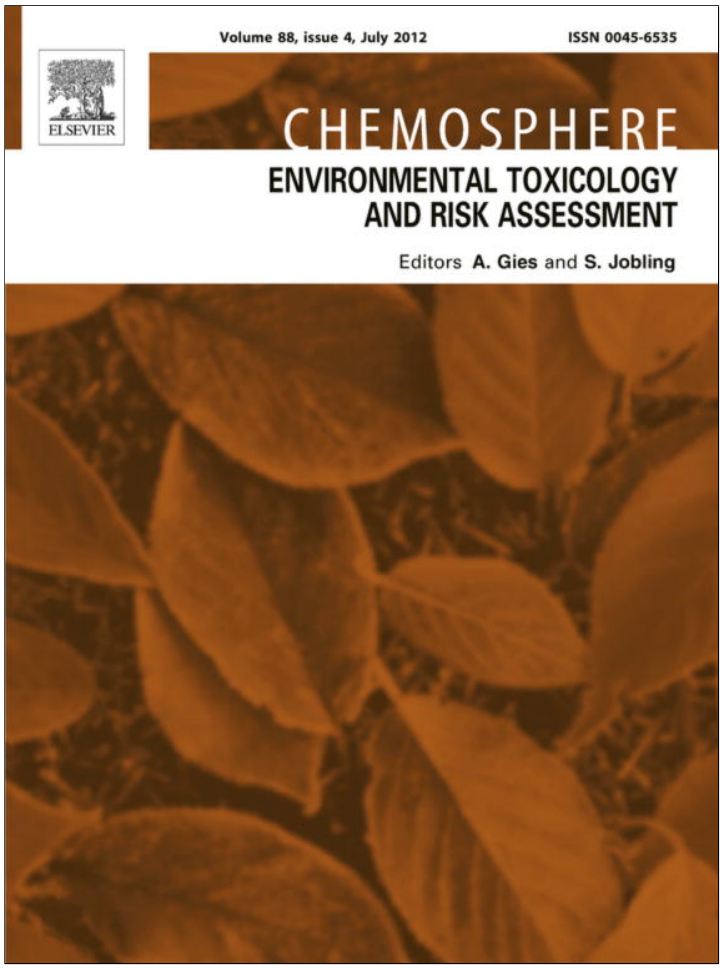

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Binary mixtures of azinphos-methyl oxon and chlorpyrifos oxon produce *in vitro* synergistic cholinesterase inhibition in *Planorbarius corneus*

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ABSTRACT

In this study, the cholinesterase (ChE) and carboxylesterase (CES) activities present in whole organism homogenates from *Planorbarius corneus* and their *in vitro* sensitivity to organophosphorous (OP) pesticides were studied. Firstly, a characterization of ChE and CES activities using different substrates and selective inhibitors was performed. Secondly, the effects of azinphos-methyl oxon (AZM-oxon) and chlorpyrifos oxon (CPF-oxon), the active oxygen analogs of the OP insecticides AZM and CPF, on ChE and CES activities were evaluated. Finally, it was analyzed whether binary mixtures of the pesticide oxons cause additive, antagonistic or synergistic ChE inhibition in *P. corneus* homogenates. The results showed that the extracts of *P. corneus* preferentially hydrolyzed acetylthiocholine (AcSCh) over propionylthiocholine (PrSCh) and butyrylthiocholine (BuSCh). Besides, AcSCh hydrolyzing activity was inhibited by low concentrations of BW284c51, a selective inhibitor of AChE activity, and also by high concentrations of substrate. These facts suggest the presence of a typical AChE activity in this species. However, the different dose–response curves observed with BW284c51 when using PrSCh or BuSCh instead of AcSCh suggest the presence of at least another ChE activity. This would probably correspond to an atypical BuChE. Regarding CES activity, the highest specific activity was obtained when using 2-naphthyl acetate (2-NA), followed by 1-naphthyl acetate (1-NA); p-nitrophenyl acetate (p-NPA), and p-nitrophenyl butyrate (p-NPB). The comparison of the IC_{50} values revealed that, regardless of the substrate used, CES activity was approximately one order of magnitude more sensitive to AZM-oxon than ChE activity. Although ChE activity was very sensitive to CPF-oxon, CES activity measured with 1-NA, 2-NA, and p-NPA was poorly inhibited by this pesticide. In contrast, CES activity measured with p-NPB was equally sensitive to CPF-oxon than ChE activity. Several specific binary combinations of AZM-oxon and CPF-oxon caused a synergistic effect on the ChE inhibition in *P. corneus* homogenates. The degree of synergism tended to increase as the ratio of AZM-oxon to CPF-oxon decreased. These results suggest that synergism is likely to occur in *P. corneus* snails exposed *in vivo* to binary mixtures of the OPs AZM and CPF.

1. Introduction

Traditionally, the evaluation of the quality of the aquatic environment has been undertaken at the chemical level, identifying and quantifying chemical residues in the different compartments of the ecosystem. However, to make an accurate assessment of the environmental risk, the quantification of chemical residues should be complemented with the study of biomarkers that serve as early warning signals (Domingues et al., 2010).

Acetylcholinesterase (AChE) is an enzyme that has been widely used as a sensitive biomarker of exposure to organophosphorous (OP) and carbamate pesticides (Timbrell, 2000; Walker et al., 2001). Since AChE hydrolyzes acetylcholine at cholinergic synapses and neuro-muscular junctions, the persistent inhibition of its activity causes neurotoxic effects. On the other hand, carboxylesterases (CES) are serine hydrolases that hydrolyze a wide range of exogenous and endogenous esters. They are assumed to provide protection against OP and carbamate toxicity through two main mechanisms: (1) direct hydrolysis of ester bonds in OPs and carbamates and (2) as alternative stoichiometric phosphorylation/carbamylation sites, which might reduce the amount of pesticide available for AChE inhibition (Sogorb and Vilanova, 2002). It has

Abbreviations: AChE, acetylcholinesterase; AcSCh, acetylthiocholine iodide; AZM-oxon, azinphos-methyl oxon; BuChE, butyrylcholinesterase; BuSCh, butyrylthiocholine iodide; BW284c51, 1,5-bis(4-allyldimethyl-ammoniumphenyl) pentan-3-one dibromide; CES, carboxylesterases; ChE, cholinesterase; CPF-oxon, chlorpyrifos oxon; DTNB, 5,5′-dithio-2-bis-nitrobenzoate; iso-OMPA, tetraiso propyl pyrophosphoramide; 1- and 2-NA, 1- and 2-naphthyl acetate; p-NPA, p-nitrophenyl acetate; p-NPB, p-nitrophenyl butyrate; OPs, organophosphorous; PrSCh, propionylthiocholine iodide.

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been suggested that in many aquatic and terrestrial species, the combined monitoring of CES and cholinesterase (ChE) activities may provide a more useful indication of OP and carbamate pesticide exposure than the measurement of AChE alone (Wheelock et al., 2005; Sanchez-Hernandez and Wheelock, 2009; Kristoff et al., 2010). However, a carefully characterization of these enzymes in the species selected as bioindicator is required before their use as biomarkers to monitor chemical exposure (Sanchez-Hernandez and Wheelock, 2009; Domingues et al., 2010).

Some species of the phylum mollusca have been postulated as powerful model systems to assess sub-lethal impacts of contaminants and mixtures of contaminants in aquatic ecosystems (Rittschof and McClellan-Green, 2005). Besides, a considerable decrease in the diversity of mollusks as consequence of anthropogenic activities has been reported (Strong et al., 2008). In particular, freshwater gastropods represent about 20% of recorded mollusk extinctions (Strong et al., 2008). Considering the role that these species play in trophic chains as grazers and as preys (Wojdak and Trexler, 2010), the declination in snail population can cause changes in the ecosystems. The gastropod *Planorbarius corneus* is a hermaphroditic snail that usually inhabits small temporary ponds and streams. This species belongs to the Planorbidae family, the largest family of aquatic pulmonate gastropods which is distributed all over the world (Jopp, 2006). It has been reported that *P. corneus* population declines constantly (Jopp, 2006). The reasons are not clear and may involve a combination of biotic and abiotic factors. *P. corneus* has already been used by several authors in toxicity testing (Pavlica et al., 2000; Otludil et al., 2004; Benstead et al., 2011). Despite this, to our best knowledge, no reports are available in the literature concerning the characterization of ChE and CES enzymes in this species. Moreover, only a few freshwater mollusk species have been studied in relation to the response of ChEs and CES activities to OPs. Most of the studies have been performed in bivalves. For example, inhibition of ChE and/or CES following exposure to OPs has been observed for the bivalves: *Amblema plicata* (Doran et al., 2001), *Elliptio complanata* (Moulton et al., 1996), and *Corbicula fluminea* (Basack et al., 1998; Mora et al., 1999; Oneto et al., 2005). In contrast, there are only few studies that report ChE inhibition by OP exposure in freshwater gastropods. Kristoff et al. (2006, 2008) reported *in vivo* ChE inhibition in the pulmonate snail *Biomphalaria glabrata* exposed to azinphos-methyl (AZM), whereas Gagnaire et al. (2008) reported *in vivo* ChE inhibition in the prosobanch snail *Potamopyrgus antipodarum* exposed to chlorpyrifos (CPF). In contrast, an increase in ChE activity was shown in the prosobanch snail *Valvata piscinalis* exposed to CPF (Gagnaire et al., 2008).

It is often assumed that the combined effects of chemicals that act by a common mechanism of action, in the absence of interactions, can be predicted by dose (or concentration) addition. That is, the concentration of each of the chemicals may be replaced by an equally effective concentration of one of them (Bosgra et al., 2009).

Mixtures of OPs might be expected to have an additive effect since they all inhibit AChE. In an aquatic vertebrate species, the Chinook salmon, concentration-additive inhibition of brain AChE activity by mixtures of OP and carbamate insecticides has been demonstrated in *in vitro* studies (Scholz et al., 2006). In contrast, *in vivo* studies demonstrated that salmon exposed to mixtures of OPs and carbamates showed either concentration-additive or synergistic inhibition of brain AChE activity, as well as unpredicted mortality (Laetz et al., 2009). Studies on the effects of mixtures of OPs on the ChEs of freshwater macroinvertebrates are scarce or null (Domingues et al., 2010).

This study focuses on ChE and CES activities present in *P. corneus* homogenates and their *in vitro* sensitivity to OP pesticides. Firstly, a characterization of ChE and CES activities using different substrates and selective inhibitors was performed. Secondly, the effects of azinphos-methyl oxon (AZM-oxon) and chlorpyrifos oxon (CPF-oxon), the potent oxygen analogs of the OP insecticides AZM and CPF, on ChE and CES activities were evaluated. Finally, it was analyzed whether binary mixtures of the pesticide oxons cause additive, antagonistic or synergistic ChE inhibition in *P. corneus* homogenates.

2. Materials and methods

2.1. Chemicals

Acetylthiocholine iodide (AcSCh), butyrylthiocholine iodide (BuSCh), propionylthiocholine iodide (PrSCh), 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 hemisulfat salt (eserine), sodium dodecil sulfate (SDS), Fast Blue RR salt, 1,5-bis (4-allyldimethyl-ammoniumphenyl) pentan-3-one dibromide (BW284c51), and tetraisopropyl pyrophosphoramide (iso-OMPA) were obtained from Sigma (St. Louis, MO). Azinphos-methyl oxon (AZM-oxon, CAS N° 961-22-8, 99% pure) was purchased from ChemService (West Chester, PA). Chlorpyrifos oxon (CPF-oxon, CAS N° 5598-15-2, 98.5% pure) was purchased from Chem-Lab (Buenos Aires, Argentina). All other chemicals used were of analytical reagent grade.

2.2. Snails

Adult *P. corneus* snails were purchased from Discus Morón S.R.L., Buenos Aires, Argentina. Afterwards the snails were reared in our Laboratory under standard conditions in aerated glass aquaria (17–20 L), at a temperature of 22 ± 2 °C, and under a 14:10 (L:D) h artificial photoperiod regime. Animals were fed lettuce leaves *ad libitum*. For all the experiments, adult snails of similar size (10 ± 2 mm of shell length, 300 ± 36 mg of wet weight) were used.

Water quality characteristics were as follows: total hardness = 67 ± 3 mg $CaCO_3\ L^{-1}$; alkalinity = 29 ± 2 mg $CaCO_3\ L^{-1}$; pH 7.0 ± 0.2 and conductivity = 250 ± 17 $\mu S\ cm^{-1}$.

2.3. Enzyme preparation

Animals were placed on ice for 6–8 min. The shells were carefully removed and the soft tissue isolated at 0 °C. The soft tissues were washed in distilled water, placed on filter paper to drain extra fluids, and weighed. Approximately 1 g was homogenized in 10 mL of cold 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 and the supernatants were immediately used as enzymatic source.

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

2.4. General ChE activity assay

ChE activity was measured, in duplicate, in 100 mM phosphate buffer, pH 8.0, 0.2 mM DTNB, and 1.5 mM AcSCh, PrSCh or BuSCh as substrate according to the method of Ellman et al. (1961). Activity was recorded continuously at 412 nm and specific activity was expressed as nmol min^{-1} mg $protein^{-1}$. The enzymatic activity was corrected for spontaneous hydrolysis of the substrate and non-specific reduction of the chromogen by tissue extracts.

2.5. General CES activity assay

CES 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 in duplicate, 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}).

CES activity using 1-NA or 2-NA as substrate was determined according to van Asperen (1962) with modifications. Reactions were performed in 2.5 mL 40 mM phosphate buffer pH 7.0 containing 5% acetone and 1 mM 1-NA or 2-NA. After 15 min incubation at 25 °C, reaction was stopped by the addition of 500 μL of freshly prepared SDS–Fast Blue solution (two parts of 1% Fast Blue RR salt in acetone and five 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 performed and specific activity was calculated using 13.6 and 11.8 mM^{-1} cm^{-1} as molar extinction coefficients, respectively.

2.6. Kinetic constants

Rates of hydrolysis of 12 AcSCh, PrSCh, or BuSCh concentrations (0.06–3.3 mM), 8 p-NPA concentrations (0.1–4 mM), 12 p-NPB concentrations (0.01–4 mM), and 12 1- and 2-NA concentrations (0.03–4 mM) were used to calculate K_m and V_{max}. The values of K_m and V_{max} were estimated from hyperbolic regression analysis using OriginPro 7.5 (OriginLab, Northampton, MA).

2.7. Eserine, BW284c51 and iso-OMPA inhibition

To assay the *in vitro* effects of eserine (a selective ChE inhibitor), BW284c51 (a selective inhibitor of mammalian AChE activity) and iso-OMPA (a specific inhibitor of mammalian BuChE activity) on enzyme activities, the compounds were pre-incubated with the enzyme extract for 15 min at 25 °C before substrate addition. Eserine and iso-OMPA were dissolved in ethanol, while BW284c51 dilutions were prepared in distilled water. Pre-incubation concentrations of the inhibitors ranged from 1 nM to 0.1 mM for eserine, and from 10 nM to 0.5 mM for BW284c51 and iso-OMPA. Three independent replicates of each inhibitor concentration were performed. Controls with ethanol and distilled water were also included.

2.8. Determination of the 50% inhibition concentration (IC_{50}) values

Absolute and relative IC_{50} values of ChE and CES inhibitors were calculated using the 4-parameter logistic model using OriginPro 7.5 (OriginLab, Northampton, MA). The model equation is specified by:

$$y = A_2 + \frac{(A_1 - A_2)}{1 + (x/x_0)^p} \quad (1)$$

In this equation, "y" is the enzyme activity (expressed as control %) at concentration "x" of the inhibitor. Parameters "A_1" and "A_2" are the upper and lower bound for function values "y" in the equation. The relative IC_{50} is the parameter "x_0" and represents the concentration corresponding to a response midway between the estimates of the bottom (A_2) and the top (A_1) plateaus. Absolute IC_{50} was calculated from the logistic Eq. (1) considering $y = 50$ and is defined as the concentration giving exactly a 50% response (Laguerre et al., 2009).

2.9. Azinphos-methyl oxon and chlorpyrifos oxon inhibition

To study the effects of the pesticides on ChE and CES activities, single-chemical and binary-mixtures trials were performed.

The individual toxic potential for the two pesticides was empirically obtained from the concentration–response relationship for enzyme inhibition in single-chemical studies. To calculate the IC_{50} values of the oxons on ChE and CES activities, stock AZM-oxon (100 μg mL^{-1}) and CPF-oxon (0.2 μg mL^{-1}) solutions were prepared in acetone and diluted to the desired concentrations with acetone. Samples (490 μL) were pre-incubated with 10 μL of the pesticide dilution before substrate addition, for 30 min at 25 °C, with agitation. A vehicle control was included. Six replicates were used for each pesticide concentration.

The effects of mixtures of the pesticides on ChE activity were determined following the method described by Laetz et al. (2009). In brief, the concentration of each pesticide was normalized to the respective IC_{50} concentration. In other words, each pesticide concentration was divided by the concentration estimated to produce a 50% decrease in ChE activity relative to carrier controls. The IC_{50}-normalized data for the two pesticides were subsequently plotted in the same graph and fit with a single regression to Eq. (1). Therefore a single curve with a 95% prediction band was obtained. If the concentration–effect curves of the two OPs are parallel, this curve can be used for detecting interactions between them in mixtures. If concentration addition occurs, ChE inhibition for a mixture would fall on the curve or within the 95% prediction band for the regression. Results falling significantly above the curve (less than expected inhibition) would be antagonistic, and results falling significantly below the curve (more than expected inhibition) would be synergistic. In this way, the curve fit to the data from single-chemical trials can be used as a basis for detecting interactions between OP pesticides in mixtures.

The effects of several combinations of AZM-oxon and CPF-oxon on ChE activity were analyzed. To this purpose, samples (490 μL) were pre-incubated with 10 μL of the pesticide combinations before substrate addition, for 30 min at 25 °C, with agitation. At the same time, single pesticide controls consisting of concentrations corresponding to 0.3 IC_{50}, 0.5 IC_{50}, and 1.0 IC_{50} were performed. A vehicle control was also included. Four replicates were used for each pesticide concentration.

2.10. Statistical analysis

Data were analyzed by one-way ANOVA followed by Tukey's post-test by using OriginPro 7.5 (OriginLab, Northampton, MA). The level of significance used was 0.05.

3. Results

3.1. Characterization of ChE and CES activities

3.1.1. Comparative activity against different substrates

Measurements of ChE activity from the soft tissue of *P. corneus* were performed using three substrates: AcSCh, PrSCh, and BuSCh. The results of the effects of substrate concentration are shown in Fig. 1A. ChE activity towards the three substrates showed a Michaelis–Menten kinetic, without inhibition by substrate in the range of concentrations 0–3.3 mM. Almost equal conversion rates were obtained with PrSCh and BuSCh, but they were lower than those obtained with AcSCh. The apparent K_m and V_{max} values are depicted in Table 1. Both parameters were approximately twice for AcSCh than for PrSCh and BuSCh.

Significant inhibition of ChE activity by excess of AcSCh and PrSCh was observed at 30 mM of substrates (Fig. 1B). In this case, ChE activity measured with 30 mM AcSCh or PrSCh was 49% and 39% of the activity registered at 1.5 mM, respectively. On the other hand, no substrate inhibition was found up to 30 mM of BuSCh.

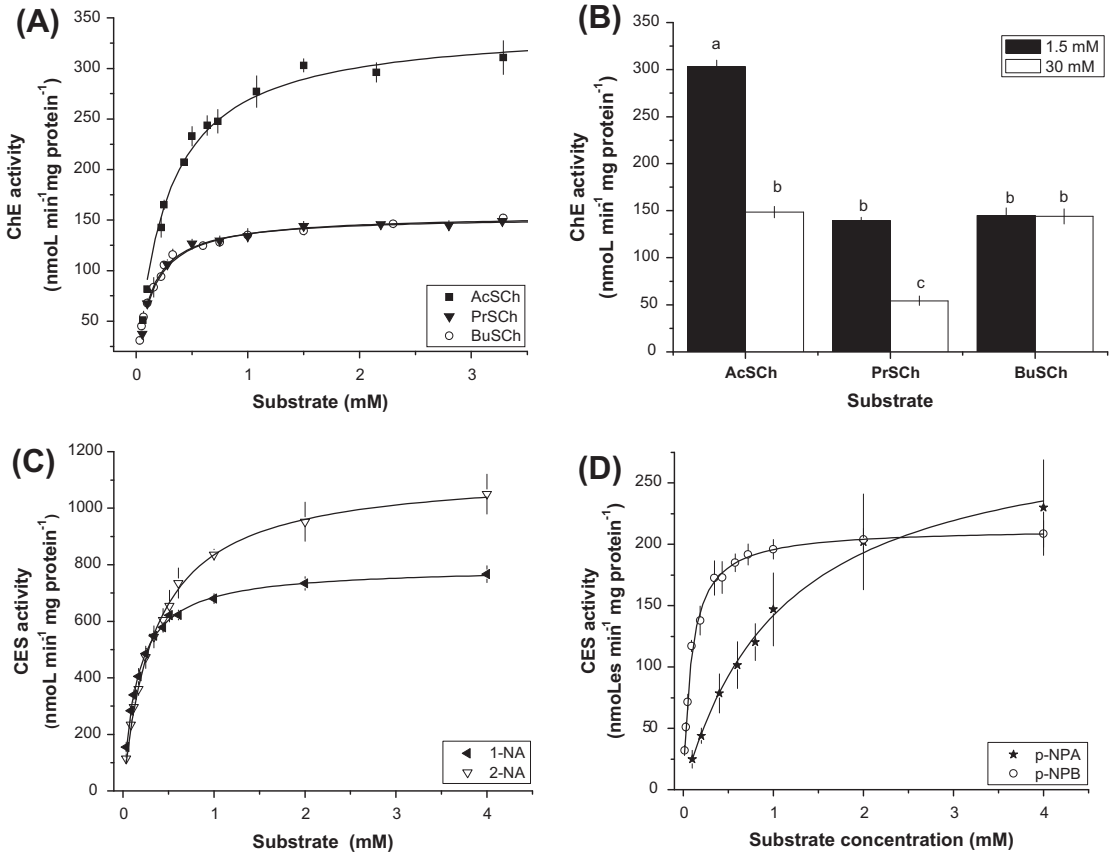

Fig. 1. Cholinesterase (ChE) and carboxylesterase (CES) activities towards different substrates: (A) Concentration–response curves of ChE activity towards acetylthiocholine iodide (AcSCh), propionylthiocholine iodide (PrSCh), and butyrylthiocholine iodide (BuSCh), (B) ChE activity at low (1.5 mM) and high (30 mM) substrate concentrations, (C) CES activity towards the substrates 1- and 2-naphthyl acetate (1- and 2-NA), (D) CES activity towards the substrates p-nitrophenyl acetate (p-NPA) and p-nitrophenyl butyrate (p-NPB). In (A), (C) and (D), data points show means ± S.D. for three or more determinations, and curves are fits of the Michaelis–Menten equation, $V = V_{max}[S]/(K_m + [S])$, to the data using OriginPro version 7.5. In (B), each bar represents the mean ± S.D. of four determinations. Means not followed by the same uppercase are significantly different at $p < 0.05$.

Table 1
Kinetic parameters for hydrolysis of different cholinesterase (ChE) and carboxylesterases (CES) substrates.

	ChE			
	Substrate			
	AcSCh		PrSCh	BuSCh
K_m (mM)	0.26 ± 0.04		0.12 ± 0.01	0.13 ± 0.01
V_{max} (nmol min^{-1} mg protein^{-1})	336 ± 11		153 ± 3	154 ± 4
	CES			
	Substrate			
	1-NA	2-NA	p-NPA	p-NPB
K_m (mM)	0.15 ± 0.01	0.35 ± 0.05	0.78 ± 0.28	0.08 ± 0.02
V_{max} (nmol min^{-1} mg protein^{-1})	787 ± 17	1119 ± 69	298 ± 53	203 ± 11

Fig. 1C and D shows the concentration–response curves of CES activity towards the substrates 1-NA, 2-NA, p-NPA and p-NPB. The extracts of *P. corneus* showed activity towards the four substrates. In all cases, CES activity showed a Michaelis–Menten kinetic, and the corresponding values for the apparent K_m and V_{max} for each substrate are shown in Table 1. The highest specific activity was obtained when using 2-NA, followed by 1-NA; p-NPA, and p-NPB. On the other hand, K_m value for 2-NA was twice the value for 1-NA, and K_m value for p-NPA was about 9-fold higher than for p-NPB. The ratios of V_{max}/K_m (mL min^{-1} mg protein^{-1}) for each substrate, based on estimated values of V_{max} and K_m shown in Table 1, were as follows: 5 (1-NA); 3 (2-NA); 0.4 (p-NPA) and 2.5 (p-NPB).

3.1.2. Effects of eserine, BW284c51 and iso-OMPA

The extent of ChE activity inhibition caused by different concentrations of eserine is presented in Fig. 2A while the enzyme inhibitions caused by BW284c51 and iso-OMPA are presented in Fig. 2B.

ChE activity was not significantly inhibited by iso-OMPA regardless the substrate assayed. In contrast, both eserine and BW284c51 induced significant inhibitions of ChE activity. In the case of eserine, a similar pattern of inhibition was observed with

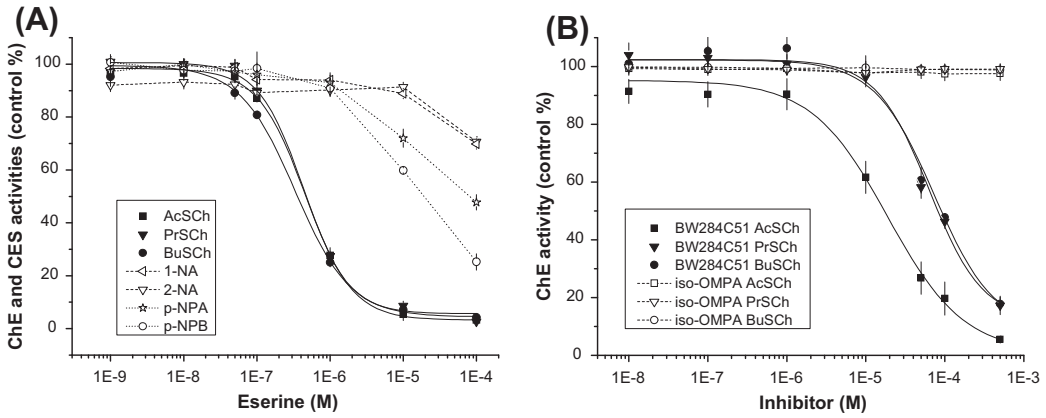

Fig. 2. Effects of eserine, BW284c51 and iso-OMPA on enzyme activities. (A) Effects of different eserine concentrations on cholinesterase (ChE) and carboxylesterase (CES) activities. (B) Effects of different BW284c51 and iso-OMPA concentrations on ChE activity. ChE activity was assayed using acetylthiocholine iodide (AcSCh), butyrylthiocholine iodide (BuSCh), and propionylthiocholine iodide (PrSCh) as substrates. CES activity was assayed using p-nitrophenyl acetate (p-NPA), p-nitrophenyl butyrate (p-NPB), and 1- and 2-naphthyl acetate (1- and 2-NA) as substrates. Data points show means ± S.D. for three or more determinations. The curves corresponding to eserine and BW284c51 effects on ChE activity are fits of the logistic sigmoid equation $y = A_2 + (A_1\text{-}A_2)/(1 + (x/x_0)^p)$, to the data using OriginPro version 7.5.

the three substrates. The concentrations of eserine causing inhibitions of 50% of ChE activity (IC_{50}) were: 0.49 ± 0.05 μM, 0.49 ± 0.07 μM and 0.39 ± 0.05 μM for AcSCh, PrSCh, and BuSCh, respectively. At a concentration of 10 μM of eserine, ChE activity was only 5% of control activity for the three substrates. In the case of BW284c51, the inhibition pattern was dependant of the substrate assayed. The highest sensitivity to the inhibitor was obtained using AcSCh as substrate, whereas no significant differences were obtained between PrSCh and BuSCh. The IC_{50} values for BW284c51-induced ChE inhibition were: 20 ± 3 μM, 80 ± 9 μM and 80 ± 10 μM for AcSCh, PrSCh, and BuSCh, respectively. At 500 μM of BW284c51, the enzyme inhibitions were 94%, 82% and 83%, respectively.

CES activity was less sensitive to eserine inhibition than ChE activity (Fig. 2A). In contrast to ChE, at a concentration of 10 μM of eserine CES activity measured with 1- or 2-NA was not inhibited, while the activity measured with p-NPA or p-NPB was around 60–72% of control values. At a high eserine concentration (100 μM) inhibition of CES activity was obtained with all the substrates assayed (30% for 1- and 2-NA, 52% for p-NPA, and 75% for p-NPB).

3.2. Effects of azinphos-methyl oxon and chlorpyrifos oxon on ChE and CES activities

3.2.1. Single pesticides

Since ChE activity was higher with AcSCh than with PrSCh and BuSCh, the effects of different concentrations of the pesticide oxons on this enzyme activity were studied only with AcSCh as substrate. Results of AChE inhibition by increasing concentrations of AZM-oxon and CPF-oxon are shown in Fig. 3A and B. Responses, expressed as a percentage of the control activity, were fitted to the logistic sigmoid equation $y = A_2 + (A_1 - A_2)/(1 + (x/x_0)^p)$. The corresponding IC_{50} values and the parameters of the non-linear regressions are shown in Table 2.

Pre-incubation of enzyme extract with increasing concentrations of AZM-oxon from 2 to 12 mg L^{-1} (Fig. 3A) or CPF-oxon from 0.01 to 0.05 mg L^{-1} (Fig. 3B) resulted in decreased AChE activity. The highest inhibition relative to the controls achieved with the pesticides was 66% at 12 mg AZM-oxon L^{-1}, and 90% at 0.05 mg CPF-oxon L^{-1}. IC_{50} of CPF-oxon was 264-fold lower than that of AZM-oxon (Table 2).

In contrast to AChE activity, CES activity measured with 1-NA, 2-NA, p-NPA and p-NPB was almost completely inhibited at 1 mg AZM-oxon L^{-1} (Fig. 3C). However, at low pesticide concentrations, activity towards p-NPB was less inhibited than towards the other three substrates. The comparison of the IC_{50} values (Table 2) reveals that, regardless of the substrate used, CES activity was approximately one order of magnitude more sensitive to AZM-oxon than AChE activity.

Although AChE activity was very sensitive to CPF-oxon, CES activity measured with 1-NA, 2-NA, and p-NPA was poorly inhibited by this pesticide (Fig. 3D). CFP-oxon at 0.05 mg L^{-1} inhibited 31% of 1-NA activity, 39% of 2-NA activity, and 50% of p-NPA activity. Due to these low inhibition responses, the CPF-oxon IC_{50} values for CES activity towards the above mentioned three substrates could not be calculated. In contrast, CES activity measured with p-NPB was more sensitive to CPF-oxon than the activity determined with the other three CES substrates and equally sensitive than AChE activity (Fig. 3D and Table 2).

3.2.2. Effects of mixtures on ChE activity

To study the effects of binary mixtures of the pesticides on AChE activity, the concentration of each pesticide normalized to the respective IC_{50} concentration (IC_{50} normalized data) was combined and fitted with a single regression (Fig. 4A). This curve ($y = 24.74 + 60.72/(1 + (x/0.91)^{4.21})$, $r^2 = 0.91462$) with its 95% prediction band was used as a basis to determine whether specific binary combinations of the oxons deviate from concentration addition.

Firstly, three combinations of AZM-oxon and CPF-oxon were performed: mixture 1: 0.15 IC_{50} units of AZM-oxon (1.2 mg L^{-1}) + 0.15 IC_{50} units of CPF-oxon (5 μg L^{-1}), mixture 2: 0.25 IC_{50} units of AZM-oxon (2 mg L^{-1}) + 0.25 IC_{50} units of CPF-oxon (8.5 μg L^{-1}), and mixture 3: 0.5 IC_{50} units of AZM-oxon (4 mg L^{-1}) + 0.5 IC_{50} units of CPF-oxon (17 μg L^{-1}). At the same time, single pesticide controls consisting of concentrations corresponding to 0.3 IC_{50}, 0.5 IC_{50}, and 1.0 IC_{50} were also performed. The results are depicted in Fig. 4.

The activity of AChE in single pesticide controls (Fig. 4C) and in mixture 1 (Fig. 4B) fell on the curve $y = 24.74 + 60.72/(1 + (x/0.91)^{4.21})$, or within the 95% prediction band for the regression. This indicates that for mixture 1, cumulative AChE inhibition was equivalent to 0.3 IC_{50} units and consequently, the expected concentration addition occurred. In contrast, mixtures 2 and 3 showed AChE inhibitions higher than the expected inhibitions, since results fell significantly below the curve (Fig. 4B). Main AChE inhibition in mixtures 2 and 3 were 60.4% and 95.1%, respectively, whereas the

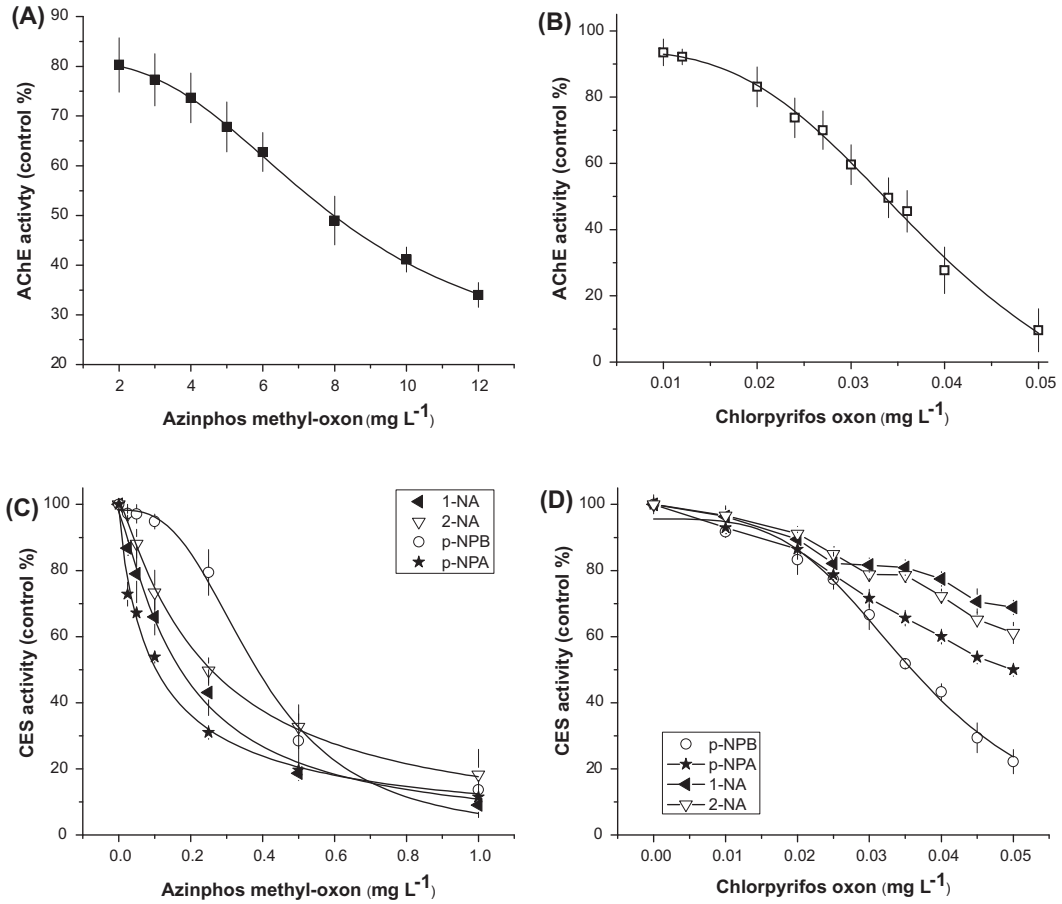

Fig. 3. Effects of increasing concentrations of azinphos-methyl oxon (A and C) and chlorpyrifos oxon (B and D) on acetylcholinesterase (AChE) and carboxylesterase (CES) activities. AChE activity was assayed using acetylthiocholine iodide (AcSCh) as substrate. CES activity was assayed using p-nitrophenyl acetate (p-NPA), p-nitrophenyl butyrate (p-NPB), 1- and 2-naphthyl acetate (1- and 2-NA) as substrates. Data points show means ± S.D. for three or more determinations. Except for the effects of chlorpyrifos oxon on 1-NA-, 2-NA-, and p-NPA-CES activities, all other curves are fits of the logistic sigmoid equation $y = A_2 + (A_1 - A_2)/(1 + (x/x_0)^p)$, to the data using OriginPro version 7.5.

Table 2
Concentrations of azinphos-methyl oxon and chlorpyrifos oxon causing inhibitions of 50% (IC_{50}) on acetylcholinesterase and carboxylesterases activities and the four parameter values of the non-linear regressions shown in Fig. 3.

Substrate[a]	A_1 (%)	A_2 (%)	x_0 (mg L^{-1})	p	r^2	IC_{50} (μM)[b]
Azinphos-methyl oxon						
AcSCh	81.21 ± 0.94	20.69 ± 4.31	7.80 ± 0.41	2.90 ± 0.29	0.9989	26.45 ± 1.49
1-NA	97.73 ± 2.96	0	0.18 ± 0.02	1.20 ± 0.11	0.9940	0.56 ± 0.06
2-NA	101.73 ± 1.62	0	0.25 ± 0.01	1.12 ± 0.06	0.9978	0.82 ± 0.03
p-NPA	98.96 ± 2.87	0	0.11 ± 0.01	0.87 ± 0.06	0.9944	0.37 ± 0.02
p-NPB	100	11.80 ± 3.67	0.34 ± 0.02	3.71 ± 0.50	0.9956	1.22 ± 0.07
Chlorpyrifos oxon						
AcSCh	93.98 ± 2.11	−30.06 ± 18.77	0.04 ± 0.01	3.46 ± 0.52	0.9954	0.10 ± 0.03
p-NPB	91.94 ± 1.90	−10.48 ± 15.22	0.04 ± 0.01	3.62 ± 0.54	0.9975	0.11 ± 0.01

[a] Cholinesterase activity was assayed using acetylthiocholine (AcSCh) as substrate and carboxylesterase activity was assayed using p-nitrophenyl acetate (p-NPA), p-nitrophenyl butyrate (p-NPB), and 1- and 2-naphthyl acetate (1- and 2-NA).
[b] The chlorpyrifos oxon IC_{50} values for carboxylesterase activity towards 1-NA, 2-NA, and p-NPA could not be calculated because of the low inhibition responses observed.

corresponding expected inhibitions were 20% and 50%. This indicates that these mixtures were synergistic.

Finally, to further study whether the proportion of each pesticide in the mixture affected the degree of AChE inhibition, pesticides were combined as it is shown in Fig. 5. Among the pairings corresponding to 0.5 IC_{50} units (Fig. 5A) and to 1.0 IC_{50} units (Fig. 5B), the combinations 80:20 of AZM-oxon:CPF-oxon resulted in concentration addition. All other combinations showed synergistic effects. It is noteworthy that the degree of synergism tended to increase as the ratio of AZM-oxon to CPF-oxon decreased.

4. Discussion

In this study, ChE and CES activities present in whole organism homogenates from the freshwater gastropod *P. corneus* and their *in vitro* sensitivity to OP pesticides were investigated. This snail has already been used as a model organism for toxicity studies because it is an important non-target species that can be found in the pools surrounded by agricultural lands in several European countries (Otludil et al., 2004). In particular, histopathological alterations after exposure of *P. corneus* to endosulfan (Otludil et al.,

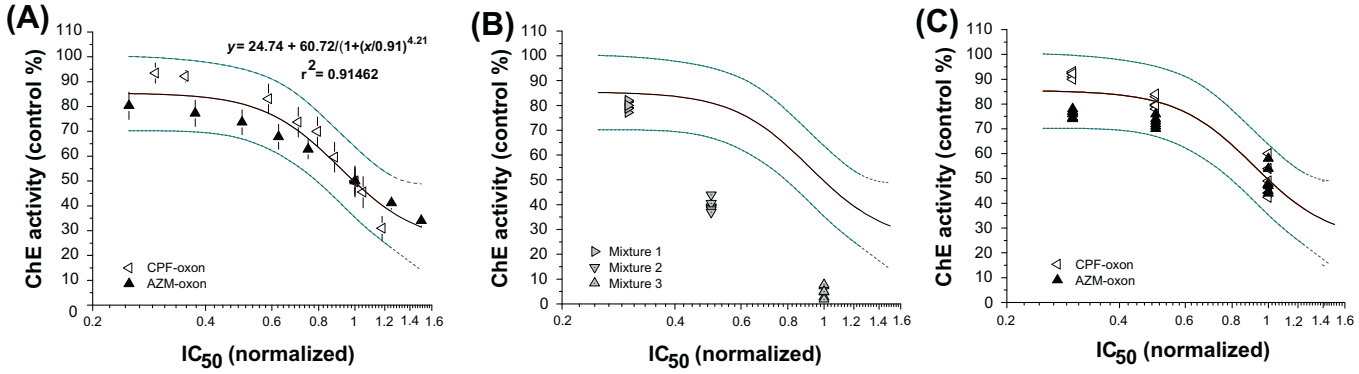

Fig. 4. Acetylcholinesterase (AChE) inhibition by single pesticides and 50:50 binary mixtures of azinphos-methyl (AZM) oxon and chlorpyrifos (CPF) oxon after normalization to their respective IC_{50}. (A) Plot of the concentration–response data shown in Fig. 3A and B after normalization to their respective IC_{50} concentrations and collectively fitting with the four parameter logistic sigmoid equation $y = A_2 + (A_1 - A_2)/(1 + (x/x_0)^p)$. Data points show means ± S.D. for three or more determinations. The solid line shows the result from the non-linear regression and dashed lines are the 95% prediction band. (B) Effects of 50:50 binary mixtures of AZM and CPF oxons on AChE activity. Based on a default assumption of concentration addition, the pairings were predicted to yield ChE inhibitions of 15% (0.3 IC_{50}, mixture 1), 20% (0.5 IC_{50}, mixture 2), and 50% (1.0 IC_{50}, mixture 3). Lines show the curve and the 95% prediction band depicted in A. Triangles represent individual determinations ($n = 6$ for each mixture). (C) Single pesticide reference controls consisting of concentrations corresponding to 0.3 IC_{50} (2.4 mg L^{-1} or 10 μg L^{-1}, for AZM-oxon and CPF-oxon, respectively), 0.5 IC_{50} (4 mg L^{-1} or 17 μg L^{-1}, for AZM-oxon and CPF-oxon, respectively), and 1.0 IC_{50} (8 mg L^{-1} or 34 μg L^{-1}, for AZM-oxon and CPF-oxon, respectively). These controls were performed at the same time that the assays with the binary mixtures shown in B. Lines show the curve and the 95% prediction band depicted in A. Triangles represent individual determinations ($n = 6$ for each condition).

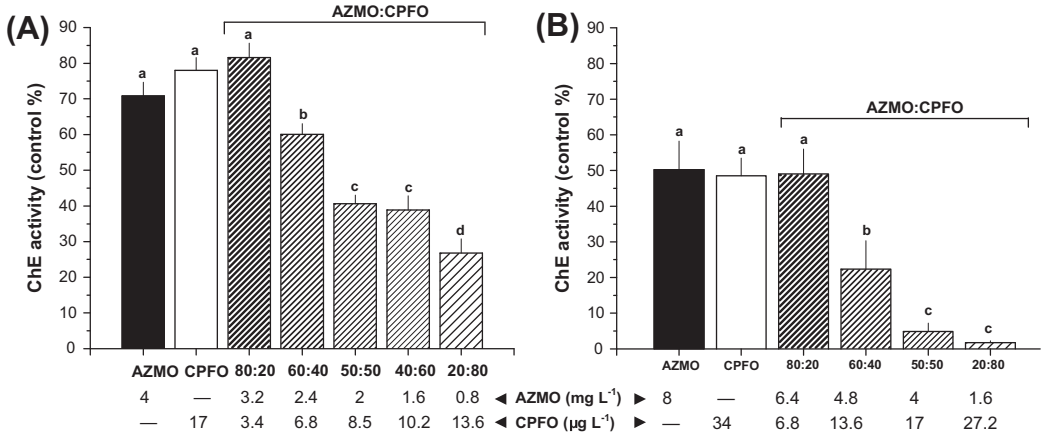

Fig. 5. Acetylcholinesterase (AChE) inhibition by different binary combinations of azinphos-methyl-oxon (AZMO) and chlorpyrifos-oxon (CPFO). Based on a default assumption of concentration addition, the pairings were predicted to yield ChE inhibitions of 20% (A) and 50% (B). Each bar represents the mean ± S.D. of four determinations. Black and white bars are single pesticide controls. Striped bars are the different AZM-oxon and CPF-oxon combinations assayed. The concentration of the oxons in the different combinations is depicted in the table below the graphic. Means not followed by the same uppercase are significantly different at $p < 0.05$.

2004) and disturbances in the reproductive performance after exposure to sewage treatment works (STWs) effluents (Clarke et al., 2009) have been reported.

The group of enzymes called B-esterases comprises the hydrolases ChEs and CES that are inhibited by OPs and carbamates. Higher vertebrates possess two ChEs, corresponding to two different genes: AChE (E.C. 3.1.1.7) and BuChE (E.C. 3.1.1.8) (Massoulié et al., 1993). AChE hydrolyzes AcSCh maximally, PrSCh adequately, but not BuSCh at all; while BuChE hydrolyzes the three substrates more or less equally (Pezzementi et al., 2011). Invertebrates possess ChEs that differ in many aspects from either vertebrate AChE or BuChE, and extensive variation in ChE biochemistry exists among species (Sanchez-Hernandez, 2007).

In the current study, ChE activity in *P. corneus* was partially characterized using the whole organism and the thiocholine esters AcSCh, PrSCh, and BuSCh. By means of the use of the ChEs inhibitor eserine, it was established that measured activities with the three substrates were due to ChEs and not to unspecific esterases. The extracts of *P. corneus* preferentially hydrolyzed AcSCh over PrSCh and BuSCh. Besides, AcSCh hydrolyzing activity was inhibited by low concentrations of BW284c51, a selective inhibitor of AChE activity, and by high concentrations of substrate. These facts indicate the presence of a typical AChE activity in this species. However, the different dose–response curves observed with BW284c51 when using PrSCh or BuSCh instead of AcSCh suggests the presence of at least another ChE. This ChE would be the responsible for the high BuSCh hydrolyzing activity of *P. corneus* extracts. According to the classification proposed by Whittaker (2010), this BuSCh hydrolyzing activity would probably correspond to an atypical BuChE since although it was not inhibited by excess of substrate, it was insensitive to iso-OMPA, a specific inhibitor of higher vertebrates BuChE activity. Atypical BuChEs have been described for several vertebrate species (Pezzementi et al., 2011), with high heterogeneity among them. The heterogeneity resides basically in substrate hydrolysis specificities, the patterns of substrate inhibition and the patterns of sensitivity to inhibitors.

In *P. corneus*, the ratios K_m PrSCh/K_m AcSCh (K_m P/A) and K_m BuSCh/K_m AcSCh (K_m B/A) were both equal to 0.5. Besides, the ratios V_{max} P/A and V_{max} B/A were also equal to 0.5. The analysis

of these ratios allows the comparison with other authors, since the differences between the methodologies used to measure ChE activity by different laboratories preclude the direct comparison of the values of K_m y V_{max}. In *B. glabrata*, a snail corresponding to the same family as *P. corneus*, the values of K_m P/A y V_{max} P/A were very similar to those observed in *P. corneus* (Kristoff, 2010). In contrast, in the snails *P. antipodarum* (Hydrobiidae), *V. piscinalis* (Valvatidae), and *Helix aspersa* (Helicidae), the reported values of K_m P/A and V_{max} P/A were approximately 4–5 and 1.4–2.6 times higher than those calculated in *P. corneus*, respectively (Talesa et al., 1995; Gagnaire et al., 2008). With respect to BuSCh, the freshwater snails *B. glabrata*, *P. antipodarum*, and *V. piscinalis* did not show significant activity with this substrate (Kristoff et al., 2006; Gagnaire et al., 2008); whereas the terrestrial snails *H. aspersa* and *Xeropicta derbentina* (Hygromiidae) showed a ratio K_m B/A and V_{max} B/A lower or equal than *P. corneus*, respectively (Gagnaire et al., 2008; Laguerre et al., 2009).

The CES family of enzymes is a key participant in the phase I drug metabolism process, catalyzing the hydrolysis of a wide range of ester- and amide-containing compounds. In crude homogenates, it is common to encounter different CES isoenzymes with broad and overlapping substrate specificity (Wheelock et al., 2005). It has also been reported that CES enzymes exhibit species differences (Kristoff et al., 2010). In the extracts of *P. corneus*, esterase activity towards four substrates commonly used to test CES activity (1-NA, 2-NA, p-NPA and p-NPB) was detected. This activity was virtually insensitive to low concentrations of eserine, indicating that it was not due to ChEs. Considering the four substrates assayed, the highest specific activity was seen for 2-NA. Besides, the catalytic efficiencies, represented by the values of V_{max}/K_m showed that 1-NA, 2-NA, and p-NPB were better substrates than p-NPA. This may suggest that the p-NPA substrate differ substantially from the natural substrates of the CES enzymes present in *P. corneus*.

Following the characterization of ChE and CES activities in the extracts of *P. corneus*, the *in vitro* effects of AZM and CPF oxons on these enzymes were evaluated using both single pesticide trials and binary mixtures of the pesticides.

Richardson et al. (2001) observed that CPF-oxon was a more potent inhibitor of rat serum and brain ChE than AZM-oxon. Similarly, a marked difference in the ability of these two compounds to inhibit ChE activity was also found in the current study in *P. corneus* extracts. Kousba et al. (2004) have suggested that the differences in the binding affinities of the OP oxons for a peripheral binding site in AChE may play an important role in determining their inhibitory potency. In addition, it has to be considered that the extracts of *P. corneus* probably contain more than one ChE and several CES enzymes, raising the possibility that other factors could also contribute to the differences observed in CPF- and AZM-oxon potencies as ChE inhibitors. At this respect, several studies have reported that CES enzymes may be more sensitive than ChE enzymes to OP pesticides (Escartín and Porte, 1997; Wheelock et al., 2005). Similarly, in the present study it was found that the ability of AZM-oxon to inhibit CES activity was markedly greater than the ability of this compound to inhibit ChE activity. However, CPF-oxon was equally or less potent inhibitor of CES than ChEs, depending on the substrate assayed. These differences between both compounds in the ability of inhibiting CES enzymes would be another factor to be considered when trying to explain the lower potency of AZM-oxon with respect to CPF-oxon to inhibit the ChEs in *P. corneus* extracts. This is because CES enzymes can act as scavengers, leaving less OP available to inhibit AChE (Sogorb and Vilanova, 2002).

Studies performed *in vitro* with AChE from human blood, rat brain or AChE extracted from the olfactory nervous system of Chinook salmon, showed that it was possible to estimate the cumulative AChE inhibition of mixtures of certain OPs by simple dose addition (Richardson et al., 2001; Scholz et al., 2006; Bosgra et al., 2009). However, when the studies were performed *in vitro* using rat serum instead of rat brain or *in vivo* exposing the salmons to the OP mixtures, deviation from the additive effects on ChE inhibition were observed (Richardson et al., 2001; Laetz et al., 2009). The departure from concentration addition in these last studies was adjudicated, at least partly, to the action of the pesticides on biochemical targets other than ChEs. Among these alternative targets, the A-esterases and the CES may have an important role. This is because the calcium dependent A-esterases can catalytically inactivate a number of OPs without being inhibited, and the CES participate in stoichiometric binding of OP molecules (Sogorb and Vilanova, 2002). It is worth to mention that in the present study, tissues were homogenized in the presence of EDTA to block the A-esterases from inactivating the oxons (Karanth et al., 2004).

The data reported herein indicate that, at concentrations equivalent to 0.5 y 1.0 IC_{50} units, only the combinations 80:20 of AZM-oxon:CPF-oxon showed concentration addition. All the other combinations assayed showed synergistic effects, with the degree of synergism increasing as the ratio of AZM-oxon:CPF-oxon decreased. This deviation from expected concentration addition in ChE inhibition would be due, at least in part, to the presence of CES enzymes in *P. corneus* extracts. Given the concentrations of AZM-oxon present in all 0.5 and 1.0 IC_{50} mixtures, CES activity in the extracts would be totally abolished by this compound. Therefore, CPF-oxon would not be sequestered by the CES, in particular by the p-NPB-hydrolyzing enzymes which are more sensitive to this compound. In this way, the blockage of this protective mechanism would generate that in the presence of AZM-oxon there would be a greater number of CPF-oxon molecules with high affinity and potency capable of irreversibly inhibiting more molecules of ChE than in its absence, thus producing the synergistic effect.

In conclusion, the characterization of ChE and CES activities in extracts of *P. corneus* and the determination of the *in vitro* inhibition of ChE by mixtures of the potent oxon metabolites of AZM and CPF performed in the current investigation could be valuable tools in the interpretation of future *in vivo* studies with mixtures of OP insecticides. In particular, this study indicates that synergism is likely to occur in *P. corneus* snails exposed to binary mixtures of OPs such as AZM and CPF.

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