hatching success

The exposure to AI did not cause negative effects on the time or the hatching rate (Fisher Test and ANOVA, $p < 0.05$, Table 2). Instead, CF increased the time of hatching with respect to WC and decreased the percentage of hatching (ANOVA, $p < 0.05$, Table 2).

3.2.6. Offspring' survival and malformations

Chorpyrifos CF 1 µg L^{-1} and both concentrations of AI caused a significant decrease in juveniles' survival with respect to each control group (ANOVA, $p < 0.05$, Table 2). After the exposure to chlorpyrifos 1 µg L^{-1} CF the survival was 83.1 %, decreasing 17 % with respect to WC. 0.1 and 1 µg L^{-1} AI decreased juveniles' survival in 19 and 11 % with respect to SC, respectively.

Ten juveniles that hatched from egg masses exposed to both

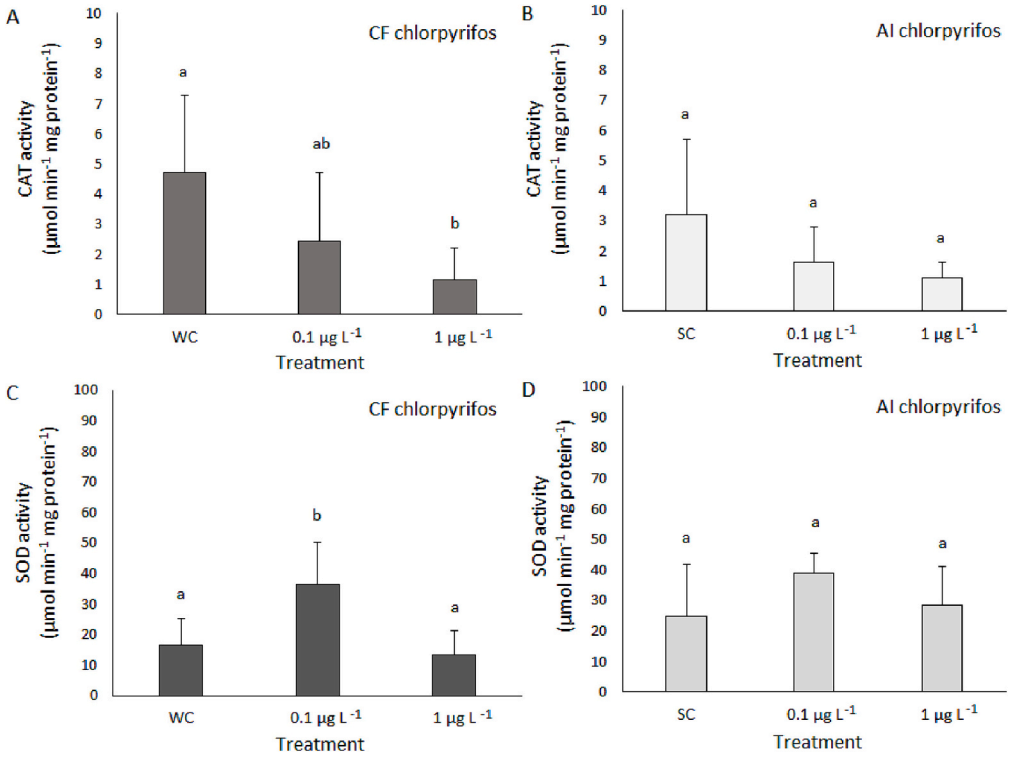

Fig. 3. Catalase (CAT) and superoxide dismutase (SOD) activities of *Biomphalaria straminea* after 14 days of exposure to a commercial formulation (CF) and the active ingredient (AI) of chlorpyrifos, dechlorinated tap water (WC) and solvent control (0.001 % acetone, SC). (A) CAT activity in WC group and in snails exposed to two concentrations of chlorpyrifos in CF: 0.1 and 1 µg L^{-1}. (B) CAT activity in SC group and in snails exposed to two concentrations of AI: 0.1 and 1 µg L^{-1}. (C) SOD activity in WC group and in snails exposed to two concentrations of chlorpyrifos in CF: 0.1 and 1 µg L^{-1}. (D) SOD activity in SC group and in snails exposed to two concentrations of AI: 0.1 and 1 µg L^{-1}. Results represent mean ± SD Different letters indicate statistical differences between treatments ($p < 0.05$).

concentrations of chlorpyrifos AI and to both concentrations of CF showed unspecified malformations or abnormalities in the tentacles. Malformations in the tentacles can be classified into cephalic malformations according to Caixeta et al. (2022) and include lack of tentacles, asymmetrical tentacles (1 shorter than the other) and tentacle atrophy (deformed). The three types of tentacle malformations were observed in each exposed group. Snails of WC and SC did not present any malformation. Control juveniles, malformed/amorphous and noticeably smaller than the rest are shown in Fig. 5.

4. Discussion

In this study, we employed a battery of endpoints including biochemical responses, inmunotoxicity, reproductive success and adult and offspring' survival to assess the toxicity of chlorpyrifos on *B. straminea*.

Although there are some works that report effects of chlorpyrifos in aquatic gastropods, most of them are limited only to the evaluation of the acute toxicity of the AI in adult snails (Cacciatore et al., 2015; Garate et al., 2020; Herbert et al., 2021; Ibrahim and Hussein, 2022). In this work, the assessment of the toxicity of a formulation and sub-chronic exposures was also carried out.

Our study shows that both the AI and the CF of chlorpyrifos elicited acute and long-term toxic effects on *B. straminea*, presenting differences between them.

Regarding the AI, chlorpyrifos was more toxic than other OP studied in *B. straminea* (Cossi et al., 2018). In this sense, in adult snails, lethality and AChE inhibition were not observed after the acute and sub-chronic exposure to azinphos-methyl. Moreover, *B. straminea* CEs had a protective role being inhibited by azinphos-methyl but not by chlorpyrifos. In other gastropods such as *Chilina gibbosa,* sensitivities variations of ChE and CEs to azinphos-methyl AI and chlorpyrifos AI were also observed (Bianco et al., 2013; Herbert et al., 2021). Results indicate that B-esterase responses have a high dependence of the kind of OP showing variations within the same species and between species. Chlorpyrifos and azinphos-methyl have a thiophosphoryl bond (P=S) instead of a phosphoryl bond (P=O) and requires metabolic activation by cytochrome P450 (CYP) enzymes to its oxon analogs to inhibit AChE. In addition, oxons can be hydrolyzed by A-esterases or may be "sequestered" by CEs (Tang et al., 2006). Therefore, variations of metabolic activities, either quantitative or qualitative, may account for differences in the toxicity of OPs, including ChE inhibition (Kristoff et al., 2006). Besides toxicokinetic processes, toxicodynamic processes can contribute to the differential susceptibility of B-esterases. In this sense, the difference in the affinity of the target enzymes to oxons explains the results observed in in vitro exposures (Cacciatore et al., 2012).

In *B. straminea*, some differences in B-esterase responses were observed between snails exposed to chlorpyrifos AI and CF. AChE resulted in more sensitivity to AI than to CF, while CEs were only decreased by the CF. These results suggest a possible action of the excipient substances on the enzymatic activities. It has been described that in addition to OPs and carbamates, other compounds can inhibit ChEs (Guilhermino et al., 1998) and/or increase the activity of CEs due to its role in the detoxification of xenobiotics (Cossi et al., 2020). Taking into account that these activities can be inhibited or induced by chemical compounds, the exposure to complex mixtures can lead to antagonistic, additive or synergistic effects. In previous reports of the effect of CFs in comparison with the AIs on ChEs and CEs activities, different responses were observed. For example, Souza da Silva et al. (2003) described that *Phyllocaulis soleiformis* ChEs were insensible to carbofuran AI, glyphosate AI and malathion AI and CF, but the CF of carbofuran decreased ChE activity and the CF of glyphosate increased it. Cossi et al. (2020) described that ChE activity was not altered while CEs (with p-NPB) were increased with both AI and a CF of acetamiprid. Iummato

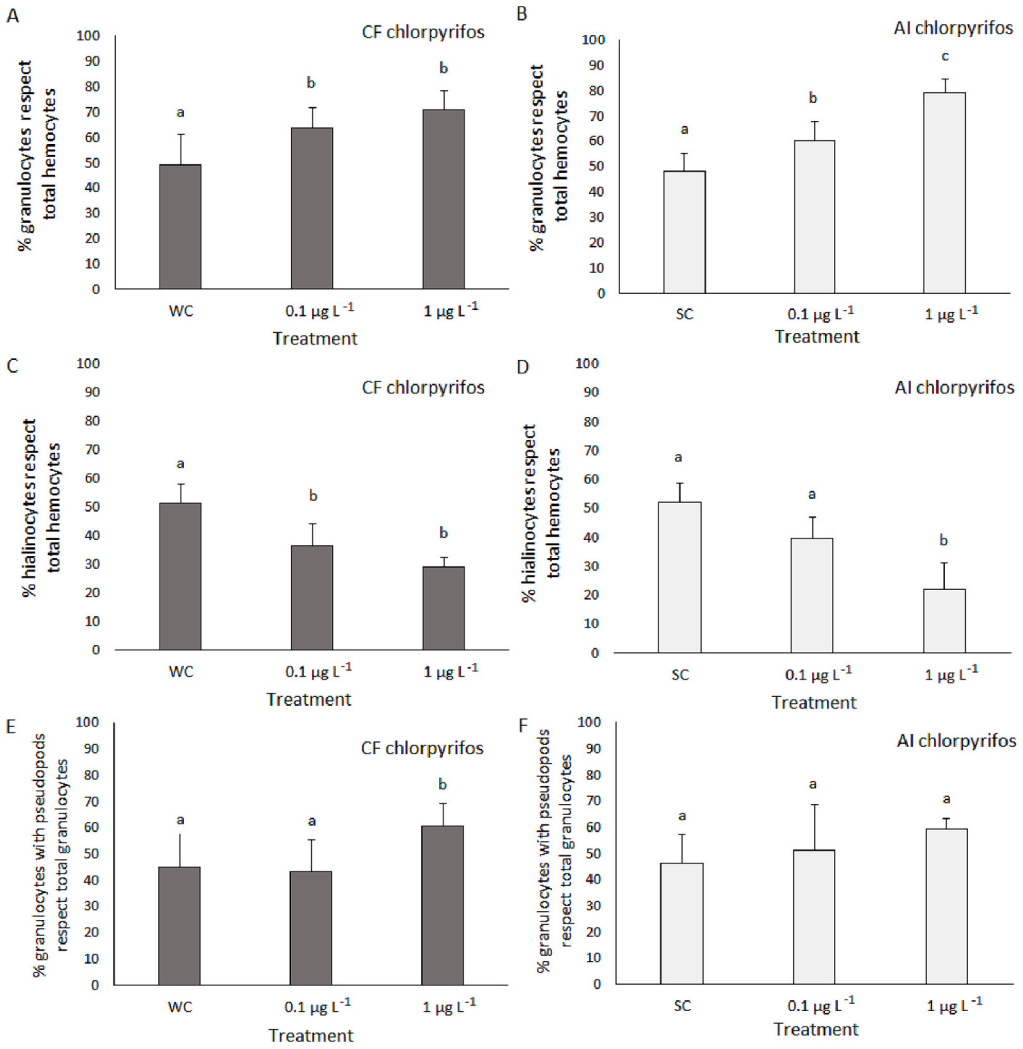

Fig. 4. Proportion of sub-populations of hemocytes of *Biomphalaria straminea* after 14 days of exposure to a commercial formulation (CF) and the active ingredient (AI) of chlorpyrifos, dechlorinated tap water (WC) and solvent control (0.001 % acetone, SC). Percentage of total granulocytes with respect to the total hemocytes in snails exposed to (A) WC and CF, (B) SC and AI; percentage of hyalinocytes with respect to the total hemocytes in snails exposed to (C) WC and CF, (D) SC and AI; percentage of granulocytes with pseudopods with respect to the granulocytes on snails exposed to (E) WC and CF, and (F) with SC and AI. Two concentrations of chlorpyrifos in the CF and AI were used: 0.1 and 1 µg L^{-1}. Results represent mean ± SD Different letters indicate statistical differences between treatments ($p < 0.05$).

et al. (2018) reported an inhibition of CEs activities in the mussel *Limnoperna fortunei* fed with glyphosate CF exposed algae. The controversial results show that new investigations are necessary to clarify how the excipients and their mixtures with pesticides affect these enzymes.

It was probed that OPs cause oxidative stress modifying antioxidant levels or activities and/or increasing EROS in invertebrate species. However, responses are non-specific and depend on the species, the exposure time and the kind and concentration of the pesticides (Kristoff et al., 2008). Different results on antioxidant responses were observed in other freshwater snails exposed to chlorpyrifos. In this sense, Cacciatore et al. (2015) have reported in *Planorbarius corneus* exposed 48 h to chlorpyrifos AI (7.5 µg L^{-1}) a decrease in GSH content and increases of CAT and GST activities. Despite that, a 14 days exposure to 5 µg L^{-1} did not modify GST in the same species (Rivadeneira et al., 2013). On the other hand, *C. gibbosa* GST did not vary after a 48 h exposure (Herbert et al., 2021), but decreased in *B. alexandrina* exposed 24 h to a CF (Ibrahim and Hussein, 2022). In this work the effect of chlorpyrifos on different *B. straminea* antioxidant defenses (CAT, SOD, GSH, GR and GST) were evaluated. Only CAT and SOD activities were altered. Several authors indicated that CAT and SOD activities may increase or decrease in stress conditions (Cao et al., 2003; Kristoff et al., 2008; Wang et al., 2016) frequently related to the levels of EROS. Generally, high increases in EROS content were related to high pesticides concentrations or long time exposures and can lead to dysfunction of the enzymes decreasing their activities. On the contrary, a slight increase of EROS levels can induce CAT and SOD activities as defense mechanisms (Cossi et al., 2018). In *B. straminea* exposed to CF, an increase of SOD activity occurred at 0.1 µg L^{-1} chlorpyrifos, while a decrease of CAT was evident after 1 µg L^{-1}, suggesting higher levels of EROS related to a higher concentration of the pesticide. However, EROS were not measured in this work, so we cannot confirm if their levels increased or not after chlorpyrifos exposure. EROS as well as oxidative damage indicators (such as lipid peroxidation and genotoxicity) will be evaluated in the future, allowing us to conclude about the role of oxidative stress in chlorpyrifos mediated toxicity in this species.

It is notable that CAT and SOD responses were only observed after the exposure to CF indicating that the excipients could be participating in the oxygen metabolism perturbation. In the same species, Cossi et al. (2020) found that only the formulation of acetamiprid decreased CAT and GST activities.

Immunotoxicity due to the exposure to OPs has been reported in some invertebrate species. Pruett et al. (1993) suggested that anticholinesterase agents cause a disruption of immune cell function and regulation by excessive cholinergic stimulation and as an indirect

Table 2
Reproductive parameters evaluated on egg masses laid by *Biomphalaria straminea* and offspring' survival after 14 days exposure.

Treatment	Eggs per mass	Embryonated eggs (%)	Hatching Time (days)	rate (%)	Offspring' survival (%)
WC	13.6 ± 4.9[a]	100 ± 0[a]	11.0 ± 1.4[a]	99.3[a]	98.5%[a]
0.1 μg L^{-1} CF	12.0 ± 7.3[a]	99 ± 3[a]	14.2 ± 2.9[b]	89.3[b]	97.3%[a]
1 μg L^{-1} CF	12.8 ± 4.6[a]	100 ± 0[a]	13.7 ± 1.1[b]	84.4[b]	83.1%[b]
SC	12.5 ± 2.6[a]	99 ± 3[a]	12.8 ± 1.0[a]	93.3[a]	98.6%[a]
0.1 μg L^{-1} AI	12.7 ± 3.2[a]	100 ± 0[a]	12.9 ± 0.8[a]	98.0[a]	81.3%[b]
1 μg L^{-1} AI	12.1 ± 4.0[a]	100 ± 0[a]	12.0 ± 1.3[a]	96.7[a]	88.9%[b]

Adult snails and egg masses were exposed to 0.1 and 1 μg L^{-1} of a commercial formulation (CF) and the active ingredient (AI) of chlorpyrifos. Dechlorinated tap water (WC) and solvent control (0.001 % acetone, SC) were included. Percentage of hatching was calculated on the total of embryonated eggs and percentage of offspring' survival (at 14 days after hatching) was calculated on the total of hatched juveniles. Results represent mean ± SD. Different letters indicate statistical differences ($p < 0.05$).

response to oxidative stress. Chlorpyrifos, AI and FC, did not decrease the hemocytes viability of *B. straminea* but modified its sub-population proportions increasing granulocytes and decreasing hyalinocytes. In coincidence, Russo and Lagadic (2004) have reported an increase of the proportion of granulocytes and a decrease in hyalinocytes in the gastropod *Lymnaea stagnalis* exposed to the herbicide atrazine. Our results indicate that the induction of a defense mechanism occurs: the increase of the cell population with greater phagocytic activity (granulocytes) and its activation by the emission of pseudopods. This mechanism occurs generally at low concentrations of xenobiotics. On the contrary, at high concentrations of OPs (Kristoff, 2010, unpublished PhD thesis) a decrease in hemocytes with pseudopods in *B. glabrata* exposed to azinphos-methyl was described. In addition, variations in the sub-populations of hemocytes depending on the concentration of chlorpyrifos were described in *B. alexandrina* exposed 24 h to a CF. Authors reported an increase of hyalinocytes and granulocytes with 2.1 mg L^{-1} and a decrease in the proportion of granulocytes at a high concentration (5.5 mg L^{-1}) (Ibrahim and Hussein, 2022). A slightly higher toxicity of the CF with respect to the AI was also found in adult organisms of other species such as *Daphnia magna* and *Rana pipiens* (Swann et al., 1996; Demetrio et al., 2014). It is worth noting that in other species like *Caenorhabditis elegans* and *Oreochromis niloticus* the CFs exposures evidenced more significant effects (Jacques et al., 2023; Majumder and Kaviraj, 2019). In *B. straminea* adult snails, no differences in lethality NOEC, LOEC and 48 h-CL_{50} between AI and CF were observed. However, CF causes more sublethal effects than the AI alone. In this sense, CF decreased CEs, CAT activity and the proportion of hyalinocytes with respect to the total hemocytes and increased SOD activity and the activation of the granulocytes.

Moreover, in *B. straminea* embryos, a notable higher toxicity of CF was observed, evidenced by the increase of the time of hatching and the decrease of the percentage of hatched juveniles. This effect can be related to developmental delays, interactions with gelatinous membranes and enzyme activities inhibitions. Inside the eggs, the embryos are protected by different membranes and substances (Cossi et al., 2018). The excipients present in the CF would be increasing membrane permeability and the entry of chlorpyrifos into de eggs causing the toxic effects on the embryos. In addition, the excipients could negatively affect embryos per se. In coincidence with our observations, in *B. glabrata* and *B. straminea,* a low embryotoxicity related to the protective role of the membranes was observed when were exposed to other AIs such as ivermectin and azinphos-methyl (Katz et al., 2017; Cossi et al., 2018).

After hatching the juveniles exposed to both the AI and CF presented higher mortality than the controls. Cossi et al. (2018) also reported a significant decrease of offspring' survival after the exposure to azinphos-methyl AI due to their direct contact with the pesticides after hatching. The presence of malformations in the offspring could be one of the causes of mortality. Malformations, mainly abnormalities in the tentacles, were observed in *B. straminea* juveniles exposed to chlorpyrifos (AI and CF). This effect has been reported in other snails such as *Bellamya aeruginosa* collected from an eutrophic lake (eastern China) (Lei et al., 2017), *Stramonita haemastoma* collected from the Bizerta Chanel (North-Eastern Atlantic Ocean) (El Ayari et al., 2018) and in *Marisa cornerarietis* exposed to a mixture of Zn and Ni (Sawasdee and Köhler, 2009). Due to the functions of aquatic *Biomphalaria* spp. tentacles, mainly related to sensory capacities and orientation, severe tentacle malformations, such as the absence of them, lead to less efficiency in orientating to food (Townsend, 1974), among other effects, which could reduce survival in their natural habitats.

It is important to note that the toxic effects observed in *B. straminea* reproduction occurred at low concentrations of chlorpyrifos, even lower than the concentrations used in previous studies. In this sense, Rivadeneira et al. (2013) observed egg masses without eggs or without hatched eggs and a higher toxic effects on the embryos and in the offspring of *P. corneus* exposed 14 days to 5 μg L^{-1}. Thus, it is probable that higher concentrations of chlorpyrifos would elicit more severe effects on the reproduction of *B. straminea.*

Sub-chronic tests with low concentrations of the CF of pesticides are the ones that come closest to real scenarios. Results indicate that at environmental concentrations and even at lower concentrations than those detected in water bodies from Argentina and other countries (Marino and Ronco, 2005; Otieno et al., 2012; Hasanuzzaman et al., 2018; Mac Loughlin et al., 2022; Bianco et al., 2023), the OP chlorpyrifos causes toxic effects at different levels of organization in *B. straminea*. This species is naturally distributed in Argentina including productive agricultural areas where pesticides, such as chlorpyrifos, are applied (Rumi et al., 2008; Bianco et al., 2023). Therefore, chlorpyrifos could be affecting the survival of this species in their natural habitats and therefore its abundance and distribution endangering the population.

The harmful effects on the reproduction of a species have great ecological relevance since they affect the species, indirectly the other species that coexist and, ultimately, the entire ecosystem.

Our results reinforce the importance of the use of embryos and juveniles in toxicological tests as a good strategy for gastropods protection due to the higher sensitivity to contaminants observed in the earliest stages in comparison with the adult organisms (Agrelo et al., 2019; Caixeta et al., 2022).

AChE and SOD activities, hemocytes sub-population proportion, embryotoxicity and offspring' survival resulted to be the most sensitive biomarkers (biomarkers modified at the lower used concentration) in *B. straminea* exposed to chlorpyrifos. In this work, effects on the defense immune systems of *B. straminea* were reported for the first time, resulting in useful and sensitive biomarkers to assess the impact of pesticides.

B. straminea snails are probed to be useful bioindicator species to evaluate the acute and sub-chronic toxicity of pollutants in laboratory conditions and to perform environmental biomonitoring in Argentina and in other countries in which they were distributed.

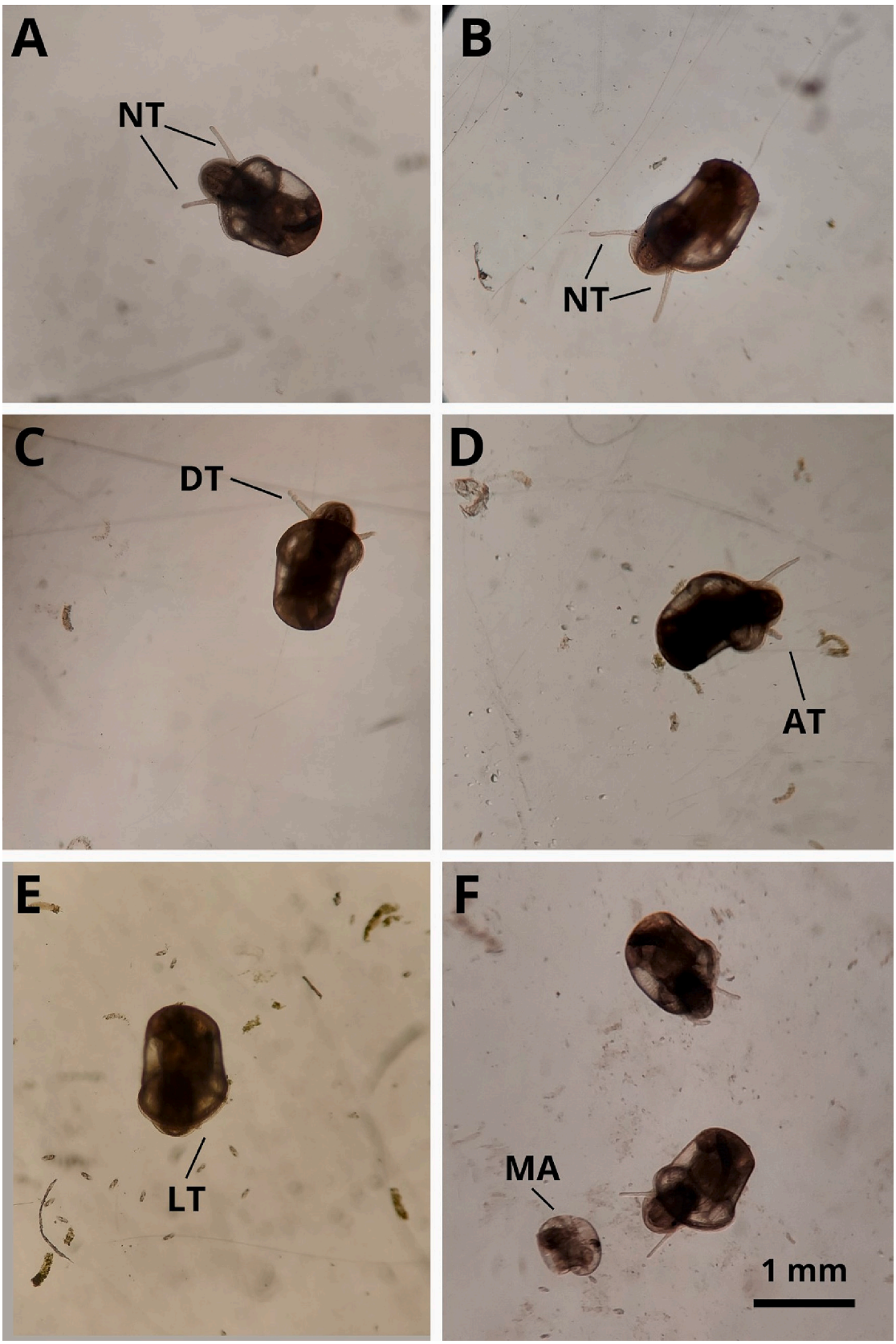

Fig. 5. Newly hatched juveniles of *Biomphalaria straminea* exposed for 14 days. **(A,B)** Normal tentacle morphology in a juvenile hatched from a WC egg mass. **(C)** Tentacle atrophy in a juvenile hatched from an egg mass exposed to 1 μg L^{-1} chlorpyrifos AI. **(D)** Asymmetrical tentacle in a juvenile hatched from an egg mass exposed to 0.1 μg L^{-1} chlorpyrifos AI. **(E)** A juvenile with lack of tentacles hatched from an egg mass and exposed to 1 μg L^{-1} CF. **(F)** A juvenile malformed/amorphous hatched from an egg mass exposed to 0.1 μg L^{-1} chlorpyrifos AI. WC: water control; AI: active ingredient; CF: commercial formulation; NT: normal tentacles; DT: deformed tentacle; AT: asymmetrical tentacle; LT: lack of tentacles; MA: malformed/amorphous juvenile.

5. Conclusions

The exposure of *B. straminea* snails to chlorpyrifos (AI and a CF) caused toxic effects on adult organisms, embryos and juveniles. The CF was more toxic compared to the AI, mainly to the embryos, probably due to the increase of embryos' membrane permeability. In adult snails, CF produced more sub-lethal effects than the AI alone, altering antioxidant and detoxificant defenses. AChE, SOD, the modification of hemocytes sub-populations, the time and percentage of juveniles' hatching and their survival becomes the most sensitive biomarkers. We proposed the use of several biomarkers at different levels of organization highlighting the inclusion of embryotoxicity tests, juvenile quality and immunological responses in environmental biomonitoring due to their high sensitivity to pollutants and their ecological relevance. Also, we emphasize the importance of the assessment of the toxicological effects of the formulations in order to not underestimate the effects of pesticides on the environment.

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CRediT authorship contribution statement

Karina Alesia Bianco: Writing – review & editing, Writing – original draft, Visualization, Investigation, Formal analysis, Data curation. **Claudia Noemí Martini:** Writing – review & editing, Writing – original draft, Visualization, Resources, Methodology, Investigation, Formal analysis, Data curation. **María José Tejedor:** Formal analysis, Investigation. **María Gimena Paredes:** Investigation. **Gisela Kristoff:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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