ARTICLE IN PRESS

Pesticide Biochemistry and Physiology xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Pesticide Biochemistry and Physiology

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

Effects of azinphos-methyl on enzymatic activity and cellular immune response in the hemolymph of the freshwater snail *Chilina gibbosa*

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ARTICLE INFO

Keywords:
Insecticide
B-esterases
Glutathione S-transferase
Immunotoxicity
Freshwater gastropod

ABSTRACT

The use of a battery of biomarkers, especially those more closely related to species integrity, is desired for more complete ecotoxicological assessments of the effects of pesticide contamination on aquatic organisms. The phosphorodithioate azinphos-methyl has been intensively used in agriculture worldwide and have been found in the habitat of *Chilina gibbosa*, a freshwater snail endemic to South America. This snail has been proposed as a good model organism for ecotoxicity bioassays on the basis of studies focused mainly on enzymatic responses in whole tissue homogenates. Our aim was to evaluate the effect of an acute 48 h exposure to an environmental concentration of azinphos-methyl on *C. gibbosa* hemolymph enzymatic activity and cellular immune response. Our results show that cholinesterase activity was strongly inhibited (94%) in hemolymph of exposed snails. Carboxylesterase activity measured with *p*-nitrophenyl butyrate and glutathione S-transferase activity were augmented 47% and 89% respectively after exposure. No differences were found for hemolymph carboxylesterase activity measured with *p*-nitrophenyl acetate. These results differ from those reported for whole tissue homogenates and reveal that tissue-specific responses of enzymatic biomarkers exist in this species. Regarding immune cell response, hemocytes were identified for the first time for *C. gibbosa*. Their viability and phagocytic activity decreased after azinphos-methyl exposure although total number of circulating cells did not differ between treatments. We conclude that concentrations of azinphos-methyl that can be found in the environment can compromise both hemolymph cholinesterase activity and the immune system of *C. gibbosa*. Furthermore, we propose that carboxylesterase and glutathione S-transferase activities measured in hemolymph and hemocyte viability and phagocytic activity could be incorporated as sensitive biomarkers to evaluate the effects of pesticide exposure on this and related species.

1. Introduction

Chilina gibbosa (Sowerby 1841) is a freshwater snail from the family Chilinidae (Pulmonata) endemic to southern Argentina and Chile [6, 21, 51]. Adults are usually found aggregated in shallow areas and have limited mobility, which makes them easy to collect and handle for bioassays [6, 41]. The family Chilinidae is considered vulnerable due to the deterioration of its habitat related to different anthropogenic perturbations, such as the presence of toxic contaminants [61]. In Argentina, *C. gibbosa* is commonly found in rivers, lakes and reservoirs of the Río Negro and Neuquén provinces, North Patagonia [6, 51, 61]. This snail plays an important role in the native ecosystem as food source for birds and different fish species, some of which have commercial value like the native silverside *Odontesthes hatcheri* [1]and the rainbow trout *Oncorhynchus mykiss*.

One of the main economic activities in the Upper Valley of Río

Abbreviations: AcSCh, acetylthiocholine iodide; AZM, azinphos-methyl; CE, carboxylesterase; ChE, cholinesterase; CDNB, 2,4-dinitrochlorobenzene; CR, Congo Red; DTNB, 5,5-dithio-2-bis-nitrobenzoate; GS, Giemsa solution; GS-DNB, S-(2,4-dinitrobenzyl)glutathione; GST, Glutathione S-transferase; OP, organophosphate insecticide; p-NPA, *p*-nitrophenyl acetate; p-NPB, *p*-nitrophenyl butyrate; TB, Trypan Blue

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https://doi.org/10.1016/j.pestbp.2018.07.005
Received 26 April 2018; Received in revised form 2 July 2018; Accepted 12 July 2018
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Negro and Neuquén is fruit production, which involves the use of large amounts of pesticides. The phosphorodithioate insecticide azinphos-methyl (AZM) has been one of the most commonly and intensively applied in this area. This insecticide is converted by oxidation to the organophosphate (OP) oxon-derivate. Maximum recorded concentrations of AZM in subsurface water vary from 0.25 $\mu g\,L^{-1}$ to 79.30 $\mu g\,L^{-1}$ between control and application periods, respectively [35, 36]. Previous studies in our laboratory have characterized the effects of acute exposures to AZM on *C. gibbosa* and evaluated recovery responses, based on the neurotoxicity, activity of B-esterases and oxidative stress parameters [5, 13]. Bianco et al. [5] provided a thorough evaluation of *C. gibbosa* whole tissue enzymatic response to a 48 h exposure to AZM. They reported an IC_{50} of 0.02 ± 0.01 $\mu g\,L^{-1}$ for cholinesterases (ChEs) and higher than 1000 $\mu g\,L^{-1}$ for carboxylesterases (CEs), with no effect of 20 $\mu g\,L^{-1}$ AZM on the activity of glutathione S-transferase (GST). Hence, it has been suggested that *C. gibbosa* could be included as a sentinel species in monitoring programs due to its sensitive ChE response to AZM.

ChEs are most commonly used as sensitive biomarkers for OP exposure [60], as they constitute the target of the mechanism of action of these pesticides. OPs inhibit ChEs by phosphorylation of a serine residue in the enzyme's active site, preventing it from hydrolyzing the neurotransmitter acetylcholine. The subsequent accumulation of acetylcholine leads to overstimulation of cholinergic receptors followed by depression or paralysis and eventual death. CEs have been postulated as participating in the metabolism and detoxification of many agrochemicals including OPs [45, 63], either by binding to the insecticide and thus removing large amounts of it, or by hydrolyzing carboxylester bonds present in some OPs [27, 28, 55]. Hence, the combined use of ChEs and CEs as biomarkers has been proposed as a more suitable strategy to obtain a detailed evaluation of the effect of exposure to OPs [56, 64]. Another important enzyme that is habitually measured in toxicological studies that pursue a multi biomarker approach is GST [15]. It plays a vital role in detoxification of OPs by catalyzing the conjugation of xenobiotics or their metabolites with glutathione, which favors their excretion [30, 59].

There is evidence that both enzyme basal activity and sensitivity of ChEs, CEs and GST vary between tissues, therefore the response to compounds such as OPs can be tissue-specific. Thus, a more accurate assessment of pesticide effects could be obtained by determining biomarkers in different tissues [43]. For example, in the mussel *Mytilus edulis*, ChE activity is higher in hemolymph than in whole tissue homogenates, whilst the opposite is true for CE activity [23]. In the bivalve mollusk *Scapharca inaequivalvis* higher GST activity has been found in the digestive gland than in foot tissue or gills [4]. Regarding tissue-based differences in enzyme sensitivity, Cacciatore et al. [10] and Otero and Kristoff [43], have found variations in ChE and CE activities between whole tissue homogenates, pulmonary region, digestive gland and hemolymph of the freshwater snail *Planorbarius corneus* exposed to the OPs AZM and chlorpyrifos.

The study of other biomarkers directly related to species integrity and survival, such as effects on immune system or reproduction, is especially relevant. Nevertheless, no studies on this kind of biomarkers have been carried out yet for *C. gibbosa*. Invertebrates present a relatively simple immune system, which makes them good models for immunotoxicity studies [19, 20, 22]. Although a wide variety of specific host defense strategies exist, they are mainly based on phagocytosis by freely circulating blood cells such as hemocytes or coelomocytes [14, 38, 46, 50]. In this sense, different immune parameters such as quantity, viability and phagocytic activity of circulating cells can be considered as potential biomarkers of pesticide exposure [3, 7, 8, 22, 44]. For instance, OP and carbamate compounds have been shown to impair lysosome activity or integrity and inhibit phagocytosis in different invertebrates [17, 48, 62]. AZM specifically, resulted an important modulator of immune and detoxification responses in the Patagonian freshwater mussel *Diplodon chilensis*, enhancing responses it was challenged with *E. coli* [12]. Thus, our aim was to study hemolymph enzymatic and cellular immune responses of *C. gibbosa* after an acute 48 h exposure to AZM. We focused on ChE, CE and GST activities and total hemocyte number, hemocyte viability and phagocytic activity as enzymatic and immune response biomarkers, in order to provide more comprehensive knowledge of the acute toxic effects of AZM on non-target organisms.

2. Materials and methods

2.1. 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), azinphos-methyl PESTANAL® (97.2% pure) were purchased from Sigma–Aldrich. Trypan Blue (TB), Giemsa solution (GS) and Congo Red (CR) were purchased from Biopack (Argentina). All other chemicals used were also of analytical reagent grade.

2.2. Organisms

Adult *C. gibbosa* individuals were hand collected from a site on the river Chimehuin (39°54′57.15"S 71°06′23"W; province of Neuquén, Argentina). The sampling site can be considered free from agrochemical or other kinds of pollution since there are no population centers upstream from it and the river Chimehuin originates in the Lanín National Park, where agricultural and industrial activities are banned [5, 13, 34].

2.3. Bioassays

Two bioassays were carried out in order to study hemolymph enzymatic activity and cellular immune response. Snails were exposed for 48 h to Chimehuin water with 0.002% acetone, as solvent control, or with 20 $\mu g\,L^{-1}$AZM, in 1 L glass vessels containing 500 mL of the corresponding solution. The concentration of 20 $\mu g\,L^{-1}$AZM represents possible real case scenarios by being within the range of pesticide found in freshwater bodies of Argentina (see Introduction). It was also chosen to match the concentration used in previous studies for evaluating enzymatic activity in whole tissue homogenates [5, 13], for comparison purposes. Acetone concentration was set at 0.002%, which is 5-fold lower than the concentration recommended by the Organization for Economic Cooperation and Development (OECD) [40] for aquatic toxicity testing. A Chimuehuin water control without acetone was included for the cellular immune response bioassay as there are no previous records of such studies in this species.

AZM working solution was obtained by diluting the stock solution of the insecticide prepared in acetone with Chimehuin water. Solutions were not renewed during exposure based on the results of stability studies of our laboratory in which concentration values measured at time 0 remained constantly after 48 h [9]. Always the AZM concentrations measured were within 97–102% of the nominal values.

In both bioassays, $N = 6$ (6 glass vessels per each treatment). Each replicate consisted of a pool of hemolymph from 12 snails for enzymatic activity (144 snails in total)or 3 snails (54 snails in total)for cellular immune response according to the amount of sample needed for measuring biomarkers. Snails were not fed during the bioassays.

After 48 h of exposure lethality and neurotoxicity were recorded. Snails were considered dead when they failed to respond mechanical stimuli or if they remained constantly retracted into the shell. The signs of neurotoxicity registered were the decrease or lack of adherence and the abnormally protruded head-foot region from the shell.

2.4. Hemolymph extraction

Snails were anesthetized 6 to 8 min on ice, wiped clean and blotted dry before extraction. A small hole was pierced in the anterior ventral region of the shell using a sharp pointed scalpel under a dissecting microscope. The needle of a disposable hypodermic syringe (0.5 × 16 mm 25G 5/8)was then inserted in the hole, taking care not to puncture any major organs. Hemolymph was suctioned as the snail slowly retracted into the shell, transferred to microcentrifuge tubes and kept on ice until extraction was finished. For enzymatic activity assays, hemolymph was centrifuged at 5000 x g for 10 min and the resulting supernatants were separated. For immunotoxicity assays, hemolymph was used immediately after extraction.

2.5. Enzymatic activity

ChE activity was measured according to the method of Ellman et al. [18], with the characterization previously performed for this species [5], using 100 mM phosphate buffer pH 8, 0.2 mM DTNB, 1.5 Mm AcSCh as substrate and 75–100 μL of the supernatant fraction. Absorbance was recorded continuously for 60 s at 412 nm. Rates were corrected for spontaneous substrate hydrolysis and non-specific reduction of the chromogen by tissue extracts. Specific activity was calculated using the molar extinction coefficient for AcSCh (13.6 $mM^{-1} cm^{-1}$).

CE activity was measured through hydrolysis of both p-NPA and p-NPB according to Kristoff [29] using the characterization previously performed for *C. gibbosa* [5], since CE activity and sensitivity to pesticides depend on the substrate used [31, 33]. Reactions were performed in 2.5 mL 100 mM phosphate buffer pH 8.0 containing 5% acetone, 1.5 mM p-NPA or p-NPB and 75 μL of the supernatant fraction. Absorbance was continuously recorded for 60 s at 400 nm. Specific activity was calculated using the molar extinction coefficient for p-nitrophenol (18.6 $mM^{-1} cm^{-1}$).

GST activity was measured by the method of Habig et al. [24]. The reaction mixture contained 1 mM GSH, 1 mM CDNB, 100 mM phosphate buffer pH 6.5 at a final volume of 3.0 mL and 10 μL of the supernatant fraction. The formation of the GS-DNB complex was evaluated by monitoring the increase in absorbance for 60 s at 340 nm. Specific activity was calculated using the molar extinction coefficient for GS-DNB (9.6 $mM^{-1} cm^{-1}$).

Protein content was determined according to the method of Lowry et al. [37], using bovine serum albumin as standard. Enzyme activity results were expressed as μmol of substrate hydrolyzed per min per mg of protein.

2.6. Cellular immune response

Total number of hemocytes was determined by counting the circulating cells from fresh hemolymph in a Neubauer chamber in four replicates. The results were expressed as number of hemocytes per mL of hemolymph [1].

Hemocyte viability was determined by mixing 30 μL of fresh hemolymph with 15 μL of TB 0.2% in anticoagulant solution [12] and incubated for 5 min at 4 °C. After incubation, live and dead cells (undyed and dyed, respectively) were counted in a Neubauer chamber in four replicates. Hemocyte viability was expressed as proportion of viable hemocytes per total number of hemocytes.

Phagocytic activity was determined by mixing 30 μL of fresh hemolymph with a volume of a CR stained yeast suspension that contained double the number of cells than the number of viable hemocytes in the sample, and incubating at room temperature for 30 min. After incubation, 30 μL were loaded onto clean microscope slides and cells were allowed to adhere during 30 min at room temperature in a wet chamber. Cells were then stained with 10% GS for 10 min and carefully washed and mounted with distilled water. Around 90 cells per sample were counted under light microscopy (100–400 X). Nuclei were identified in blue, cytoplasm light blue or violet; yeast cells appeared black or red. Phagocytic activity was expressed as phagocyted yeast cells per total number of viable hyalinocytes (modified from [32]). Pictures of cells were taken with a Leica DM 500 microscope at 400 X. Image edition was carried out using the open access software ImageJ 1.51n.

2.7. Data analysis

The assumption of normality was tested by the F test and the Kolmogorov-Smirnov test. Homogeneity of variances was tested by Bartlett's tests for all variables. Differences in enzymatic activity in hemolymph of solvent control snails and AZM exposed snails were tested using Student's *t*-test for independent samples. A log10 transformation was applied on ChE activity data in order to meet the normality assumption.

Immunotoxicity variables were tested for differences between control, solvent control and AZM exposed snails using one-way ANOVA. Significant differences were further analyzed by Tukey's multiple comparisons. An arcsine (√) transformation was applied on cell viability and hyalinocyte phagocytic activity data in order to meet assumptions. The level of significance used was 0.05 for all analyses. Statistical analyses were performed with GraphPad InStat 3.01 software.

3. Results

3.1. Lethality and neurotoxicity

No mortality was observed either in control and exposed organisms. However, all the exposed snails showed signs of neurotoxicity.

3.2. Protein content

Protein content did not vary between solvent control and exposed snails (Student's *t*-test, $t = 0.998$, df = 10, $P = .342$). Mean protein content in hemolymph was 9.58 ± 2.15 $mg\,mL^{-1}$ in solvent control snails and 8.23 ± 2.51 $mg\,mL^{-1}$ in exposed snails.

3.3. Cholinesterase and carboxilesterase activity

ChE activity was strongly inhibited in hemolymph of snails exposed to AZM, with mean activity dropping 94% with respect to solvent control animals (Fig. 1; Student's *t*-test, $t = 10.17$, df = 10, $P < .0001$). CE response in the hemolymph of exposed organisms varied according

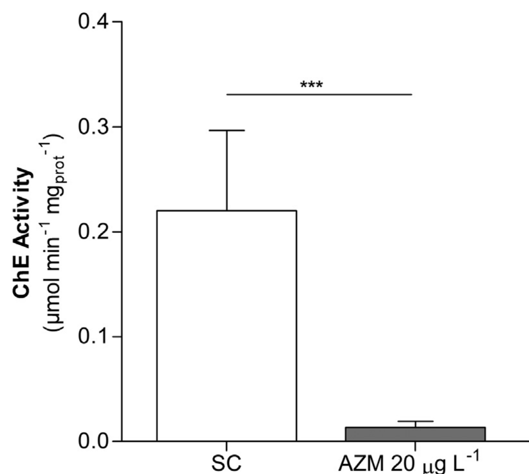

Fig. 1. Cholinesterase (ChE) activity [mean ± SD] in *Chilina gibbosa* hemolymph after a 48 h exposure. SC = solvent control (0.002% acetone) and AZM = azinphos-methyl (20 $\mu g\,L^{-1}$); ***$P < .001$.

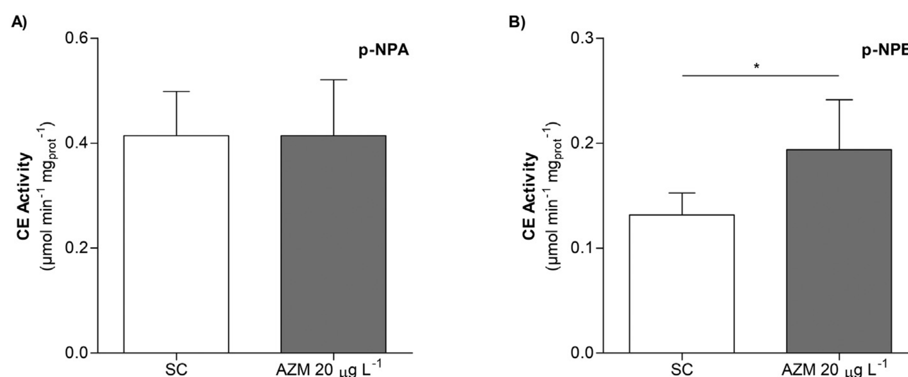

Fig. 2. Carboxylesterase (CE) activity determined using (a) *p*-nitrophenyl acetate (p-NPA) or (b) *p*-nitrophenyl butyrate (p-NPB) as substrates [mean ± SD] in *Chilina gibbosa* hemolymph after a 48 h exposure. SC = solvent control (0.002% acetone) and AZM = azinphos-methyl (20 μg L^{-1}); *$P < .05$.

to the substrate used. When measured using p-NPA as substrate, CE activity did not vary between control and exposed animals (Fig. 2a; Student's *t*-test, $t = -0.01$, df = 10, $P = .996$). When measured using p-NPB as substrate, CE activity was 47% higher in hemolymph of exposed snails than in control snails (Fig. 2b; Student's t-test, $t = -2.93$, df = 10, $P < .05$).

3.4. Glutathione S-transferase activity

GST activity strongly augmented by 89% in the hemolymph of AZM exposed snails when compared with solvent control snails (Fig. 3; Student's t-test, $t = -4.38$, df = 9, $P < .01$).

3.5. Cellular inmune response

No significant differences were found between the water and the solvent control for the measured parameters, with the exception of cell viability (acetone caused a decrease of 4% with respect to the water control). Henceforth, only solvent control results are shown in the figures.

The total number of circulating hemocytes in *C. gibbosa* hemolymph did not differ between control, solvent control (acetone) or AZM exposed snails (Fig. 4a; One-way ANOVA, $F = 0.34$, df = 2, $P > .05$).

Exposure to AZM caused a 28% reduction in cell viability (Fig. 4b; One-way ANOVA, $F = 162.99$, df = 2, Tukey's Multiple Comparisons Test, $P < .001$). Acetone had a minor effect on this variable, causing a 4% reduction with respect to control snails (Tukey's Multiple Comparisons Test, $P < .05$) that differed from AZM effects (Tukey's Multiple Comparisons Test, $P < .0001$).

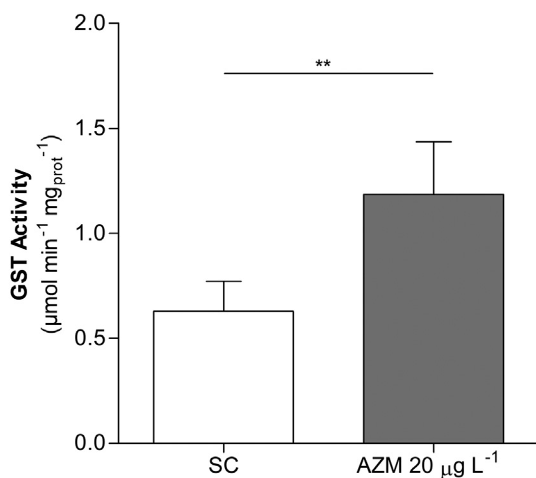

Fig. 3. Glutathione S-transferase (GST) activity [mean ± SD] in *Chilina gibbosa* hemolymph after a 48 h exposure. SC = solvent control (0.002% acetone) and AZM = azinphos-methyl (20 μg L^{-1}); **$P < .01$.

Chilina gibbosa hemolymph contained clearly differentiated cells that were able to ingest CR stained yeast. These cells were identified as hyalinocytes based on the following morphological characteristics: round or polymorphic basophilic nuclei and non-granular cytoplasm (Fig. 5A, B). Hyalinocyte phagocytic activity in *C. gibbosa* hemolymph was observed in all treatments. Fig. 5 shows an example of hyalinocytes in hemolymph of a control snail, without (C) and with (D) ingested yeast. Exposure to AZM reduced phagocytic activity by 72% compared to control snails (Fig. 6; One-way ANOVA, $F = 29.11$, df = 2, $P < .001$; Tukey's Multiple Comparisons Test, $P < .05$). There was no solvent effect on this variable.

4. Discussion

Chilina gibbosa has been suggested as a possible sentinel species for monitoring programs of freshwater pesticide contamination, specifically in southern Argentina and Chile [5, 13]. This has been based on the fact that it presents distinct and sensitive neurotoxic signs and very sensitive ChE activity in whole tissue homogenates, which could readily serve as biomarkers of acute exposure to AZM. Our study is the first to assess hemolymph enzymatic and cellular responses to AZM in *C. gibbosa*. Furthermore, it is, to the best of our knowledge, the first record of immunotoxicity biomarkers in this species.

The acute 48 h exposure to 20 μg L^{-1} AZM produced severe signs of neurotoxicity and 94% inhibition of ChE activity in *C. gibbosa* hemolymph. This inhibition coincides with the results of Cossi et al. [13], who reported an 85% inhibition in *C. gibbosa* ChE activity in whole tissue homogenates after the same exposure time and AZM concentration. Our results suggest that ChE activity is a reliable biomarker of AZM exposure in *C. gibbosa*, both in whole tissue homogenates and in hemolymph. In this sense, ChE sensitivity has also been reported to be higher in hemolymph than in whole tissue homogenates for other gastropods such as *P. corneus* [10].

Regarding CE activity, when measured with p-NPA as substrate, we found no differences between exposed and control animals in hemolymph. However, when using p-NPB as substrate, we measured a 47% increase in activity in the hemolymph of exposed animals. This result contrasts with Cossi et al. [13], who found no differences between treatments in whole tissue homogenates after exposure to the same concentration of AZM and during the same time as the present study. According to the classification proposed by Aldridge [2], *C. gibbosa* CEs can be considered B-esterases as they can be inhibited by OPs [5]. This inhibition, produced by the irreversible binding of the insecticide to the serine residue of the active site of this enzyme, could protect the organism from the effect of ChE inhibition by sequestering circulating OP [64]. Alternatively, CEs actively participate in detoxification pathways of different xenobiotics [64]. Increased CE activity such as that found in

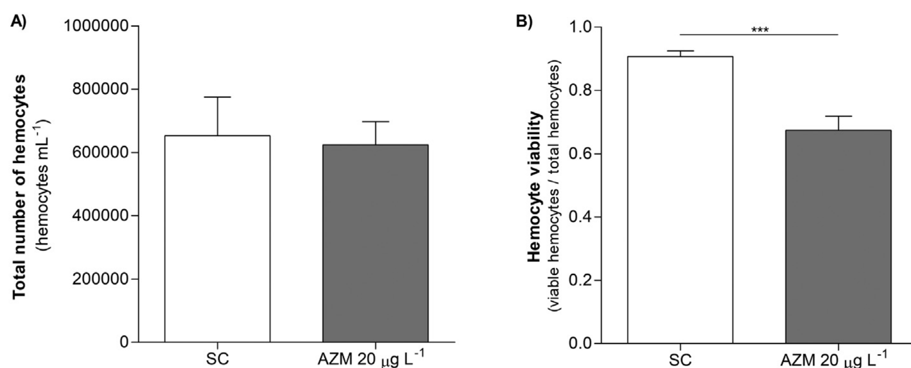

Fig. 4. (a) Total number of circulating hemocytes per mL [mean ± SD] and (b) cell viability calculated as viable cells per total number of circulating hemocytes observed [mean ± SD], in *Chilina gibbosa* hemolymph after a 48 h exposure. SC = solvent control (0.002% acetone) and AZM = azinphos-methyl (20 µg L^{-1}); ***$P < .001$.

this study in *C. gibbosa* hemolymph suggests that these enzymes participate in the metabolism of low concentrations of AZM after an acute exposure. An increase in CEs as a result of exposure to different contaminants has been reported in invertebrates, including honeybees, mosquitoes and mussels [11, 39, 57]. Variation in CE response according to the tissue studied has been observed in many aquatic invertebrates [56, 64]. For example, Cacciatore et al. [10] determined CE inhibition to be much more sensitive to low concentrations of AZM in soft tissues than in hemolymph of *P. corneus* when measured using p-NPB. Furthermore, our results confirm that CE response can vary according to the substrate used to measure CE activity. This has been attributed to CEs being a group of enzymes with low substrate specificity with multiple isozymes whose presence varies from tissue to tissue. In this sense, both Kristoff et al. [31] with *Biomphalaria glabrata* and Otero and Kristoff [43] with *P. corneus* reported differences in p-NPB and p-NPA CE responses to 48 h exposures to AZM depending on the tissue analyzed.

Interestingly, the effect of a 48 h exposure to 20 µg L^{-1} AZM on *C. gibbosa* hemolymph GST activity was also different from that found in whole tissue homogenates. We found a significant increase of 89% whilst Bianco et al. [5] did not find any differences between treatments, after the same exposure time to the same AZM concentration. In invertebrates, different GST responses to anticholinesterase-insecticides have been reported. For example, Oneto et al. [42] found that fenitrothion induced GST activity in *Corbicula fluminea,* while in *B. glabrata* and *P. corneus* no effects after AZM or chlorpyrifos exposure were observed [30, 49]. Alternatively, a 96 h exposure to chlorpyrifos inhibited GST activity in *Hyalella azteca* [58]. An increase in GST activity has been associated with resistance or tolerance to OPs and carbamates [15, 55]. Therefore, we suggest that GST pathways could be involved in defense mechanisms of *C. gibbosa* against AZM.

Gastropods appear as a promising sentinel group for monitoring freshwater environments, since several species have been shown to display potent immunological responses upon pesticide exposure [52–54]. In this snail, total number of circulating hemocytes was not affected by a 48 h AZM exposure. In contrast, total hemocyte number decreased in armyworm fed acutely with hexaflumuron sprayed leaves [26] and increased in different snails exposed to pyrethroids [47] or

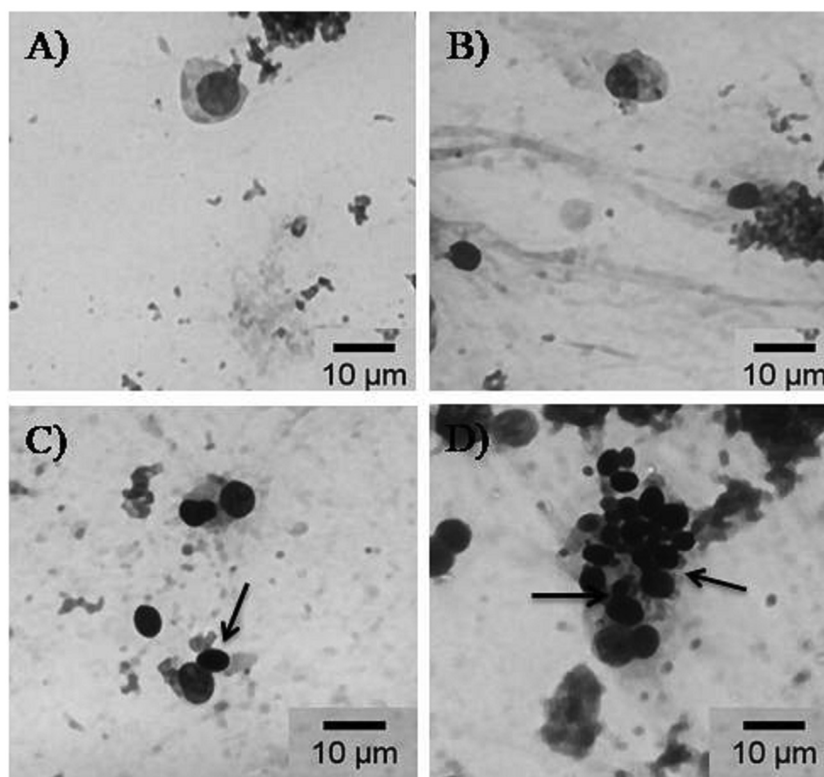

Fig. 5. Light micrograph of circulating hyalinocytes in *Chilina gibbosa* hemolymph, stained with GS 10% (400 X).Cells without (A, B, C) and with (D) phagocytic activity. Yeast cells are indicated by arrowheads.

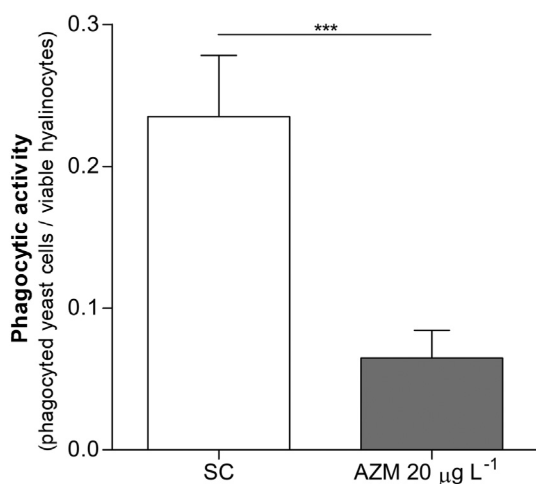

Fig. 6. Phagocytic activity calculated as number of yeast cells phagocyted per total number of viable hyalinocytes [mean ± SD] in *Chilina gibbosa* hemolymph. SC = solvent control (0.002% acetone) and AZM = azinphos-methyl (20 μg L^{-1});***P < .001.

hexachlorobenzene and atrazine [52, 53].

Hemocyte viability in *C. gibbosa*, on the other hand, was impaired by 28% by AZM exposure. Similar effects on immune cells were observed in earthworms exposed to chlorpyrifos contaminated soil [17] and in mice exposed to diazinon [25]. In the present study, a decrease of approximately 4% in cellular viability was also observed in snails exposed to 0.002% acetone. This effect was minimal compared with that caused by the pesticide but its significance highlights the sensitivity of the immune biomarkers to this solvent. This should be taken into account for future immunotoxicity assays. Nevertheless, it is worth noting that the concentration of acetone used in this study was 5-fold lower than that used by Castro et al. [12] who did not find any effect on the viability of *D. chilensis* hemocytes.

Phagocytic activity also decreased after a 48 h AZM exposure in hyalinocytes of *C. gibbosa*, in accordance with results observed in the freshwater snail L. *stagnali* exposed to atrazine [53] and in earthworms exposed to different OPs. It has been suggested that lipophilic organic contaminants such as AZM are likely to affect phagocytosis through cell membrane disruption [16].

There are scarce antecedents dealing with immunotoxic effects of AZM on invertebrates and, in general terms, specific mechanisms of action linking neurotoxic and immunotoxic consequences deserve more attention. AZM effects seem to depend on exposure time, concentration and target organism. For example, the freshwater mussel *D. chilensis* exposed for 72 h to a ten-fold higher AZM concentration (200 μg L^{-1}) than the one used for *C. gibbosa* showed increased number of hemocytes with no change in cell viability [12]. These authors suggest that the mussel's immune system is responding in order to maintain homeostasis against possible tissue damage. In this regard, Russo and Lagadic [53] argue that hemocyte density may be seen as a way to maintain a constant level of global phagocytic activity when individual cell phagocytosis is inhibited by a xenobiotic. Such a response seems to be lacking in *C. gibbosa*, since hemocyte number remains at basal levels, while viability and hyalinocyte phagocytosis are decreased by 28% and 72%, respectively.

Our results support the idea of considering immunological variables in *C. gibbosa* as good biomarkers for low concentrations of AZM that could be included when working with this and related species as bioindicators in freshwater environments. Furthermore, we provide valuable information to help bridge the gap between biomarker studies and physiological effects related to the well-being of sensitive aquatic species, important in order to incorporate them in ecotoxicological studies.

5. Conclusions

Our results show that CE and GST responses measured in *C. gibbosa* hemolymph to AZM exposure differ from those found in whole tissue homogenates. We suggest that both CEs and GST are involved in *C. gibbosa* defense mechanisms. Furthermore, we show that AZM has a detrimental effect on the immune system of *C. gibbosa*. ChE, CE and GST activities in hemolymph and hemocyte viability and phagocytosis of *C. gibbosa* are sensitive biomarkers to concentrations of AZM found in the environment.

Acknowledgments

This research was supported by grants UBACYT 20020130200055BA, CONICET PIP11220120100680CO, CONICET PIP 11220130100529CO, ANPCYT PICT 2013-1415 and ANPCYT PICT201-0797. L. T. Herbert, J. M. Castro and P. F. Cossi are on scholarships from CONICET, Argentina. Wewouldlike to thank QB (Departamento de Química Biológica, FCEyN, UBA), DBBE (Departamento de Biodiversidad y Biología Experimental, FCEyN, UBA), IQUIBICEN (Instituto de Química Biológica de la Facultad de Ciencias Exactas y Naturales) and CEAN (Centro de Ecología Aplicada del Neuquén). We would also like to thank Julio Painefilú for the photograph of *C. gibbosa* used in the graphical abstract.

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