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In vitro and *in vivo* studies of cholinesterases and carboxylesterases in *Planorbarius corneus* exposed to a phosphorodithioate insecticide: Finding the most sensitive combination of enzymes, substrates, tissues and recovery capacity

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ABSTRACT

Organophosphate insecticides (OPs) continue to be an important class of agrochemicals used in modern agriculture worldwide. Even though these pesticides persist in the environment for a relatively short time, they show a high acute toxicity that may represent a serious hazard for wildlife. Sub-lethal effects on non-target species are a focus in pest management programs and should be used as biomarkers. Cholinesterases (ChEs) are the most used biomarker of OP exposure in vertebrate and invertebrate species. However, the combined monitoring of ChE and carboxylesterase (CE) activities may provide a more useful indication of exposure and effect of the organisms. The objective of the present work was to find the most sensitive combination of enzyme, substrate, tissue and capacity to recovery of B-esterases in the freshwater gastropod *Planorbarius corneus* exposed to the OP azinphos-methyl. For this purpose, ChE and CE activities in different tissues of *P. corneus* (head-foot, pulmonary region, digestive gland, gonads and whole organism soft tissue) were studied. Measurements of ChE activity were performed using three substrates: acetylthiocholine, propionylthiocholine and butyrylthiocholine and CE activity using four different substrates: p-nitrophenyl acetate, p-nitrophenyl butyrate, 1-naphthyl acetate, and 2-naphthyl acetate in control and exposed organisms. Finally, the recovery rates of ChE and CE activities following 48 h exposure to azinphos-methyl were analyzed. Our results show a preference for acetylthiocholine as substrate, a high inhibition with eserine (a selective ChE inhibitor) and inhibition with excess of substrate in all the analyzed tissues. The highest ChE and CE activity was found in the pulmonary region and in the digestive gland, respectively. The highest CE V_{max} was obtained with 1 and 2-naphthyl acetate in all the tissues. CEs were more sensitive than ChE to azinphos-methyl exposure. The highest sensitivity was found using p-nitrophenyl acetate and butyrate as substrates. On the other hand, CEs of the digestive gland and the pulmonary region were more sensitive than CEs of the whole organism soft tissue. Regarding the recovery of enzyme activities after 48 h exposure, ChE and CEs with p-nitrophenyl butyrate reached control values after 14 days in the digestive gland and after 21 days in the pulmonary region. Our results show marked differences in *P. corneus* basal ChE and CE activities depending on substrates and the tissue. Also, both tissue-dependent and substrate-dependent variations in sensitivity to azinphos-methyl exposure and recovery were obtained. CEs measured with p-nitrophenyl butyrate in the pulmonary region were the best combination to be used as biomarker of exposure to azinphos-methyl due to their sensitivity and low recovery capacity. Environmental concentrations of azinphos-methyl inhibited CE activity so they could be used as effective biomarkers of aquatic contamination.

Abbreviations: AChE, acetylcholinesterase; AsCh, acetylthiocholine iodide; AZM, azinphos methyl; BuChE, butyrylcholinesterase; BsCh, butyrylthiocholine iodide; CE, carboxylesterase; ChE, cholinesterase; DG, digestive gland; DTNB, 5,5ᐟdithio-2-bis-nitrobenzoate; G, gonad; HF, head-foot; 1 and 2-NA, 1 and 2-naphthyl acetate; OP, organophosphate insecticide; p-NPA, p-nitrophenyl acetate; p-NPB, p-nitrophenyl butyrate; PsCh, propionylthiocholine iodide; PR, pulmonary region; WO, whole organism soft tissue.

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1. Introduction

Organophosphate insecticides (OPs) continue to be an important class of agrochemicals used in modern agriculture worldwide. Even though these pesticides persist in the environment for a relatively short time, they show a high acute toxicity that may represent a serious hazard for wildlife. Their main action mechanism is the acetylcholinesterase (AChE) inhibition. Since AChE hydrolyzes acetylcholine at cholinergic synapses and neuromuscular junctions, the inhibition of its activity causes neurotoxicity (Timbrell, 2000; Walker et al., 2001).

Sub-lethal effects on non-target species are a focus in pest management programs (Desneux et al., 2007) and should be used as biomarkers. Biomarkers are defined as a biochemical, cellular, physiological or behavioural change which can be measured in body tissues or fluids or at the level of the whole organism that reveals the exposure at/or the effects of one or more chemical pollutants (Depledge, 1994).

AChE is the most used biomarker of OP exposure and effect in vertebrate and invertebrate species (Fulton and Key, 2001; van der Oost et al., 2003). Inhibition of AChE was related with neurotoxic signals in several organisms (Bianco et al., 2013; Cossi et al., 2015; Ferrari et al., 2004a; Kristoff et al., 2006). Cholinesterases (ChEs) and carboxylesterases (CEs) belong to the group of hydrolases which Aldridge (1953) classified as B-type esterases, being inhibited by OPs. In the case of CEs, these enzymes catalyze the hydrolysis of a wide range of exogenous and endogenous carboxylesters. CEs are assumed to play a protective role by removing a significant amount of the OP prior to reaching AChE and hydrolyzing carboxylesters presented in some pesticides (Sanchez-Hernandez, 2007). Several authors have suggested that the combined monitoring of CE and ChE activities may provide a more useful indication of exposure and effect of aquatic invertebrates to OP pesticides than the measurement of ChE inhibition alone (Cossi et al., 2015; Sanchez-Hernandez and Wheelock, 2009; Wheelock et al., 2005). Some studies have reported the patterns of inhibition and recovery of ChEs and/or CEs in different organisms (Cossi et al., 2015; Kristoff et al., 2006; Ferrari et al., 2004a,; Rivadeneira et al., 2015), which, however, are still unknown for most species (Barata et al., 2004).

In using B-esterases as biomarkers an important issue is to characterize ChEs and CEs that are present in the different tissues of the species postulated as sentinels. Not only basal ChE and CE activities but also sensitivity to pesticides and recovery might vary with the tissue and the substrate used in their measurements (Bianco et al., 2013; Kristoff et al., 2012; Laguerre et al., 2009; Malagnoux et al., 2014; Sanchez-Hernandez and Wheelock, 2009).

Azinphos-methyl is an OP insecticide-acaricide containing a phosphorotioate group (P=S). Azinphos-methyl has to undergo metabolic activation to its corresponding oxygen analog (oxon) to inhibit AChE. Its half-life in water is 26 days at 30 °C and pH 7, and its water solubility is 28 mg L^{-1} at 20 °C (US EPA, 2001). Azinphos-methyl is one of the most used OP in the fruit-horticultural activity of the North Patagonia Argentina and it has been reported as the most frequently detected OP in groundwater and surface waters of the region (Loewy et al., 2003, 2011). Around the world, concentrations up to 0.42 mg L^{-1} have been found in shallow waters adjacent to agricultural fields (Granovsky et al., 1996; Klosterhaus et al., 2003; Loewy et al., 2011; Schulz, 2004; Wan et al., 1995).

A considerable decrease in the diversity of mollusks as a consequence of anthropogenic activities has been recently noticed (Fuentealba et al., 2010; Strong et al., 2008). The gastropod *Planorbarius corneus* is a hermaphroditic snail that usually inhabits ponds and streams. This species belongs to the Planorbidae family which is distributed all over the world (Jopp, 2006). Jopp (2006) and Wiese (2005) reported that *P. corneus* population declines constantly. Since aquatic contamination would be one of the causes of the declination, studies of exposure to several toxicants are necessary to improve the knowledge of this species. Different responses to xenobiotics such as 17 β-Oestradiol, chlorpyrifos, endosulfan, pentachlorophenol and treated sewage effluents were reported in *P. corneus* (Benstead et al., 2011; Clarke et al., 2009; Pavlica et al., 2000; Otludil et al., 2004; Rivadeneira et al., 2013, 2015). Since gastropods are an important item in aquatic food chains their population declines would imply a possible damage at higher trophic levels.

In previous works, a partial characterization of ChEs and CEs present in whole organism soft tissue and haemolinph and their responses to azinphos-methyl were performed. Also, toxic effects on growth and survival of the offspring were reported (Agrelo, 2012; Agrelo and Kristoff, 2012; Cacciatore et al., 2012, 2013).

The objective of the present work was to find the most sensitive combination of enzyme, substrate, tissue and capacity of B-esterases to recover in *P. corneus* exposed to azinphos-methyl. For this purpose, ChE and CE activities in different tissues of *P. corneus* (head-foot, pulmonary region, digestive gland, gonads and whole organism soft tissue) were studied. Measurements of ChE activity were performed using three substrates: acetylthiocholine (AsCh), propionylthiocholine (PsCh) and butyrylthiocholine (BsCh) and CEs using four different substrates: p-nitrophenyl acetate (p-NPA), p-nitrophenyl butyrate (p-NPB(*p*-NPB), 1-naphthyl acetate (1-NA), and 2-naphthyl acetate (2-NA) in control and exposed organisms. Finally, the recovery rates of ChE and CE following 48 h exposure to azinphos-methyl were analyzed.

2. Materials and methods

2.1. Chemicals

Acetylthiocholine iodide (AsCh), butyrylthiocholine iodide (BsCh), propionylthiocholine iodide (PsCh), p-nitrophenyl acetate (p-NPA), p-nitrophenyl butyrate (p-NPB), 1- and 2-naphthyl acetate (1- and 2-NA), 5,5′-dithio-2-bis-nitrobenzoate (DTNB), physostigmine (eserine) and Azinphos-methyl PESTANAL® [S(3,4-dihydro-4-oxobenzo-[1,2,3-d]triazin-3-ylmethyl) O-dimethyl phosphorodithioate] were purchased from Sigma-Aldrich. All chemicals used were of analytical reagent grade.

2.2. Organisms selected

Snails (*Planorbarius corneus*) were cultured in the laboratory in aerated glass aquarias (20 L), at a temperature of 22 ± 2 °C, and under a 14–10 h artificial light-dark photoperiod regime. Water quality characteristics were as follows: total hardness 67 ± 3 mg $CaCO_3$ L^{-1}; alkalinity 29 ± 2 mg $CaCO_3$ L^{-1}; pH 6.9 ± 0.2 and conductivity 250 ± 17 μS cm^{-1}. Animals were fed lettuce leaves *ad libitum* (Fried et al., 1992). For all the experiments, adult snails of similar size (18 ± 2 mm) were used.

2.3. Bioassays

Bioassays were carried out in 3 L containers with 1 L of each test solution. Each vessel contained 9 snails randomly selected. Six vessels were used for each treatment condition (controls and different concentration of the pesticide). During the bioassays animals were not fed. All the tests were performed at a temperature of 22 ± 2 °C and under a photoperiod of 14:10 h light/dark without aeration. No mortality was observed in either control or exposed organisms. Azinphos-methyl was dissolved in acetone and its concentrations were measured as described Bianco et al. (2013). Snails were exposed to 7 pesticide concentrations (0.005; 0.025; 0.05; 0.1; 0.5; 1.5; 3 mg L^{-1}) prepared by dissolving the original solution of pesticide in dechlorinated water. Solvent (0.05% of acetone) and

water control were included. The measured pesticide concentrations were within 97–102% of the nominal values. The constancy of azinphos-methyl concentrations during the bioassay (48 h) was probed in stability studies performed previously by Cacciatore (2009). ChE and CE activities and protein content were recorded at 48 h of exposure.

In the case of exposure-recovery studies, the organisms were exposed to 1.5 mg L^{-1} azinphos-methyl for 48 h (six containers) and after 48 h, pesticide and acetone solutions were replaced by dechlorinated water. ChE and CE activities and protein content were recorded after 7, 14 and 21 days of the recovery period (in water free of pesticide).

2.4. Enzymatic determinations

Animals were anaesthetized with ice for 8 min. The shells were carefully removed and head-foots (HF), pulmonary regions (PR), digestive glands (DG) and gonads (G) or whole organism soft tissue (WO) were dissected on ice, washed in distilled water, placed on filter paper to drain extra fluids, and weighed. Homogenates (1:10 w:v) were made in 20 mM Tris/HCl buffer, pH 7.5, plus 0.5 mM EDTA, and centrifuged at 11,000 x *g* for 20 min at 4 °C. The supernatants were immediately used as enzyme source. Each tissue homogenate consisted of a pool of 3–4 snails except for WO homogenates which had tissue from one snail and G which, due to their small size, had tissue from 7 to 8 snails.

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

2.4.1. General ChE activity

ChE activity was measured in 100 mM phosphate buffer, pH 8.0, 0.2 mM DTNB, and 0.75 mM AsCh, PsCh or BsCh as substrate according to the method of Ellman et al. (1961). Activity was recorded continuously at 412 nm and specific activity was expressed as μmol min^{-1} mg $protein^{-1}$. The enzymatic activity was corrected for spontaneous hydrolysis of substrate and non-specific reduction of the chromogen by tissue extracts.

2.4.2. General CE activity

CE activity was determined using four different substrates: p-NPA, p-NPB, 1-NA, and 2-NA.

Hydrolysis of p-NPA and p-NPB by CEs was determined according to Kristoff et al. (2010). Reactions were performed in 2.5 mL 100 mM phosphate buffer pH 8.0 containing 5% acetone and 1 mM p-NPA or p-NPB. Activity was continuously recorded at 400 nm. Specific activity was calculated using the molar extinction coefficient for p-nitrophenol (18.6 mM^{-1} cm^{-1}).

CE activity using 1-NA or 2-NA as substrate was determined according to van Asperen (1962) with modifications (Kristoff et al., 2012). Supernatants were diluted 1:8 (WO, HF, PR and G) or 1:27 (DG) in 20 mM Tris/HCl buffer, pH 7.5, containing 0.5 mM EDTA. Reactions were performed in 2.5 mL 40 mM phosphate buffer, pH 7.0, containing 5% acetone, 1 mM 1-NA or 2-NA, and sample. Sample volumes of 20–40 μL and 80–160 μL were chosen for the determinations with 1-NA and 2-NA, respectively. After 15 min incubation at 25 °C, reaction was stopped by the addition of 500 μL of freshly prepared SDS-Fast Blue solution (2 parts of 1% Fast Blue RR salt in acetone and 5 parts of a 5% solution of SDS in 50% acetone). The solutions were allowed to stand at room temperature for 15 min, and the absorbance of the naphthol-Fast Blue RR complex was read at 600 nm (1-NA) or 550 nm (2-NA). Calibration curves for 1- and 2-naphthol were constructed and specific activity was calculated using 13.6 and 11.8 mM^{-1} cm^{-1} as molar extinction coefficients, respectively.

2.5. Kinetic constants

Rates of hydrolysis of seven AcSCh and BuSCh concentrations (0.075–3 mM), nine PrSCh concentrations (0.019–3 mM), nine p-NPA concentrations (0.02–1 mM), eight *p*-NPB concentrations (0.2–1 mM), and seven 1- and 2-NA concentrations (0.01–1 mM) were used to calculate K_M and V_{max}. The values of K_M and V_{max} were estimated from hyperbolic regression analysis ± 95% confidence intervals, using Hyper (Hyperbolic Regression Analysis of Enzyme Kinetic Data 1.1w, 1992–2003, J.S. Easterby).

2.6. In vitro assays with eserine and excess of substrates

To assay the *in vitro* effects of eserine (a selective ChE inhibitor) on ChE activity, the enzyme extracts (40 μL) of each tissue were incubated with four concentrations of eserine (0.001; 0.01; 0.1; 1 mM) for 20 min at 25 °C before substrate addition. Six different replicates were performed. Controls with ethanol (solvent) and water were also included.

To assay the *in vitro* effects of excess of each substrate on ChEs, the activity was measured with AsCh (30 mM), PsCh (30 mM) and BsCh (30 mM). Six different replicates were performed.

2.7. No observed effect concentration (NOEC)

NOEC was determined as the highest azinphos-methyl concentration that did not produce ChE and CE inhibition (activity not statistically different from the control group; one-way ANOVA, $p > 0.05$) (Kristoff et al., 2006).

2.8. Half maximal inhibitory concentration (IC_{50})

The azinphos-methyl concentration needed to inhibit 50% of ChE and CE activity was determined using non linear regression (Bianco et al., 2013; Cacciatore et al., 2013).

2.9. Data analysis

Results were expressed as mean ± S.D. Data were analyzed by one-way ANOVA followed by Tukey HSD post-test using VassarStats (http://faculty.vassar.edu/lowry/VassarStats.html). Prior to any analysis, data were tested for normality (Shapiro-Wilk test) and variance homogeneity (Bartlett's test). The level of significance used was 0.05.

3. Results

3.1. Characterization of ChE

Measurements of ChE activity from the WO, HF, PR, DG, G were performed using three substrates: AsCh, PsCh, and BsCh. Table 1 shows the results of the estimations of apparent K_M and V_{max} values and Fig. 1 shows ChE activity towards a fixed concentration (0.75 mM) of the three substrates. Regardless of the tissue analyzed, the highest ChE activity was obtained when using AsCh as substrate, followed by PsCh, except in G where activity was higher with BsCh as substrate than with than PsCh. The highest K_M values where obtained with AsCh. K_M values with BsCh were higher than the ones obtained with PsCh. The highest ChE activity was obtained in PR, regardless of the substrate. In the case of AsCh hydrolysis, the activity measured in the PR represented 236% of the activity measured in WO (0.351 ± 0.050 μmol min^{-1} $protein^{-1}$). Furthermore, the activities measured in HF and G were similar to the one measured in WO (116% and 112% of the value in WO, respectively), while the activity measured in the DG represented only the 43% of WO activity. ChE inhibition by excess substrate was also investigated

Table 1
Kinetic parameters for hydrolysis of different cholinesterases (ChEs) substrates.

Tissue		Substrate		
		AsCh	PsCh	BsCh
Head-foot	V_{max}	0.407 ± 0.077	0.181 ± 0.029	0.105 ± 0.022
	K_M	0.262 ± 0.050	0.094 ± 0.009	0.126 ± 0.060
	Inhibition with 30 mM of substrate (%)	25 ± 2[a]	40 ± 8[a]	no inhibition
Pulmonary Region	V_{max}	0.827 ± 0.153	0.474 ± 0.107	0.274 ± 0.059
	K_M	0.405 ± 0.097	0.058 ± 0.086	0.135 ± 0.074
	Inhibition with 30 mM of substrate (%)	27 ± 4[a]	36 ± 5[a]	no inhibition
Digestive Gland	V_{max}	0.150 ± 0.049	0.082 ± 0.018	0.050 ± 0.016
	K_M	0.604 ± 0.086	0.052 ± 0.010	0.256 ± 0.113
	Inhibition with 30 mM of substrate (%)	29 ± 4[a]	65 ± 10[a]	no inhibition
Gonads	V_{max}	0.421 ± 0.064	0.112 ± 0.011	0.142 ± 0.014
	K_M	0.496 ± 0.068	0.041 ± 0.010	0.236 ± 0.097
	Inhibition with 30 mM of substrate (%)	74 ± 9[a]	72 ± 10[a]	no inhibition

Cholinesterase activity was assayed using acetylthiocholine (AsCh), propionylthiocholine (PsCh) or butirylthiocholine (BsCh) as substrate.
V_{max} and K_M values are expressed as μmol min^{-1} mg $protein^{-1}$ and mM, respectively.
[a] Indicates statiscally differences respect to the supernatant without the excess of the substrate (100% of the acitivity) ($p < 0.05$).

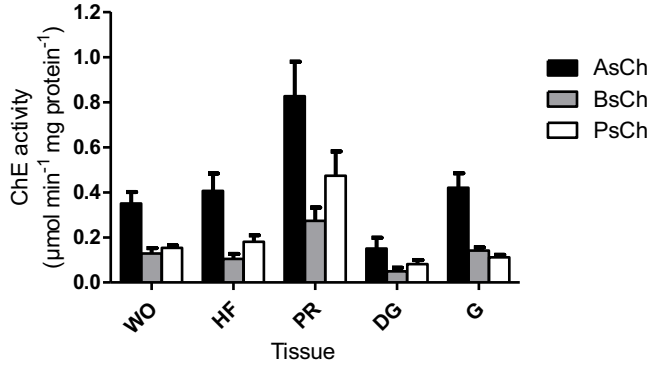

Fig. 1. Cholinesterase (ChE) activity with the substrates acetylthiocholine iodide (AsCh), propionylthiocholine iodide (PsCh), and butyrylthiocholine iodide (BsCh). Each bar represents the mean ± S.D. of 8 homogenates. WO: total soft tissue, HF: head-foot, PR: pulmonary region, DG: digestive gland, G: gonads.

for the four tissues with the three substrates. While no inhibition was observed when testing BsCh hydrolisis, both AsCh and PsCh hydrolisis were inhibited, in all the tissues tested, by the addition of 30 mM of each substrate (Table 1). G presented the highest inhibition of ChE activity (approximately 75% with both AsCh and PsCh).

The effects of eserine (specific inhibitor of ChEs) in head-foot, pulmonary region, digestive gland and gonads were also studyed. Eserine concentrations from 0.01 to 1 mM reduced ChE activity to less than 10% independently from the tissue analyzed (Table 3). This response indicates that specific ChEs were measured in all tissues.

3.2. Characterization of CEs

Regarding to CE activity, regardless of the substrate used for the assay (p-NPA, p-NPB, 1-NA or 1-NB), the highest specific activity was registered in DG followed by G, and last HF and PR (Fig. 2A and B). DG also had the highest K_M and V_{max} values regardless of the susbtrate analyzed (Table 2). V_{max} values measured with 1-NA or 2-NA as substrate were, in general, higher than the ones measured with p-NPA or p-NPB. However, the highest K_M values where obtained using p-NPA in all tissues. CEs measured in DG represented about 120% of WO activity using p-NPA or as substrate (Fig. 2A), and 350% using 1-NA or 2-NA (Fig. 2B).

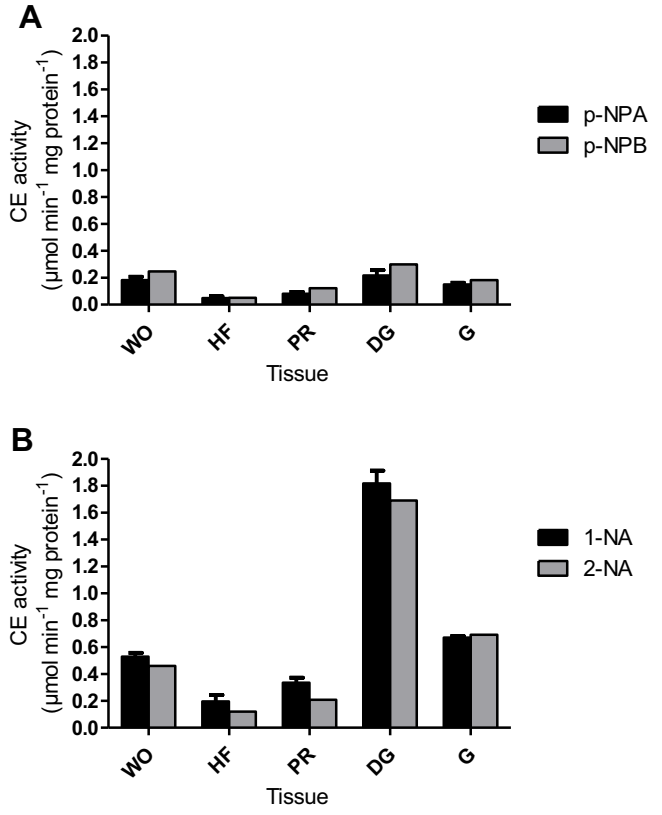

Fig. 2. Carboxylesterase (CES) activity with A: the substrate p-nitrophenyl acetate (p-NPA) and p-nitrophenyl butyrate (p-NPB), B: the substrates 1- and 2-naphthyl acetate (1- and 2-NA). Each bar represents the mean ± S.D. of 8 homogenates. WO: total soft tissue, HF: head-foot, PR: pulmonary region, DG: digestive gland, G: gonads.

3.3. Azinphos-methyl exposure

The inhibition of ChE (Fig. 3) and CEs (Figs. 4 and 5) was determined in WO, PR and DG from snails exposed for 48 h to azinphos-methyl concentrations in the range of 0.5–3 mg L^{-1}. Enzymatic activity was not determined in HF and G regions due to their small size and, in the case of HF, because it's hard to break the tissue.

ChE activity was determined using AsCh, as was recommended by Cacciatore et al. (2013), since it was the preferred substrate in all tissues. A significant difference compared to the control group was

Table 2
Kinetic parameters for hydrolysis of different carboxylesterases (CEs) substrates.

Tissue		Substrate			
		p-NPA	p-NPB	1-NA	2-NA
Head-foot	V_{max}	0.049 ± 0.014	0.050 ± 0.150	0.196 ± 0.048	0.121 ± 0.017
	K_M	0.215 ± 0.050	0.094 ± 0.009	0.050 ± 0.005	0.089 ± 0.010
Pulmonary Region	V_{max}	0.081 ± 0.013	0.123 ± 0.016	0.336 ± 0.037	0.209 ± 0.003
	K_M	0.197 ± 0.050	0.058 ± 0.086	0.060 ± 0.009	0.072 ± 0.009
Digestive Gland	V_{max}	0.217 ± 0.040	0.299 ± 0.058	1.817 ± 0.096	1.690 ± 0.259
	K_M	0.284 ± 0.068	0.052 ± 0.010	0.052 ± 0.003	0.076 ± 0.008
Gonads	V_{max}	0.150 ± 0.013	0.182 ± 0.022	0.670 ± 0.012	0.693 ± 0.020
	K_M	0.075 ± 0.008	0.041 ± 0.010	0.059 ± 0.006	0.078 ± 0.008

Carboxylesterase activity was measured using 1- and 2-naphthyl acetate (1- and 2-NA), p-nitrophenyl acetate (p-NPA), and p-nitrophenyl butyrate (p-NPB).
K_M and V_{max} values are expressed as mM and μmol min^{-1} mg $protein^{-1}$, respectively.

Table 3
ChE activity after the *in vitro* incubation with eserine.

	ChE activity (% respect to control activity)		
Eserine	0.01 mM	0.1 mM	1 mM
Total soft tissue	4.0 ± 1.0[a]	2.0 ± 0.6[a]	1.0 ± 0.5[a]
Head-foot	7.0 ± 1.0[a]	5.5 ± 1.1[a]	2.0 ± 0.5[a]
Pulmonary region	7.5 ± 1.5[a]	5.0 ± 0.5[a]	1.0 ± 0.5[a]
Digestive gland	6.0 ± 1.0[a]	6.5 ± 1.2[a]	4.0 ± 1.0[a]
Gonads	6.5 ± 1.1[a]	6.0 ± 0.5[a]	5.0 ± 0.5[a]

Cholinesterase activity was assayed using acetylthiocholine (AsCh). Each value represents the mean ± S.D. of four determinations.
[a] Are stastically different ($p < 0.05$) to control activity (100%).

found in the exposed organism, regardless of the tissue analyzed, after the exposure to 1.5 mg L^{-1} azinphos-methyl. ChE activity was inhibited by 36, 44 and 49% in WO, PR and DR, respectively (Fig. 3A, B and C). WO, unlike PR and DR, showed a greater inhibition (aproximately 73%) after the exposure to 3 mg L^{-1} of azinphos-methyl.

Since basal activity and sensitivity to pesticide exposure of CE depends on the substrate, CE was determined using p-NPA, p-NPB, 1-NA and 2-NA. When using p-NPA as substrate the activity determined in PR (Fig. 4B) showed significant difference as compared to the control group after the exposure to 0.005 mg L^{-1} of azinphos-methyl, while in WO and DG (Fig. 4A and C respectively) inhibition was observed after the exposure to 0.025 mg L^{-1} of the pesticide. The maximum inhibition (approximately 80%) was reached after the exposure to 0.1 mg L^{-1} in WO and PR, and 0.5 mg L^{-1} in DG. When CE activity was determined using p-NPB as substrate, significant inhibition was observed after exposure to 0.025 mg L^{-1} of azinphos-methyl in WO and DG, and 0.005 mg L^{-1} in PR. The maximum inhibition (approximately 90%) was obtained with 0.05 mg L^{-1} of the pesticide in WO and with 0.1 mg L^{-1} in PR and DG. CE activity measured with 1 and 2-NA (Fig. 5) was less sensitive than the activity measured using p-NPA and p-NPB. The hydrolysis of 1-NA was significantly inhibited in WO and DG (Fig. 5A and B respectively) after the exposure to 0.025 mg L^{-1} while PR (Fig. 5B) was less sensitive. The maximum inhibition (approximately 70%) was found after the exposure to 0.5 mg L^{-1} of azinphos-methyl. Using 2-NA, inhibition was observed in WO after the exposure to 0.05 mg L^{-1} and with 0.1 mg L^{-1} of azinphos-methyl in PR and DG. The maximum CE inhibition, approximately 70%, was found after the exposure to 1.5 mg L^{-1} of azinphos-methyl in WO, PR and DG (Table 3).

3.4. NOEC and IC_{50}

CE activity proved to be more sensitive to azinphos-methyl exposure than ChE activity since the NOEC values obtained for CEs are lower than the ones obtained for ChEs (Table 4). Moreover, CE activity using p-NPA and p-NPB was more sensitive than with 1 and 2-NA as substrates. The highest sensitivity to azinphos-methyl was found in PR and DG using p-NPA or p-NPB as substrates. Also, CE activity showed lower IC_{50} values than ChEs (Table 4). Only using p-NPB as substrate difference between the tissues analized were found.

3.5. Exposure-recovery studies

To analyze recovery of enzyme activity, snails were exposed to 1.5 mg L^{-1} of azinphos-methyl for 48 h and then transferred to pesticide-free dechlorinated water. ChE and CE activities were determined after 7, 14 and 21 days in pesticide-free water.

After exposing the snails for 48 hs to 1.5 mg L^{-1} of azinphos-methyl, ChE activity was inhibited 40% in WO, PR and DG (Fig. 6A). Activity recovery was found in DG after 14 days in clean water and after 21 days in clean water in WO and PR.

CE activity was determined using p-NPA and p-NPB since with these substrates the highest sensitivity to azinphos-methyl was observed. After 48 h azinphos-methyl exposure, CE activity measured with p-NPA was inhibited by 80% in WO, PR and DG (Fig. 6B). After 14 days in clean water CE activities recovered to control values. When using p-NPB as substrate, CE activity after 48 hs of exposure was inhibited by 90% in WO, PR and DG (Fig. 6C). Complete recovery of CE activity was reached after 14 days in DG, and after 21 days in WO and PR.

4. Discussion

ChE activity has been frequently used as a specific and sensitive biomarker of OP exposure as it is the target enzyme of this kind of pesticides. Nevertheless, it seems that OPs have higher affinity to CEs than to ChEs in most invertebrates. Therefore, the measurement of both enzymes would supply more information of aquatic contamination (Cossi et al., 2015; Sanchez-Hernandez and Wheelock, 2009).

Substrate preference and tissue distribution of ChEs and CEs should be studied before they can be used as biomarkers in ecotoxicology evaluations (Kristoff et al., 2012). This is important since not only basal activities but also sensitivity to pesticides might vary with the substrate and the tissue used in enzyme activity determinations (Laguerre et al., 2009; Sanchez-Hernandez and Wheelock, 2009). A previous characterization of ChE in the whole organism soft tissue of *P. corneus* indicates the presence of a typical AChE activity (Cacciatore et al., 2012). In accordance, our results show a preference for AsCh as a substrate, a high inhibition with eserine (a selective ChE inhibitor) and inhibition with excess of substrate in all the analyzed tissues using AsCh and PsCh (30 Mm) but not using BsCh as a substrate. Kinetics parameters of head-foot ChEs were similar with those recorded for the whole organism soft tis-

Table 4
IC_{50} and NOEC for ChEs and CEs in different anatomic regions of *Planorbarius corneus*.

Tissue	ChE		CE							
	AsCh		p-NFA		p-NFB		1-NA		2-NA	
	CI_{50}	NOEC	CI_{50}	NOEC	CI_{50}	NOEC	CI_{50}	NOEC	CI_{50}	NOEC
WO	1.8 ± 0.3	0.500	0.07 ± 0.04	0.005	0.027 ± 0.007	0.005	0.20 ± 0.10	< 0.025	0.15 ± 0.07	< 0.025
PR	2.2 ± 0.2	0.500	0.04 ± 0.03	0.001	0.02 ± 0.001	0.001	0.20 ± 0.10	0.025	0.60 ± 0.30	0.025
DG	1.9 ± 0.2	0.500	0.04 ± 0.02	0.001	0.01 ± 0.005	0.001	0.30 ± 0.10	0.025	0.30 ± 0.10	0.025

IC_{50} (Half Inhibitory Concentration) and NOEC (No Observed Effect Concentration) determined for cholinesterases and carboxylesterases. WO: total soft tissue, PR: pulmonary region, DG: digestive gland.

sue (Cacciatore et al., 2012). The highest ChE activity was found in the pulmonary region of *P. corneus*, whereas the activity in the digestive gland was very low. In the freshwater gastropod *Biomphalaria glabrata* similar results were observed indicating that the pulmonary region and head-foot have the highest activities of ChE, whereas it was very low in the digestive gland (Kristoff et al., 2012). In general, ChE activity in bivalves was found to be lower in digestive glands than in other tissues (Bonacci et al., 2009; Brown et al., 2004; Mora et al., 1999; Vioque-Fernández et al., 2007). The highest CE activity in *P. corneus* was found in the digestive gland and the lowest activity in heat-foot. These results are expected considering the involvement of these enzymes in the detoxification of drugs. Regardless of the substrate used, the highest V_{max} was obtained with 1 and 2-NA in all the tissues and in the whole organism soft tissue as has been previously reported (Cacciatore et al., 2012). In other snails, different results were observed. For example, in *B. glabrata*, Kristoff et al. (2012) reported that the highest activity was observed in the digestive gland with p-NPA, 1 and 2-NA as substrates and using p-NPB in the pulmonary region.

Azinphos-methyl effects on B-esterases of several organisms such as in the vertebrates *Carassius auratus, Oncorhynchus mykiss*, *Rhinella arenarum* (Ferrari et al., 2004a,b, 2011), *Sparus aurata* (Arufe et al., 2007) and in the invertebrates *B. glabrata* (Kristoff et al., 2006, 2012), *Biomphalaria straminea* (Bianco et al., 2014), *Chilina gibbosa* (Bianco et al., 2013), *Hyalella curvispina* (Anguiano et al., 2014) and *Lumbriculus variegatus* (Kristoff et al., 2006). CEs were more sensitive to the pesticide than ChEs in most of them. Nevertheless, in *C. gibbosa* and *L. variegatus* ChE was strongly inhibited at low concentrations of azinphos-methyl, CEs were less sensitive and severe neurotoxic signals were observed (Bianco et al., 2013; Kristoff et al., 2006). The irreversible inhibition of CEs by OPs protects ChE from being inhibited, decreasing the impact on nervous system, so generally neurotoxicity appears when ChEs are more sensitive than CEs (Bianco et al., 2013).

In *P. corneus*, CE activity was more sensitive to the pesticide than ChEs in the pulmonary region and digestive gland, but in the whole organism soft tissue and the haemolymph similar sensitivity was observed (Cacciatore et al., 2013). In *P. corneus* exposed to azinphos-methyl no neurotoxicity signals were observed.

In all tissues of *P. corneus*, the degree of CE inhibition by azinphos-methyl depends on the substrate used to measure the enzymatic activity. CE activity was more sensitive using *p*-NPA and *p*-NPB than with 1 and 2-NA in all the tissues, in accordance with the results obtained in *B. glabrata* (Kristoff et al., 2012). Other authors have reported variability in CE sensitivity to OP based on the substrate used. González Vejares et al. (2010) reported that in different tissues of *Lumbricus terrestris*, a higher sensitivity to the OP chlorpyrifos was obtained using p-nitrophenyl valerate as substrate than when 1-NA was used. Also, in the snail *Xeropicta derbentina* (Laguerre et al., 2009) CEs were more sensitive to chlorpyrifos-oxon and less sensitive to dichlorvos with p-nitrophenyl valerate than with p-NPA and 1-NA as a substrate. All these studies emphasize the need to use a battery of substrates for the determination of CE inhibition by OPs. Regarding the analyzed tissues, ChE sensitivity to azinphos-methyl was similar in the digestive gland, pulmonary region and the whole organism soft tissue. *P. corneus* CEs measured with p-NPA and p-NPB were more sensitive in the digestive gland and pulmonary region than in the whole tissue. On contrary, IC_{50} values obtained were lower in the total tissue than in the others.

ChEs of *P. corneus* were more sensitive to azinphos-methyl than ChEs of *B. glabrata* and *B. straminea* and less sensitive than those of *C. gibbosa* and *L. variegatus*. *P. corneus* CE sensitivity was higher than that of *B. glabrata*, *B. straminea*, *C. gibbosa* and *L. variegatus* (Bianco et al., 2013, 2014; Kristoff et al., 2006) and similar to that of *H. curvispina* (Anguiano et al., 2014).

Before B-esterases are used as biomarkers, characterization, sensitivity and recovery studies should be performed. Conducting recovery studies is valuable, because they allow finding out whether the pesticide effects are permanent or not and also, to detect alterations in the organisms even when the pesticide is no longer present in the environment (Cossi et al., 2015). Recovery of enzyme activity depends on several factors such as the type and concentration of the pesticide, the species, the tissue, the substrate used, the initial inhibition and the synthesis capacity. In this work we observed that CE activities measured with p-NPA recovered after 14 days in all tissues while ChE activity and CE activities measured with p-NPB recovered to control values after 14 days in digestive gland and after 21 days in the pulmonary region and the whole organism. In contrast, in *B. glabrata* the pulmonary region had more capacity of recovery than the digestive gland (Kristoff et al., 2012). Studies in gonads of *P. corneus* exposed to chlorpyrifos showed that the initial CE inhibition (approximately of 70%) disappeared after 14 days while no recovery of ChE activity was observed (Rivadeneira et al., 2015).

Others authors have studied the recovery of ChE activity in the whole organism soft tissue. For example, Cossi et al. (2015) and Kristoff et al. (2006) observed in *C. gibbosa* and *L. variegatus*, respectively, that the initial inhibition of 90% did not disappear after 21 days in water.

On the other hand, our results showed an increment on ChE and CE activities at 21 days in pesticide-free water compared to control values. Some works have reported a rebound effect in B-esterase activities during the recovery period after exposure to insecticides inhibiting cholinesterase (Dorandeu et al., 2008; Duysen and Lockridge, 2011; Kristoff et al., 2010). The time required for recovery depends on the *de novo* synthesis of the enzyme (Habig and Di Giulio, 1991). The rebound effect may be related with a strong induction of enzyme synthesis.

Some authors have reported a possible relationship between B-esterases and reproduction in molluscs (Kristoff et al., 2011; Mikhailov et al., 1997; Tripathi and Singh, 2004). Previous results in our laboratory showed that a sub-chronic exposure to an environmental concentration of azinphos-methyl (0.4 mg L^{-1}) caused significant mortality of the offspring (70%) after 2 months of hatching, inhibition of ChE (60%) and inhibition of CE activity (80%) (Agrelo, 2012). Environmental concentrations of the OP chlorpyrifos also produced toxic effects on the reproduction and the survival of the offspring (Rivadeneira et al., 2013). Our results show that

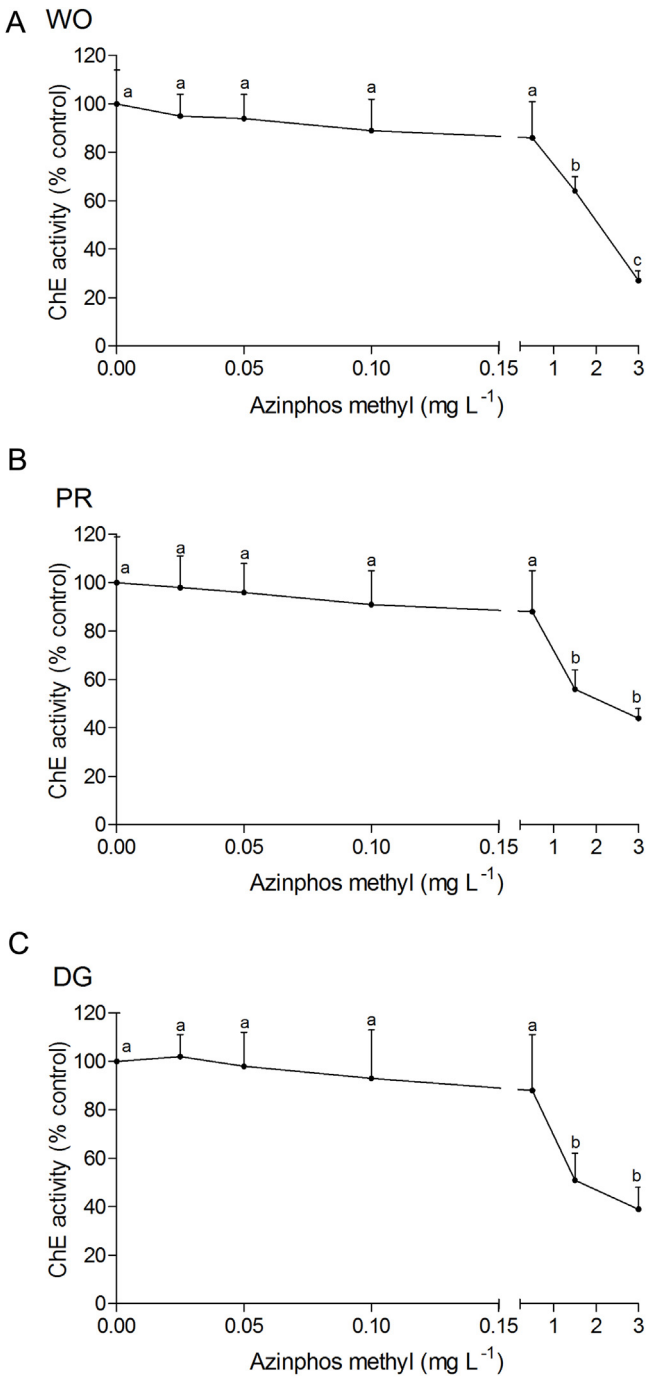

Fig. 3. Cholinesterase (ChE) activity in *P. corneus* exposed 48 h to different concentrations of azinphos-methyl. ChE activity was assayed using acetylthiocholine iodide (AsCh) as substrate in different tissues, A: total soft tissue (WO), B: pulmonary region (PR), C: digestive gland (DG). Data points show means ± S.D. of 6 homogenates. For each tissue, means not followed by the same uppercase letter are significantly different at $p < 0.05$. ChE activity of the solvent and solvent-free controls did not differ significantly.

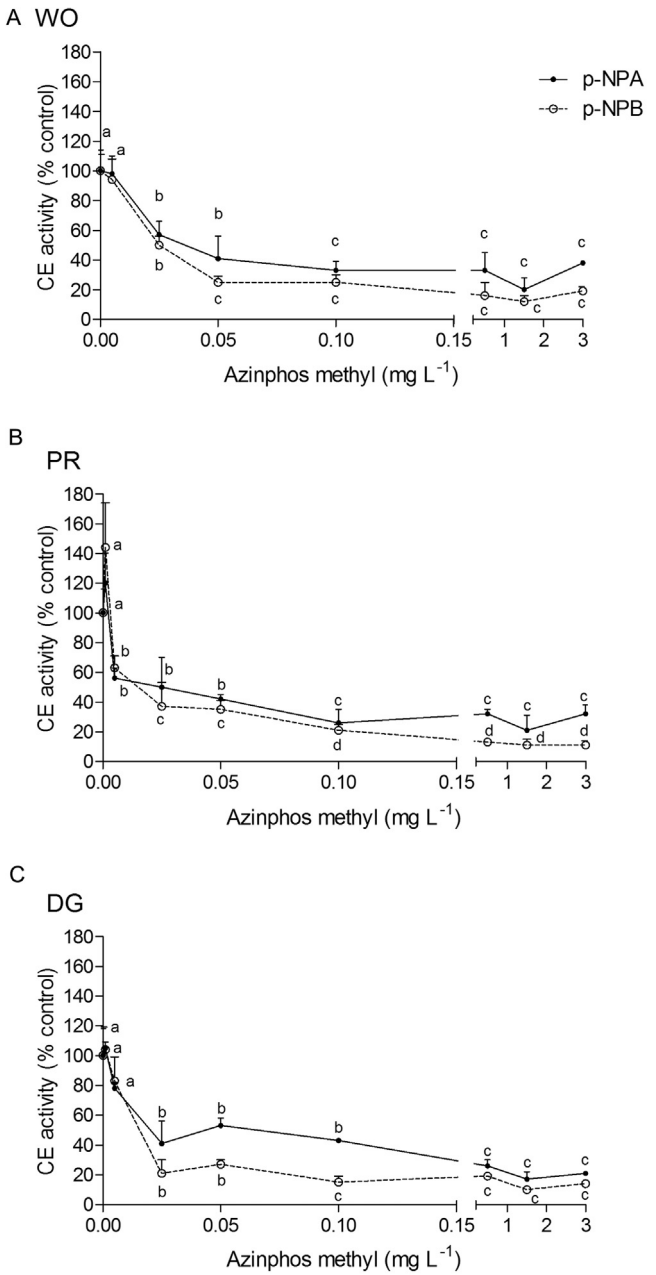

Fig. 4. Carboxylesterase (CE) activity in *P. corneus* exposed 48 h to different concentrations of azinphos-methyl. CE activity was assayed using two different substrates: p-nitrophenyl acetate (p-NPA), and p-nitrophenyl butyrate (p-NPB) and in different tissues, A: total soft tissue (WO), B: pulmonary region (PR), C: digestive gland (DG). Data points show means ± S.D. of 6 homogenates. For each tissue and substrate, means not followed by the same uppercase letter are significantly different at $p < 0.05$. CE activity of the solvent and solvent-free controls did not differ significantly.

aquatic contamination with this kind of pesticide can produce not only effects on biochemical parameters but also severe alterations in physiological processes in *P. corneus*.

5. Conclusions

Differences in basal ChE and CE activities of *P. corneus* depending on substrates and the tissue were observed. Also, both tissue-dependent and substrate-dependent variations in sensitivity to azinphos-methyl inhibition and recovery were obtained. This work showed that CEs measured with p-NPB in the pulmonary region were the best combination to be used as biomarker of exposure to azinphos-methyl due to the sensitivity and the low recovery capacity. These results depend on the species used. In another freshwater snail, the best combination was the CE activity measured in the digestive gland with p-NPA and p-NPB (Kristoff et al., 2012). Environmental concentrations of azinphos-methyl inhibited CE activity so this could be used as an effective biomarker of aquatic contamination.

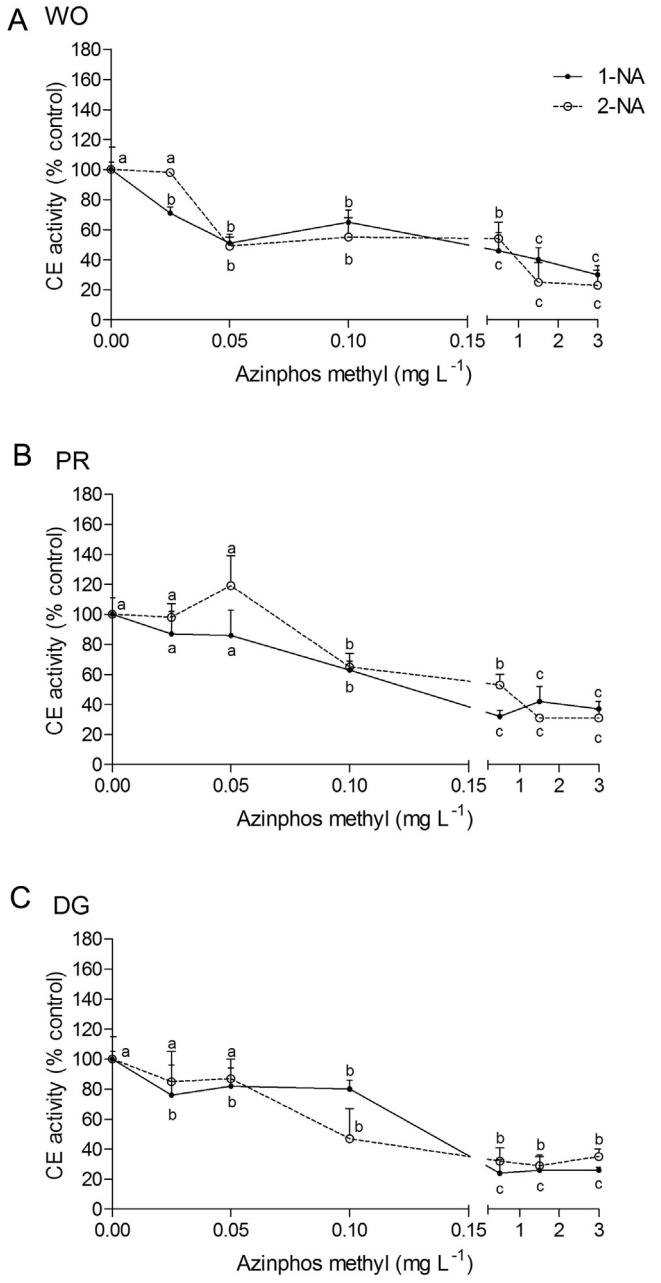

Fig. 5. Carboxylesterase (CE) activity in *P. corneus* exposed 48 h to different concentrations of azinphos-methyl. CE activity was assayed using two different substrates: 1-naphthyl acetate (1-NA), and 2-naphthyl acetate (2-NA) and in different tissues, A: total soft tissue (WO), B: pulmonary region (PR), C: digestive gland (DG). Data points show means ± S.D. of 6 homogenates. For each tissue and substrate, means not followed by the same uppercase letter are significantly different at $p < 0.05$. CE activity of the solvent and solvent-free controls did not differ significantly.

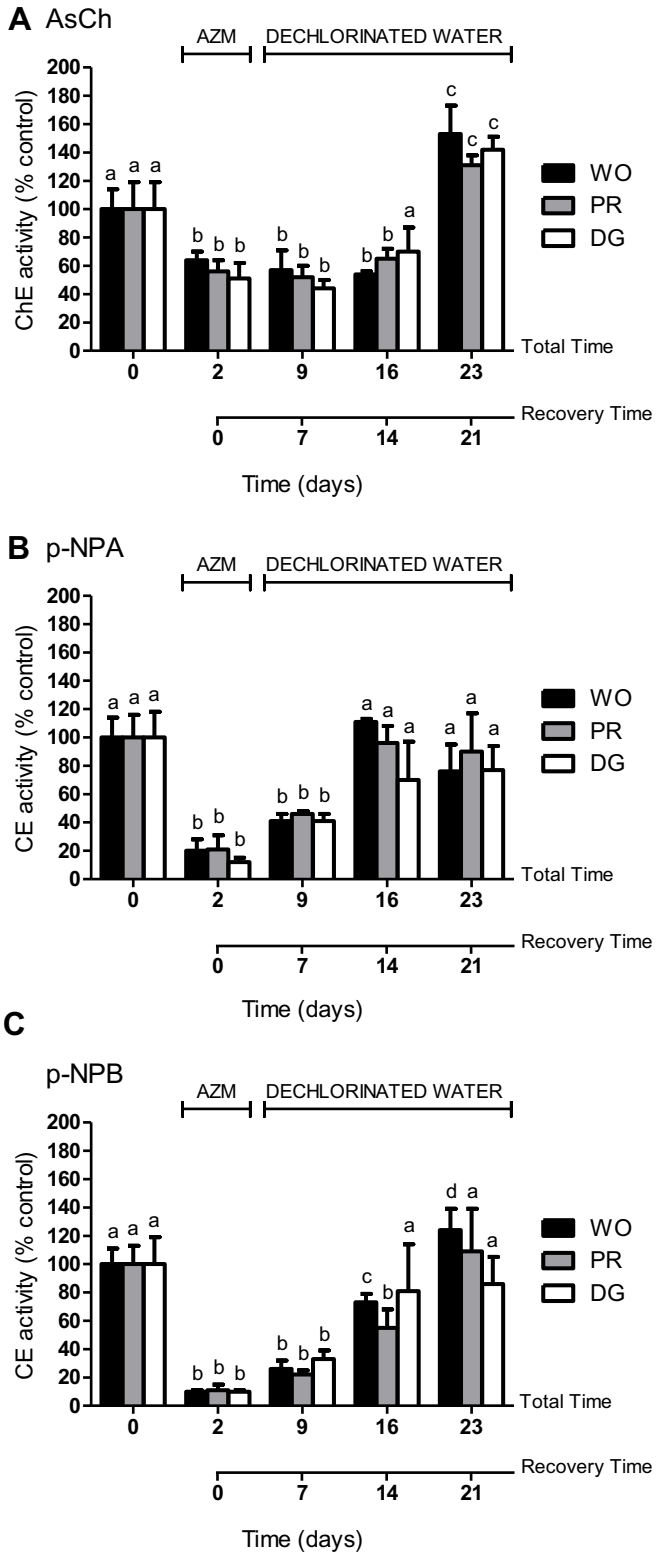

Fig. 6. Time course of recovery of the activities of cholinesterases (ChEs) and carboxylesterases (CEs) following exposure to azinphos-methyl. *P. corneus* snails were exposed to 1.5 mg L^{-1} of azinphos-methyl for 48 h and then transferred to clean water for 21 days. ChE and CE activities were determined at 0, 7, 14 and 21 days. Activities were expressed as a percentage of each control. Control values of enzyme activities remained constant during the bioassay time. ChE activity was assayed using A: acetylthiocholine iodide (AsCh) as substrate. CE activity was assayed using B: p-nitrophenyl acetate (p-NPA), and C: p-nitrophenyl butyrate (p-NPB). Each bar represents the mean ± S.D. of 6 homogenates. * indicates significantly different from the corresponding control ($p < 0.05$). WO: total soft tissue, PR: pulmonary region, DG: digestive gland.

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References

Agrelo, M., Kristoff, G., 2012. Efectos de las concentraciones ambientales del pesticida metilazinfos sobre la reproducción y descendencia de una población de gasterópodos de agua dulce. Revista da XX Jovens Pesquisadores da AUGM, 416–422.

Agrelo, 2012. Alteraciones en la reproducción y en la actividad de B-esterasas de *Planorbarius corneus* expuestos a metilazinfos. Análisis de malformaciones, reproducción, crecimiento y supervivencia de su descendencia. Tesis de Licenciatura en Ciencias Biológicas. Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires, Argentina.

Aldridge, W.N., 1953. Two types of esterases (A and B) hydrolysing p-nitrophenyl acetate propionate and butyrate, and a method for their determination. Biochem. J. 53, 110–117.

Anguiano, O.L., Castro, C., Venturino, A., Ferrari, A., 2014. Acute toxicity and biochemical effects of azinphos methyl in the amphipod *Hyalella curvispina*. Environ. Toxicol. 29, 1043–1053.

Arufe, M.I., Arellano, J.M., García, L., Albendín, G., Sarasquete, C., 2007. Cholinesterase activity in gilthead seabream (*Sparus aurata*) larvae: characterization and sensitivity to the organophosphate azinphosmethyl. Aquat. Toxicol. 84, 328–336.

Barata, C., Solayan, A., Porte, C., 2004. Role of B-esterases in assessing toxicity of organophosphorus (chlorpyrifos, malathion) and carbamate (carbofuran) pesticides to *Daphia magna*. Aquat. Toxicol. 66, 125–139.

Benstead, R.S., Baynes, A., Casey, D., Routledge, E.J., Jobling, S., 2011. 17 β-Oestradiol may prolong reproduction in seasonally breeding freshwater gastropod molluscs. Aquat. Toxicol. 101, 326–334.

Bianco, K., Yusseppone, M.S., Otero, S., Luquet, C., Ríos de Molina, Mdel C., Kristoff, G., 2013. Cholinesterases and neurotoxicity as highly sensitive biomarkers for an organophosphate insecticide in a freshwater gastropod (*Chilina gibbosa*) with low sensitivity carboxylesterases. Aquat. Toxicol. 144–145, 26–35.

Bianco, K., Otero, S., Oliver, A.B., Nahabedian, D., Kristoff, G., 2014. Resistance in cholinesterase activity after an acute and subchronic exposure to azinphos-methyl in the freshwater gastropod *Biomphalaria straminea*. Ecotoxicol. Environ. Saf. 109, 85–92.

Bonacci, S., Corsi, I., Focardi, S., 2009. Cholinesterases in the Antartic scallop *Adamussium colbecki:* Characterization and sensitivity to pollutants. Ecotoxicol. Environ. Saf. 72, 1481–1488.

Brown, M., Davies, I.M., Moffat, C.F., Redshaw, J., Craft, J.A., 2004. Characterisation of choline esterases and their tissue and subcellular distribution in mussel (*Mytilus edulis*). Mar. Environ. Res. 57, 155–169.

Cacciatore, L.C., Kristoff, G., Verrengia Guerrero, N.R., Cochón, A.C., 2012. Binary mixtures of azinphos-methyl oxon and chlorpyrifos oxon produce *in vitro* synergistic cholinesterase inhibition in *Planorbarius corneus*. Chemosphere 44, 450–458.

Cacciatore, L.C., Verrengia Guerrero, N.R., Cochón, A., 2013. Cholinesterase and carboxylesterase inhibition in *Planorbarius corneus* exposed to binary mixtures of azinphos-methyl and chlorpyrifos. Aquat. Toxicol. 128–129, 124–134.

Cacciatore, L.C., 2009. Investigación de procesos toxicocinéticos del pesticida metil azinfos en dos especies de invertebrados acuáticos. Tesis de Maestría en Ciencias Ambientales. Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires, Argentina.

Clarke, N., Routledge, E.J., Garner, A., Casey, d., Benstead, R., Walker, D., Watermann, B., Gnass, K., Thomsen, A., Jobling, S., 2009. Exposure to treated sewage effluent disrupts reproduction and development in the seasonally breeding ramshorn snail (Subclass: pulmonata, *Planorbarius corneus*). Environ. Sci. Technol. 43, 2092–2098.

Cossi, P., Boburg, F., Luquet, B., Kristoff, C., 2015. 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. Aquat. Toxicol. 167, 248–256.

Depledge, M.H., 1994. The role of biomarkers in environmental assessment (2). invertebrates. In M. C. fossi (Ed). Ecotoxicology 3, 161–172.

Desneux, N., Decourtye, A., Delpuech, J.M., 2007. The sublethal effects of pesticides on beneficial arthropods. Annu. Rev. Entomol. 52, 81–106.

Dorandeu, F., Foquin, A., Briot, R., Delacour, C., Denis, J., Alonso, A., Froment, M.T., Renault, F., Lallement, G., Masson, P., 2008. An unexpected plasma cholinesterase activity rebound after challenge with a high dose of the nerve agent VX. Toxicology 248, 151–157.

Duysen, E.G., Lockridge, O., 2011. Induction of plasma acetylcholinesterase activity in mice challenged with organophosphorus poisons. Toxicol. Appl. Pharmacol. 255 (2), 214–220.

Ellman, G.L., Courtney, K.D., Andres V.Jr. Featherstone, R.M., 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7, 88–95.

Ferrari, A., Venturino, A., Pechen de Dí Angelo, A.M., 2004a. Time course of brain cholinesterase inhibition and recovery following acute and subacute azinphos methyl, parathion and carbaryl exposure in the goldfish (Carassius auratus). Ecotoxicol. Environ. Saf. 57, 420–425.

Ferrari, A., Anguiano, O.L., Soleno, J., Venturino, A., Pechen de Dí Angelo, A.M., 2004b. Different susceptibility of two aquatic vertebrates (Oncorhynchus mykiss and Bufo arenarum) to azinphos methyl and carbaryl. Comput. Biochem. Physiol. C 139, 239–243.

Ferrari, A., Lascano, C., Pechén de DíAngelo, A.M., Venturino, A., 2011. Effects of azinphos methyl and carbaryl on *Rhinella arenarum* larvae esterases and antioxidant enzymes. Comput. Biochem. Physiol. C 153, 34–39.

Fried, B., Sundar Rao, K., Sherma, J., 1992. Fatty acid composition of *Biomphalaria glabrata* (Gastropoda: planorbidae) fed hensís egg yolk versus leaf lettuce. Comp. Biochem. Physiol. 101 A, 351–352.

Fuentealba, C., Figueroa, R., Monroe, J.J., 2010. Endemism analysis of chilean freshwater mollusks. Rev. Chil. Hist. Nat. 83, 289–298.

Fulton, M.H., Key, P.B., 2001. Acetylcholinesterase inhibition in estuarine fish and invertebrates as an indicator of organophosphorus insecticide exposure and effects. Environ. Toxicol. Chem. 20 (1), 37–45.

González Vejares, S., Sabat, P., Sanchez-Hernandez, J.C., 2010. Tissue-specific inhibition and recovery of esterase activities in Lumbriculus terrestris experimentally ezposed to chlorpyrifos. Comput. Biochem. Physiol. C 151, 351–359.

Granovsky, A.V., Ma, L., Ricaud, R., Bengtson, R.L., Selim, H.M., 1996. Fate of azinphosmethyl in a sugarcane field: distributions in canopy, soil, and runoff. J. Environ. Qual. 25, 1210–1216.

Habig, C., Di Giulio, R.T., 1991. Biochemical characteristics of cholinesterases in aquatic organisms. In: Mineau, P. (Ed.), Cholines-terase-Inhibiting Insecticides. Their Impact on Wildlife and theEnvironment. Elsevier, Amsterdam, pp. 20–30.

Jopp, F., 2006. Comparative studies on the dispersal of the Great Ramshorn (*Planorbarius corneus* L.): A modelling approach. Limnologica 36, 17–25.

Klosterhaus, S.L., Di Pinto, L.M., Chandler, G.T., 2003. A comparative assessment of azinphosmethyl bioaccumulation and toxicity in two estuarine meiobenthic harpacticoid copepods. Environ. Toxicol. Chem. 22, 2960–2968.

Kristoff, G., Verrengia Guerrero, N.R., Pechén de D'Angelo, A.M., Cochón, A.C., 2006. Inhibition of cholinesterase activity by azinphos-methyl in two freshwater invertebrates: Biomphalaria glabrata and Lumbriculus variegatus. Toxicology 222, 185–194.

Kristoff, G., Verrengia Guerrero, N.R., Cochón, A.C., 2010. Inhibition of cholinesterases and carboxylesterases of two invertebrate species, Biomphalaria glabrata and Lumbriculus variegatus, by the carbamate pesticide carbaryl. Aquat. Toxicol. 96, 115–123.

Kristoff, G., Cacciatore, L.C., Verrengia Guerrero, N.R., Cochón, A.C., 2011. Effects of the organophosphate insecticide azinphos-methyl on the reproduction and cholinesterase activity of *Biomphalaria glabrata*. Chemosphere 84, 585–591.

Kristoff, G., Chiny Barrionuevo, D., Cacciatore, L.C., Verrengia Guerrero, N.R., Cochón, A.C., 2012. In vivo studies on inhibition and recovery of B-esterase activities in Biomphalaria glabrata exposed to azinphos-methyl: analysis of enzyme, substrate and tissue dependence. Aquat. Toxicol. 112-113, 19–26.

Laguerre, C., Sanchez-Hernandez, J.C., Köhler, H.R., Triebskorn, R., Capowiez, Y., Rault, M., Mazzia, C., 2009. B-type esterases in the snail *Xeropicta derbentina:* An enzymological analysis to evaluate their use as biomarkers of pesticide exposure. Environ. Pollut. 157, 199–207.

Loewy, R.M., Kirs, V., Carvajal, G., Venturino, A., Pechén de DíAngelo, A.M., 2003. Effect of pesticide use in fruit production orchards on shallow ground water. J. Environ. Sci. Health 38 B, 317–325.

Loewy, R.M., Monza, L.B., Kiers, V.E., Savini, M.C., 2011. Pesticide distribution in an agricultural environment in Argentina. J. Environ. Sci. Health B. 46, 662–670.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193, 265–275.

Malagnoux, L., Capowiez, Y., Rault, M., 2014. Characterization of B-esterases and their inhibition responses to pesticides in the earwig *Forficula auricularia*. Chemosphere 112, 456–464.

Mikhailov, A.I., Torrado, M., Korochkin, L.I., Kopantzeva, M.A., Méndez, J., 1997. Male predominant carboxylesterase expression in the reproductive system of molluscs and insects: inmunochemical and biochemical similarity between *Mytilus* male associated polypeptide (MAP) and Drosophila sex-specific esterase S. Comput. Biochem. Physiol. 118, 197–208.

Mora, P., Michel, X., Narbonne, J.-F., 1999. Cholinesterase activity as potencial biomarker in two bivalves. Environ. Toxicol. Pharmacol. 7, 253–260.

Otludil, B., Cengiz, E.I., Yildirim, M.Z., Ünver, Ö., Ünlü, E., 2004. The effects of endosulfan on the great ramshorn snail *Planorbarius corneus* (Gastropoda, Pulmonata): a histopathological study. Chemosphere 56, 707–716.

Pavlica, M., Klobučar, G.I.V., Vetma, N., Erben, R., Papeš, D., 2000. Detection of micronuclei in haemocytes of zebra mussel and great ramshorn snail exposed to pentachlorophenol. Mutat. Res. 465, 145–150.

Rivadeneira, P.R., Agrelo, M., Otero, S., Kristoff, G., 2013. Different effects of subchronic exposure to low concentrations of the organophosphate insecticide chlorpyrifos in a freshwater gastropod. Ecotoxicol. Environ. Saf. 90, 82–88.

Rivadeneira, R., Agrelo, M., Kristoff, G., 2015. Acute Exposure of the Freshwater Gastropod *Planorbarius Corneus* to an Environmental Concentration of Chlorpyrifos. Recovery Studies of Enzyme Activities in Gonads and Effects on Reproduction in Chlorpyrifos: Toxicological Properties, Uses and Effects on Human Health and the Environment. Biochemistry Research Tronds. Nova Publishers.

Sanchez-Hernandez, J.C., Wheelock, C.E., 2009. Tissue distribution, isozyme abundance and sensitivity to chorpyrifos-oxon of carboxylesterases in the earthworm *Lumbricus terrestris*. Environ. Pollut. 157, 264–272.

Sanchez-Hernandez, J.C., 2007. Ecotoxicological perspectives of B-Esterases in the assessment of pesticide contamination. In: Plattenberg, R.H. (Ed.), Environmental Pollution: New Resarch. Nova Science Publishers, Inc. N. Y., USA, pp. 1–45.

Schulz, R., 2004. Field studies on exposure, effects, and risk mitigation of aquatic nonpoint-source insecticide pollution: a review. J. Environ. Qual. 33, 419–448.

Strong, E.E., Garcominy, O., Ponder, W.F., Bouchet, P., 2008. Global diversity of gastropods (Gastropoda; Mollusca) in freshwater. Hydrobiolgía 595, 149–166.

Timbrell, J.A., 2000. Principles of Biochemical Toxicology. Taylor and Francis, London.

Tripathi, P.K., Singh, A., 2004. Carbaryl induced alterations in the reproduction and metabolism of the freshwater snail *Lymnaea acuminata*. Pest. Biochem. Physiol. 79, 1–9.

Vioque-Fernández, A., Alves de Almeida, E., López-Barea, J., 2007. Esterases as pesticide biomarkers in crayfish (*Procambarus clarkii* Crustacea): Tissue distribution, sensitivity to model compounds and recovery from inactivation. Comput. Biochem. Physiol. 145, 404–412.
Walker, C.H., Hopkin, S.P., Sibly, R.M., Peakall, D.B., 2001. Principles of Ecotoxicology. Taylor and Francis, London.
Wan, M.T., Szeto, S.Y., Price, P., 1995. Distribution and persistence of azinphos-methyl and parathion in chemigated cranberry bogs. J. Environ. Qual. 24, 589–596.
Wheelock, C.E., Shan, G., Ottea, J., 2005. Overview of carboxylesterases and their role in the metabolism of insecticides. J. Pestic. Sci. 30, 75–83.
Wiese, V., 2005. Nonmarine mollusca of Schleswig-Holstein (northernmost Germany). (February 2005). MOLLBASE-National Database of German Land-and Freswater-Mollusks.01.06.2005. http://www.mollbase.org/sh/.
van Asperen, K., 1962. A study of housefly esterases by means of a sensitive colorimetric method. J. Ins. Physiol. 8, 401–416.
van der Oost, R., Beyer, J., Vermeulen, N.P.E., 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. Environ. Toxicol. Pharmacol. 13, 57–149.