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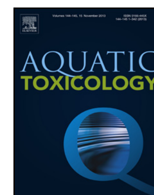

Recovery study of cholinesterases and neurotoxic signs in the non-target freshwater invertebrate *Chilina gibbosa* after an acute exposure to an environmental concentration of azinphos-methyl

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ABSTRACT

Azinphos-methyl belongs to the class of organophosphate insecticides which are recognized for their anticholinesterase action. It is one of the most frequently used insecticides in the Upper Valley of Río Negro and Río Neuquén in Argentina, where agriculture represents the second most important economic activity. It has been detected in water from this North Patagonian region throughout the year and the maximum concentration found was 22.48 $\mu g\,L^{-1}$ during the application period. *Chilina gibbosa* is a freshwater gastropod widely distributed in South America, particularly in Patagonia, Argentina and in Southern Chile. Toxicological studies performed with *C. gibbosa* in our laboratory have reported neurotoxicity signs and cholinesterase inhibition after exposure to azinphos-methyl for 48 h. Recovery studies together with characterization of the enzyme and sensitivity of the enzyme to pesticides can improve the toxicological evaluation. However, little is known about recovery patterns in organisms exposed to organophosphates. The aim of the present work was to evaluate the recovery capacity (during 21 days in pesticide-free water) of cholinesterase activity and neurotoxicity in *C. gibbosa* after 48 h of exposure to azinphos-methyl. Also, lethality and carboxylesterase activity were registered during the recovery period. Regarding enzyme activities, after a 48-h exposure to 20 $\mu g\,L^{-1}$ of azinphos-methyl, cholinesterases showed an inhibition of 85% with respect to control, while carboxylesterases were not affected. After 21 days in pesticide-free water, cholinesterases continued to be inhibited (70%). Severe neurotoxicity signs were observed after exposure: 82% of the snails presented lack of adherence to vessels, 11% showed weak adherence, and 96% exhibited an abnormal protrusion of the head-foot region from shell. After 21 days in pesticide-free water, only 15% of the snails presented severe signs of neurotoxicity. However, during the recovery period significant lethality (30%) was registered in treated snails. *C. gibbosa* is a very sensitive organism to azinphos-methyl. These snails play an important role in the structure and function of aquatic food webs in this region. Thus, a decline of this species' population would probably have an impact on aquatic and non-aquatic communities. Our results show that *C. gibbosa* is a relevant sentinel species for studying exposure and effects of azinphos-methyl using behavioral and biochemical biomarkers. Neurotoxic behavioral signs are very sensitive, non-destructive biomarkers, which can be easily detected for about one week after acute exposure. Cholinesterse activity is a very useful biomarker showing a high sensitivity and a slow recovery capacity increasing the possibility to indirectly detect organophosphates for long periods after a contaminant event.

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Abbreviations: AcSCh, acetylthiocholine iodide; CE, carboxylesterase; Che, cholinesterase; DTNB, 5′-Dithio-2-bis-nitrobenzoate; OP, organophosphate insecticide; p-NPA, p-Nitrophenyl; p-NPB, p-Nitrophenyl butyrate.

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1. Introduction

The use of pesticides for pest control has become a common practice around the world. Organophosphates are widely used in agriculture. Azinphos-methyl is one of the insecticides most commonly applied in the fruit-horticultural activity in the Upper Valley of Río Negro and Río Neuquén in North Patagonia, Argentina. About 35,000 h cultivated in this region correspond to pome fruit production, mainly pears and apples. Pesticides are applied as sprays with ground-based equipment and they can easily reach surface drainage, water and soil (Loewy et al., 1999, 2011). Azinphos-methyl half-life in water is 26 days at 30 °C and pH 7 (US EPA, 2001). Through surface and subsurface water monitoring in this area, azinphos-methyl was the insecticide detected most frequently and in highest concentrations. During control period a maximum concentration of 0.25 $\mu g L^{-1}$ was detected while in the application period (November–March) a concentration of 22.48 $\mu g L^{-1}$ was found (Loewy et al., 2011). Even though these concentrations are below lethal concentrations for aquatic organisms, they exceed guide levels for protection of aquatic life ($\leq$0.02 $\mu g L^{-1}$; Subsecretaría de Recursos Hídricos de la Nación, 2003).

Azinphos-methyl belongs to the class of organophosphate insecticides (OP) which are recognized for their ability to inhibit acetylcholinesterase (AChE) by phosphorylation of a serine residue in the active site of the enzyme. AChE catalyses the hydrolysis of the neurotransmitter acetylcholine. In consequence, its inhibition results in an accumulation of acetylcholine within the synaptic space, leading to overstimulation of cholinergic receptors followed by depression or paralysis and eventual death.

Several works carried out in vertebrate and invertebrate species exposed to OPs have reported neurotoxicity (Bianco et al., 2013; Couillard and Burridge, 2015; Glynn, 2006; Jokanovic and Kosanovic, 2010), inmunotoxicity (Corsini et al., 2013; Galloway and Handy, 2003; Jin et al., 2015), embryotoxicity, teratogenicity (Shafiullah, 2013), genotoxicity, DNA damage (Shadnia et al., 2005; Sinha and Kumar, 2014), alterations in energy allocation (Couillard and Burridge, 2015) and in reproduction (Kristoff et al., 2011; Miranda-Contreras et al., 2013; Rivadeneira et al., 2013).

Cholinesterase activity (ChE) and, more recently, carboxylesterase activity (CE) are evaluated in environmental monitoring. Both enzymes belong to the group of B-esterases, which are defined as a serine family of esterases that are inhibited by OP insecticides. CEs comprise a multiple isozymes family with broad substrate specificity (Satho and Hosokawa, 1998) thus; CEs sensitivity to a given insecticide might vary according to the substrate used (Laguerre et al., 2009). For example, in aquatic gastropods total soft tissue CE activity measured with p-nitrophenyl acetate (p-NPA) and p-nitrophenyl butyrate (p-NPB) are more sensitive to OPs than CEs measured with other substrates such as 1 and 2 naphthyl acetate (Cacciatore et al., 2013; Kristoff et al., 2012). It has been postulated that CEs are involved in the metabolism and detoxification of many agrochemicals (Potter and Wadkins, 2006; Wheelock et al., 2005). CEs are assumed to act by two main mechanisms in the interaction with OPs: by binding stoichiometrically to the insecticide, which protects ChEs from OP toxicity by removing large amounts of insecticide, and by hydrolyzing carboxylester bonds present in some OPs (Jokanovic 2001; Kao et al., 1984; Sanchez-Hernandez, 2007).

ChE activity is commonly used as a sensitive biomarker of OP exposure (Timbrell, 2000) whereas, few ecotoxicological studies have been performed with CEs (González Vejares et al., 2010). It has been suggested that a combined activity measurement of both enzymes may provide a more detailed scenery of organism health and exposure to OP insecticides (Sanchez-Hernandez and Wheelock, 2009; Wheelock et al., 2008). Also, the search for more specific and sensitive biomarkers is important for ecotoxicological studies in order to understand and predict possible effects of insecticides on single organisms, population sustainability and ecosystem functioning (Jemec et al., 2010; Lam, 2009; van der Oost et al., 2003).

Recovery studies together with characterization of the enzyme and its sensitivity to pesticides can improve the toxicological evaluation. However, little is known about recovery patterns in organisms exposed to OPs. Enzyme's activity recovery depends on the species, the time of exposure, the kind and concentration of the insecticide, and the degree of enzyme inhibition reached (Fleming, 1981; Ferrari et al. 2004a,b; Jindal and Kaur, 2014; Kristoff et al., 2012; Oruc, 2012). Conducting recovery studies is valuable because they allow finding out whether the pesticide effects are permanent or not and also, to detect alterations on the organisms even when the pesticide is no longer present in the environment.

Chilina gibbosa Sowerby 1841 (Chilinidae, Pulmonata) is a freshwater gastropod widely distributed in South America. The Chilinidae is an endemic family, being especially abundant in freshwater habitats of Patagonia, Argentina and Southern Chile (Bosnia et al., 1990; Fuentealba et al., 2010; Gutiérrez-Gregoric et al., 2010; Rumi et al., 2008). Many species belonging to this family are considered vulnerable due to restricted geographic distribution and habitat deterioration related to the presence of toxic contaminants, increasing number of exotic species, climate change, among other factors (Valdovinos, 2006). *C. gibbosa* is a semelparous species with annual life cycle and reproductive period in summer, when population increases. This snail is a relevant item in the benthic fauna and an important food source for ducks and fishes, some of which are commercially valuable fish species (Bosnia et al., 1990; Ferriz, 1993). Adults are easy to collect and handle, they can usually be found in shallow waters. Their limited mobility and ability to excrete pollutants may result in several negative effects at low environmental concentrations of toxicants (Oehlmann and Schulte-Oehlmann, 2003), which might endanger the survival of the species in a contaminated or potentially contaminated site.

Toxicological studies performed with *C. gibbosa* in our laboratory have reported neurotoxicity signs (entire head-foot region out of the shell, lack of adherence and spontaneous movements) after exposure to 0.01 $\mu g L^{-1}$ and higher concentrations of azinphos-methyl for 48 h. No lethality was registered in these experiments even at the highest concentration used (5 mg/L). ChE and CE activities were characterized and sensitivity to azinphos-methyl was studied. A strong ChE inhibition was observed at environmental concentrations of azinphos-methyl; on the contrary CE activity was not inhibited at those concentrations but above 500 $\mu g L^{-1}$ (Bianco et al., 2013).

The aim of the present work was to evaluate the recovery capacity of ChEs and signs of intoxication (lack of adherence, weak adherence to vessel and abnormal head-foot region protrusion) in *C. gibbosa* following exposure to azinphos-methyl for 48 h. Also, lethality and CE activity were registered after exposure to the pesticide and during 21 days of the recovery period. A concentration of 20 $\mu g L^{-1}$ azinphos-methyl caused approximately 90% of inhibition of ChEs after acute exposure (Bianco et al., 2013). Also, it corresponds to a concentration found in freshwater (Loewy et al., 2011) so, in the present work, recovery at an environmental concentration, which produced high inhibition of ChEs, was evaluated.

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), azinphos-methyl PESTANAL® (97.2% PURE)

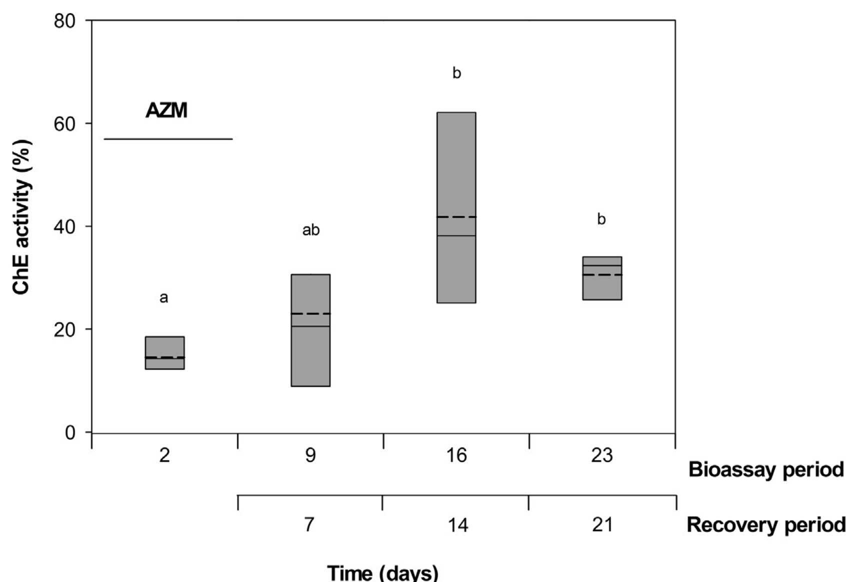


Fig. 1. Time course of inhibition and recovery of cholinesterase (ChE) activity in *Chilina gibbosa* after two days of exposure to 20 μg L^{-1} azinphos-methyl (AZM). ChE activity was expressed as% of control activity. Control values of ChEs correspond to 0.058 ± 0.005 μmoles min^{-1} mg $protein^{-1}$. Organisms were exposed for 48 h and then transferred to clean water for 21 days. Boundary of boxes closest to zero indicates 25th percentile, full line within boxes marks median while broken line represents mean, and boundary of boxes furthest from zero indicates 75th percentile. Statistical differences are indicated with different letters.

were purchased from Sigma–Aldrich. Other chemicals used were also of analytical reagent grade.

2.2. Organisms

Individuals of *C. gibbosa* were collected by hand at a depth of 5–70 cm during April and May 2014, from the Chimehuin River (39° 54′ 57.15′′ S 71° 06′ 23″ W) upstream of Junín de los Andes city, Neuquén province, Argentina and 20 km downstream from Huechulafquen Lake. This area can be considered free of agrochemical pollution given that there is no agricultural activity upstream of the sampling site (Bianco et al., 2013; Federal Law 22351, Argentina).

Individuals were transported to the Faculty of Exact and Natural Sciences, University of Buenos Aires and were acclimated in aerated glass aquaria (10 L) at 10 ± 2 °C, under a 12:12 h (L:D) artificial photoperiod regime for 20 days and fed with goldfish flakes (TetraFin). For the experiments adult snails of similar size were used.

2.3. Bioassays

Bioassays were performed in 600 mL glass vessels with 450 mL of the corresponding solution; each vessel housed between 4 and 6 snails randomLy selected. A total of 16 vessels were used, half of them with 0.05% acetone in dechlorinated tap water (solvent control) and the other half with 20 μg L^{-1} azinphos-methyl in dechlorinated tap water. Azinphos-methyl working solution was obtained by diluting the stock solution of the insecticide prepared in acetone with dechlorinated tap water. Azinphos-methyl concentration was measured by HPLC with UV detector (detection wavelength: 230 nm) (Bianco et al., 2013).

Bioassays were carried out at 10 ± 2 °C under a photoperiod of 12:12 h (L:D). Physico-chemical parameters were recorded: total hardness = 67 ± 3 mg $CaCO_3$ L^{-1}; alkalinity = 29 ± 2 mg $CaCO_3$ L^{-1}; pH 6.6 ± 0.2 and conductivity = 250 ± 17 μS cm^{-1}.

After 48 h of acute exposure, all the organisms were transferred to vessels containing dechlorinated tap water (pesticide-free water) which was renewed every week. Recovery was studied for 21 days after the acute exposure. Animals were fed once a week during the bioassay with goldfish flakes (TetraFin). At 48 h and at 7, 14 and 21 days after the exposure, visual observation of the organisms was performed according to Kristoff et al. (2006) and Bianco et al. (2013), then, soft parts of one snail per vessel were separated from the shell and homogenized (a total of eight control snails and eight treated snails per day). Activity of ChEs and CEs and protein content were also measured at 48 h and at 7, 14 and 21 days after exposure.

2.4. Homogenates

Snails were anesthetized on ice for 6–8 min. The shells were carefully removed and the soft tissue isolated at 0 °C. The body soft tissues were washed in distilled water, placed on filter paper to drain extra fluids, and weighed. One snail per homogenate was used. Tissues were homogenized in 10 V of 20 mM Tris/HCl buffer, pH 7.5, plus 0.5 mM EDTA. Homogenates were centrifuged at 11,000 × *g* for 20 min at 4 °C. The resulting supernatants were used as the enzyme source.

Protein content was determined according to the method of Lowry et al. (1951), using bovine serum albumin as standard.

2.5. Cholinesterase activity (ChE)

ChE activity was measured in the supernatant fraction (200 μL), in 100 mM phosphate buffer, pH 8, 0.2 mM DTNB and 1.5 mM AcSCh as substrate according to the method of Ellman et al. (1961) with the characterization previously performed for this species (Bianco et al., 2013). Absorbance was recorded continuously for 60 s at 412 nm. Rates were corrected for spontaneous hydrolysis of the substrate and non-specific reduction of the chromogen by tissue extracts. Specific activity was expressed as μmoles of substrate hydrolyzed per min per mg of protein.

2.6. Carboxylesterase activity (CE)

Hydrolysis of p-NPA and p-NPB was measured according to Kristoff et al. (2010) with the characterization previously performed for *C. gibbosa* (Bianco et al., 2013). Reactions were

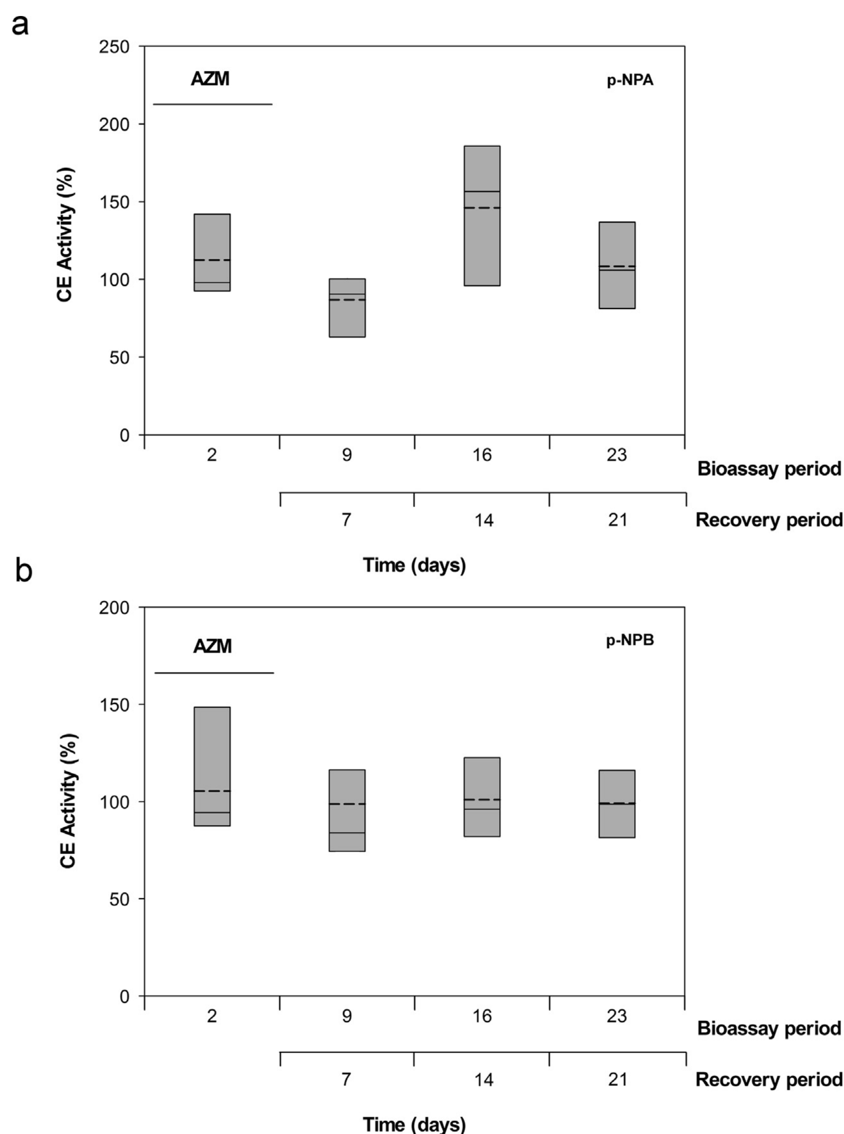


Fig. 2. Time course of carboxylesterase (CE) activity in *Chilina gibbosa* with respect to control after two days exposure to 20 μg L^{-1} azinphos-methyl (AZM), (a) using p-NPA and (b) p-NPB as substrates. Control values of CEs correspond to 0.056 ± 0.007 μmoles min^{-1} mg protein^{-1} with p-NPA and 0.086 ± 0.011 μmoles min^{-1} mg protein^{-1} with p-NPB. Organisms were exposed for 48 h and then transferred to clean water for 21 days. Activity was expressed as percentage of activity in control of each day. Boundary of boxes closest to zero indicates 25th percentile, full line within boxes marks median while broken line represents mean, and boundary of boxes furthest from zero indicates 75th percentile.

performed in 2.5 mL 100 mM phosphate buffer pH 8.0 containing 5% acetone, 1.5 mM p-NPA or p-NPB and 150 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}) and expressed as μmoles of substrate hydrolyzed per min per mg of protein.

2.7. Data analysis

The assumptions of normality and homogeneity of variances were tested by Kolmogorov–Smirnov and Levene's test, respectively.

Differences in ChE activity of solvent control snails during the bioassay (2, 7, 14, and 21 days determinations) were tested with a non-parametric test (Kruskal–Wallis) given that the assumptions required by the parametric test (normality and/or homogeneity of variances) were not reached. Differences in CE activity (with p-NPA and p-NPB) of solvent control snails during the bioassay were tested with one-way ANOVA.

Percentage of ChE and CE (with p-NPA and p-NPB) activities in treated snails with respect to the solvent controls were analyzed by the non-parametric Kruskal–Wallis test. Significant differences were analyzed by Dunn's Multiple Comparisons Test.

Neurotoxicity and lethality were tested through contingency tables (Fisher's Exact Test).

The level of significance used was 0.05. Statistical analyses were performed with Statistica 7.1 and GraphPad InStat 3.01.

3. Results

3.1. Esterase activities during exposure and recovery

To study ChE and CE activities, snails were exposed to azinphos-methyl for 48 h and then transferred to pesticide-free water for 21 days. In the present work only a solvent control was performed considering the results reported for this species by Bianco et al. (2013) who did not find differences between control and solvent control treatments.

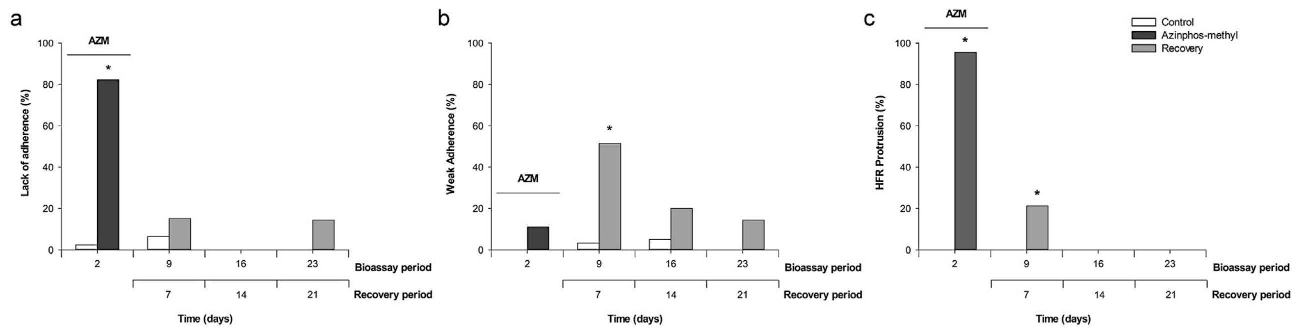
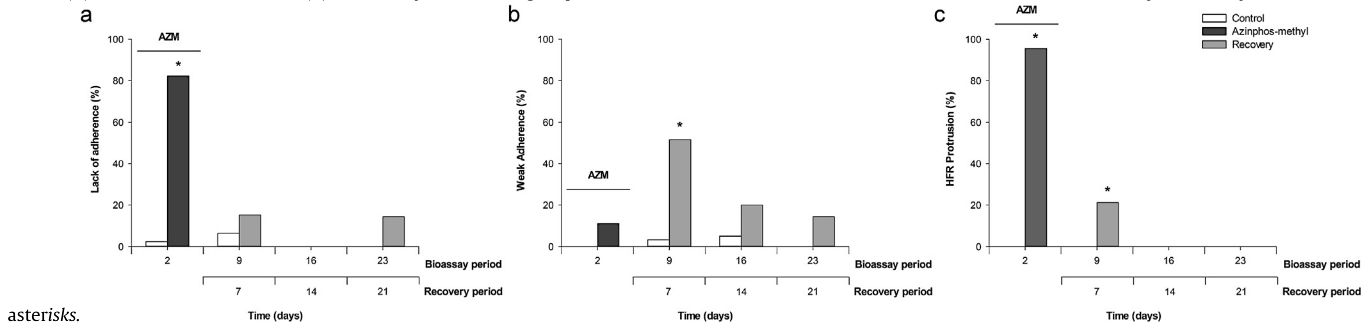


Fig. 3. Percentage of *Chilina gibbosa* showing neurotoxic effects after 48 h exposure to 20 μg L^{-1} azinphos-methyl (AZM) and during 21 recovery days, (a) Lack of adherence to vessel; (b) weak adherence to vessel; **(c)** abnormally head-foot region protrusion. Statistical differences between control and treated or recovery of each day are indicated with



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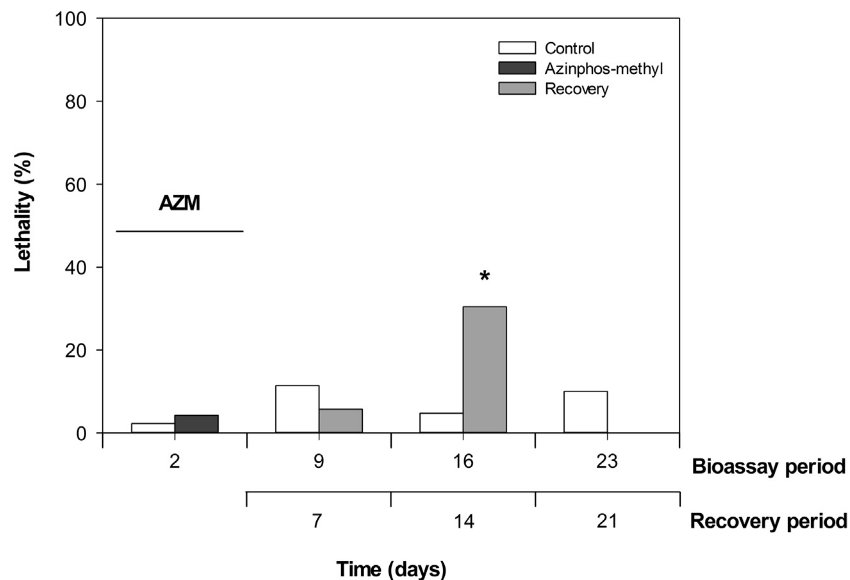

Fig. 4. Lethality of Chilina *gibbosa* after 48 h exposure to 20 μg L^{-1} azinphos-methyl (AZM) and during 21 recovery days. Statistical differences between control and treated or recovery of each day are indicated with asterisks.

ChE and CE activity of solvent controls remained constant during the bioassay period and were not statistically different between days (ChE: Kruskal–Wallis; $H = 0.12$; CE p-NPA: one-way ANOVA; $F = 2.12$; df = 3; CE p-NPB: one-way ANOVA; $F = 0.34$; df = 3; $P > 0.05$).

In treated snails, ChE and CE activities were expressed as percentages of the solvent control activity of the same day.

After 48 h of exposure to azinphos-methyl, ChEs were inhibited by 85 % with respect to control. During the following 21 days in clean water, even though the enzyme remained inhibited, there was a slight increase in the activity. At day 21ChE inhibition was approximately 70% with respect to the control (Kruskal-Wallis; $H = 13.28$; $P < 0.05$) (Fig. 1).

CE activity measured with p-NPA and p-NPB did not show any initial inhibition after 48 h exposure. During the following recovery days, activities remained almost constant and no statistically significant differences were observed with respect to the controls (CE p-NPA: Kruskal-Wallis; $H = 5.51$; CE p-NPB: Kruskal–Wallis; $H = 1.61$; $P > 0.05$) (Fig. 2a,b).

3.2. Neurotoxicity

Exposure to azinphos-methyl for 48 h induced several neurotoxicity signs in *C. gibbosa*: 11% of the snails showed weak adherence, 82% presented lack of adherence to vessels, and 96% exhibited an abnormally protruded head–foot region from the shell and absence of spontaneous movements, hardly reacting to mechanical stimuli; the latter two signs presented significant differences with respect to controls (Fisher's Exact Test; $P < 0.05$) (Fig. 3). When transferred

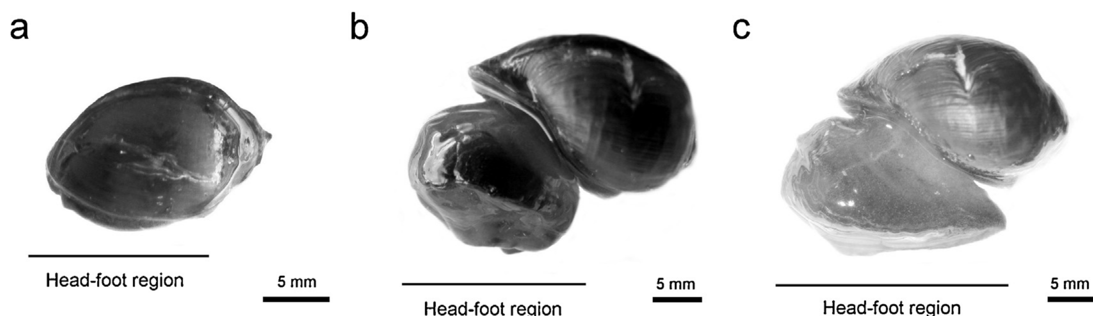

Fig. 5. Photographs of *Chilina gibbosa*, (a) control; (b), (c) exposed to 20 μg L^{-1} of azinphos-methyl for 48 h.

to clean water, at days 7 and 21 of recovery, only 15% of the snails did not adhere to the recipient (Fig. 3a). Individuals showing weak adherence, increased significantly to 53% on the 7th day of recovery (Fisher's Exact Test; $P < 0.05$), and then decreased to 20% and 14% at days 14 and 21, respectively (Fig. 3b). The high percentage of treated individuals showing protrusion of head–foot region after 48 h of exposure diminished significantly to 21% at day 7 of recovery in pesticide-free water (Fisher's Exact Test; $P < 0.05$). At days 14 and 21 none of the snails presented this neurotoxicity sign (Fig. 3c).

The external aspect of three *C. gibbosa* individuals is shown in Fig. 5. The organisms were transferred from the vessels to a Petri dish and photographed through a magnifying glass. As can be appreciated, the head–foot region on control snails (Fig. 5a) can barely be seen as it is usually fully or partially hidden inside the shell. These animals present normal locomotion, movements and adherence to recipients. Conversely, in treated organisms (Fig. 5b,c) the heat-foot region emerges completely from the shell which correlates with lack of adherence and mobility, and weak response to external stimuli.

3.3. Lethality

Organisms were considered dead when they failed to respond to mechanical stimuli or if they remained permanently retracted into the shell. After 48 h of exposure to azinphos-methyl, very low mortality was observed (4%). During the following days of recovery, on the 14th day, mortality increased significantly to 30% with respect to control (Fisher's Exact Test; $P > 0.05$) (Fig. 4).

4. Discussion

Increasingly, the extensive use of agrochemicals has led to the need to detect aquatic contamination and pollutant availability to the organisms. OP insecticides have a relatively low persistence in the environment. The irreversible binding of OPs to ChEs makes them an effective tool for detecting ChE inhibition in the tissues of sentinel species even when the OP insecticide is no longer detected in the water (Rivadeneira et al., 2013).

C. gibbosa ChE activity was strongly inhibited (85%) after 48 h of exposure to 20 μg L^{-1} of azinphos-methyl, whereas CE activity did not change significantly with p-NPB and with p-NPA as substrates. These results are in accordance with those of Bianco et al. (2013), where significant inhibition of ChEs was observed at low concentrations of this insecticide (38% inhibition with 0.005 μg L^{-1} of azinphos-methyl) and CE activity was inhibited only at high concentrations (above 1000 μg L^{-1}). Only few studies in aquatic invertebrates have reported ChEs to be more sensitive than CEs to OP exposure. For example, the freshwater oligochaete *Lumbriculus variegatus* (Kristoff, 2010) showed a significant ChE inhibition at 0.004 mg L^{-1} of azinphos-methyl and no inhibition of CEs at even higher concentrations of the pesticide (0.1 mg L^{-1}). On the other hand, in most aquatic invertebrates, CEs were more sensitive than ChEs to the insecticide (Barata et al., 2004; Basack et al., 1998; Bianco et al., 2014; Kristoff et al., 2012; Ochoa et al., 2013; Ozretic and Krajnovic-Ozretic, 1992). ChE activity has been frequently used as a specific and sensitive biomarker of OP exposure as it is the target enzyme of these pesticides. Nevertheless, it seems that OPs have higher affinity to CEs than to ChEs in most of the aquatic invertebrates studied so far, therefore the measurement of both enzymes activities would supply a more detailed information of organism health. Although the physiological role of CEs is still not well understood, several studies have postulated CEs to play a protective role against the toxicity of OPs (Maxwell 1992; Potter and Wadkins, 2006; Wheelock et al., 2005). In *C. gibbosa*, CEs were not found to be a sensitive biomarker of exposure to azinphos-methyl whereas ChEs turned out to be very sensitive. Also, *C. gibbosa* ChEs are highly sensitive to azinphos-methyl compared to ChEs of other studied invertebrate species: *Biomphalaria glabrata*, *Biomphalaria straminea*, *Hyalella curvispina*, *L. variegatus* and *Planorbarius corneus* (Anguiano et al., 2014; Bianco et al., 2013, 2014; Cacciatore et al., 2013; Kristoff et al., 2006). These species, in which CEs are more sensitive than ChEs to azinphos-methyl, show no signs of neurotoxicity. In such cases, CEs would contribute to the tolerance of the organisms to toxic effects from the OP. In *C. gibbosa* and *L. variegatus* CEs are less sensitive than ChEs, which could be associated to the appearance of neurotoxicity signs in both species.

CEs comprise a group of enzymes with low substrate specificity and their activity could be measured with many substrates. Several authors have used only one substrate for CE activity measurement. Basack et al. (1998) in *Corbicula fluminea*, Galloway et al. (2002) in *Mytilus edilus* and Vioque-Fernández et al. (2007) in *Procambarus clarkii* have used phenylthioacetate as substrate while Barata et al. (2004) and Anguiano et al. (2014) measured CE activity with 1 naphthyl acetate in *Daphnia magna* and *H. curvispina*, respectively. However, the recommended strategy is the use of more than one substrate in CE activity determination. For example, Bianco et al. (2013, 2014), Cacciatore et al. (2013), González Vejares et al. (2010), Kristoff et al. (2010, 2012), Laguerre et al. (2009), Malagnoux et al. (2014) and Rivadeneira et al. (2013), used 2 or more substrates as 1- and 2-naphtyl acetate, p-NPA, p-NPB, p-nitrophenylvalerate and phenylthioacetate. Sensitivity to OPs depends on the substrate used (Sanchez-Hernandez and Wheelock, 2009). Thus we cannot discard the possibility that in *C. gibbosa* the use of other substrates in CE determination could increase their sensitivity. However, the appearance of severe signs of neurotoxicity at low concentrations of azinphos-methyl suggests that CEs have little role over ChEs, probably due to their low affinity to the insecticide independent of the substrate used in their measurement. Nevertheless, other CE substrates should be used in future works in *C. gibbosa* in order to increase the knowledge about CEs.

The most severe signs of neurotoxicity were the lack of adherence to the recipient and the abnormal protrusion of the head-foot

region from the shell. Neurotoxicity was also reported for terrestrial invertebrates as the carabid beetle *Pterostichus cupreus* (Jensen et al., 1997) and the earthworm *Eisenia andrei* (Jordaan et al., 2012), which showed alterations in locomotion, correlated with ChE inhibition after exposure to the OP insecticides dimethoate and azinphos-methyl. Nevertheless, other species showed different patterns, *L. variegatus* exposed to azinphos-methyl presented neurotoxicity signs without ChE inhibition and at very low degrees of inhibition (Kristoff et al., 2006). The correlation between ChE inhibition and neurotoxicity is not clear yet and varies depending on the studied pesticide and species (Pope, 1999). Some species did not show signs of neurotoxicity even at high levels of ChE inhibition. For example, *B. glabrata* did not present intoxication signs despite reaching a ChE inhibition of 66 % (Kristoff et al., 2006). Ferrari et al. (2004b) did not observe neurotoxicity in the goldfish *Carassius auratus* at inhibitions of 90% of ChE activity.

Our results show that the initial ChE inhibition of about 90% was no recovered to control values after 21 days in pesticide-free water. In other organisms exposed to OP compounds a slow or no recovery has been observed depending on the initial inhibition (Ferrari et al., 2004a,b; Kavitha and Rao, 2008; Kristoff et al., 2006, 2012). Kristoff et al. (2006) described that ChE activity recovery in *L. variegatus*, with an initial inhibition of 50%, reached control values after 21 days but, when initial inhibition was 90%, ChE activities could not be recovered. Ferrari et al. (2004b) found that 35 days of depuration in pesticide-free water, with an azinphos-methyl induced inhibition between 77% and 90%, were needed to recover most of the activity in the goldfish *C. auratus*. Other factors are involved in the recovery capacity of organisms exposed to contaminants, such as the kind and concentration of the toxic compounds, species, exposure time, among others (Bretaud et al., 2002; Ferrari et al., 2004a,b; Rao 2006). The time required for recovery may be related with the rate of spontaneous reactivation and *the novo* synthesis of the enzyme (Ferrari et al., 2004a; Habig and Di Giulio 1991). In our study, besides being very sensitive, ChEs remained inhibited over an extended period of time, suggesting no spontaneous enzyme reactivation.

Regarding neurotoxicity, during the recovery period, individuals showing lack of adherence or abnormally protruded head-food diminished from 82 and 96%, respectively, to 15 and 21% after 7 days in pesticide-free water. At 21 days, 15% of the snails still presented lack of adherence by, 15% showed weak adherence to vessels and none of them had their head-foot region abnormally exposed. A considerable lethality was observed (30%) at day 14. It is probable that the individuals with severe neurotoxic signs at 48 h of exposure to azinphos-methyl (lack of adherence and head-foot region protrusion) remained intoxicated during recovery and consequently then died. When comparing ChE inhibition and neurotoxic effects at 48 h and during the recovery period we could not observe a clear correlation between the parameters; while ChE activity remained reduced, the severe intoxication signs could be reversed even though lethality was registered. During exposure, with an 85% inhibition of ChEs the snails showed important neurotoxic effects but, at 21 days, during recovery, with a 70% of ChE inhibition no severe neurotoxicity was observed. There are very few works that have studied the reversion of neurotoxic signs. Kristoff (2010) reported that *L. variegatus,* exposed for 48 h at the IC_{90} of azinphos-methyl, showed no recovery of ChE activity after 21 days in pesticide-free water, but a marked reduction of intoxication signs. Recovery from toxic effects not only depends on the interaction of the contaminant with its target enzyme, it is a much more complex process, where physiological resilience of the organism plays a very important role to overcome the induced stress. The study of biomarkers with slow recovery following exposure to a contaminant gives valuable information. It allows to detect effects of contaminants when they are no longer present in the environment, also it shows that toxic effects may last longer than the exposure time and may be underestimated in acute exposure experiments.

In some works a rebound effect has been observed in B-esterases during the recovery period after exposure to anticholinesterase insecticides. Kristoff et al. (2010) described a significant increase in *L. variegatus* ChE activity during the recovery process after 48 h exposure to 2 $mg\,L^{-1}$ of the carbamate carbaryl; while Otero (2013) observed an increment on ChE and CE activities in the aquatic gastropod *P. corneus* at 21 days in pesticide-free water after 48 h exposure to 1.5 $mg\,L^{-1}$ azinphos-methyl. Although in a previous work *C. gibbosa* CEs showed no sensitivity to acute exposure to 20 $\mu g\,L^{-1}$ azinphos-methyl (Bianco et al., 2013), we included these enzymes in the present study in order to detect possible induction effects during the recovery period. However, CEs measured with p-NPA and p-NPB remained insensitive to azinphos-methyl throughout the 23-day experiment period.

In our study mortality was registered during the recovery period. This suggests an important sensitivity of *C. gibbosa*, which has not been detected in the acute exposure assay (48 h). Organism survival varies depending on different factors. Concerning ChE activity, while some species can tolerate high levels of inhibition without mortality, others die under the same conditions (Kristoff et al., 2006; Pope, 1999; Rivadeneira et al., 2013).

C. gibbosa turned out to be a very sensitive organism to azinphos-methyl showing an ominous neurotoxicity and an important ChE inhibition after acute exposure. The neurotoxic effects are, in most cases, related with the mobility and behavior of the affected organisms, which could have an ecological relevance in a natural environment. A lack of mobility in *C. gibbosa* would probably have a negative impact on their ability to feed and reproduce, and to withstand the water current, which may endanger the survival of the species in polluted habitats. As *C. gibbosa* plays an important role in the structure and function of aquatic food webs its population decline would probably have a negative impact on the aquatic and non-aquatic communities of North Patagonia. Nevertheless, further laboratory and field research is needed to assess the possible effects of OP insecticides on natural populations of this species. In future works subchronic exposure to low concentrations of azinphos-methyl will be performed. Our results suggest that subchronic exposure could produce lethality risking survival of this species in agriculture areas. Additionally, the specificity of the neurotoxicity signs in *C. gibbosa* should be tested in future works.

5. Conclusions

This study provides valuable information about recovery capacity of B-esterases and neurotoxic signals recovery capacity in the non-target freshwater invertebrate *C. gibbosa* after exposure to an organophosphate pesticide. After a recovery period of 21 days, ChE activity could not reach control values but there was a significant reduction of neurotoxic signs. Our work shows that *C. gibbosa* ChEs were a very useful biomarker showing a strong sensitivity and a slow recovery capacity increasing the possibility to indirectly detect the presence of OP compounds for long periods. Neurotoxicity signs were also very sensitive and, after an acute exposure, they can be easily detected for about one week. *C. gibbosa* could provide relevant information about effects of pesticides used in Argentina.

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