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Linking cholinesterase inhibition with behavioural changes in the sea snail *Gibbula umbilicalis*: effects of the organophosphate pesticide chlorpyrifos

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Abstract

Inhibition of acetylcholinesterase (AChE) activity has been widely used to assess the exposure and effects of anticholinergic environmental contaminants in several species. The aim of this study was to investigate if sublethal concentrations of the organophosphorous pesticide chlorpyrifos (CPF), a well-known AChE inhibitor, would also affect cholinesterases (ChE) in *Gibbula umbilicalis* and if this inhibition would result in an alteration of its behaviour, in an attempt to link the effects observed at the cellular level with effects at higher levels of ecological relevance. The biochemical properties of ChEs in this species were first characterized through assessment of different enzymatic forms present in the sea snail, using different substrates and selective inhibitors. The results suggest that *G. umbilicalis* possess ChEs with characteristics of typical AChE, which should be the main form present. Additionally, *in vitro* and *in vivo* effects of CPF on AChE activity were investigated, along with effects on snails' behaviour: the ability of the snails to move/turn after exposure to the contaminant (flipping test). As expected, CPF inhibited AChE activity both *in vitro* and *in vivo* conditions. Moreover, the link between AChE activity inhibition and adverse effects on behavioural changes was established: AChE inhibition was positively correlated with the flipping test, indicating a mechanistic relationship between the two endpoints determined in *in vivo* exposures.

This study highlights the importance of linking biochemical endpoints such as AChE activity with higher level endpoints like behavioural alterations, increasing the ecological relevance of the effects observed.

Keywords: Acetylcholinesterase activity, Behaviour, Cholinesterases characterization, Ecotoxicology, Marine Snails

Highlights

- Cholinesterases (ChE) were characterized for the first time in *Gibbula umbilicalis*.
- Acetylcholinesterase (AChE) is the predominant ChE form in this sea snail.
- Chlorpyrifos affects *in vitro* and *in vivo* AChE activity in *G. umbilicalis*.
- A positive correlation was shown between AChE inhibition and behaviour.

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1. Introduction

Human activities introduce a myriad of contaminants in the environment on a daily basis, and pesticides, being widely used in agriculture, are some of the most frequently found (Özkara et al. 2016). Although they are usually applied on land, pesticides frequently end up affecting larger areas, with residual concentrations being found in coastal and estuarine environments due to natural processes like runoff, often affecting non-target organisms (Damalas and Eleftherohorinos 2011; Readman et al. 1992).

Organophosphorus (OPs) compounds are still among the most used pesticides in developing countries, being generally accepted as the most effective means for protecting crops against insects (Costa 2018). These compounds are extremely toxic, being able to easily permeate cells and severely modify the neurological responses of organisms (Cao et al., 1999; Saunders et al. 2012). Chlorpyrifos [CPF; O,O-diethyl O-(3,5,6-trichloro-2-pyridyl) phosphorothioate], the active ingredient in the commercial formulation Dursban®, is still one of the most sold OPs in the world having many urban and agricultural crop pest control uses (Lemus and Abdelghani 2000).

Chlorpyrifos acts by inhibiting cholinesterases (ChE) activities and in particular acetylcholinesterase (AChE), an enzyme involved in the termination of nervous impulse transmission by catalyzing the hydrolysis of the neurotransmitter acetylcholine, and therefore playing an essential role in the nervous system coordination (Fournier et al. 1992). The inhibition of this enzyme results in a continuous binding of acetylcholine to the receptor, leading to an overstimulation of this cholinergic receptors, which may result in alterations in behaviour reducing the organisms' ability to move, eat, and reproduce (Azevedo-Pereira 2011). The assessment of this enzyme activity thus confers a high level of ecological relevance potential due to its link with higher levels of biological organization (Dhadialla, Carlson, and Le 1998; Khalil et al. 2013; Lemos et al. 2010; Sandahl et al. 2005; Yen, Donerly, and Linney 2011). *Gibbula umbilicalis* (Costa, 1778) is a small marine gastropod with an extensive geographical distribution, inhabiting the upper intertidal zone on rocky shores. This species is easy to identify, collect, maintain in laboratory and possesses a convenient size, making them suitable organisms for ecotoxicological assays (Cabecinhas et al. 2015).

The main aim of this study was to assess if the inhibition of ChE in *G. umbilicalis*, by sublethal concentrations of CPF would affect its behaviour, in an attempt to link the effects observed at the cellular level with effects at higher levels of ecological relevance. As a first part of this work, a characterization of the ChE present in *G. umbilicalis* was

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performed, as there was no data available on this matter and some studies point to the existence of differences amongst species of molluscs (Talesa et al. 1993; Mora et al., 1999). Cholinesterases are a family of enzymes, traditionally divided in two classes (in vertebrates) based on their properties and functions: acetylcholinesterases (AChEs), generically designated as true cholinesterases, are involved in the regulation of neurotransmission and neuromuscular functioning; and pseudocholinesterases such as butyrylcholinesterases (BChEs) and propionylcholinesterases (PChEs). These ChE forms may be distinguished functionally both kinetically and pharmacologically: AChE has a high affinity for acetylthiocholine (ATCh), being very sensitive to eserine and selectively inhibited by BW284C51 while relatively insensitive to iso-OMPA. Pseudocholinesterases have higher affinity for propionylthiocholine (PTCh) or butyrylthiocholine (BTCh), being sensitive to eserine and selectively inhibited by iso-OMPA, while relatively insensitive to BW284C51 (Eto 1974). The characterization of ChE is important because different ChE may respond differently to anticholinesterase agents such that the measurement of this enzyme activity in a given species using one substrate instead of the other can lead to misinterpretation of results in ecotoxicological studies (Alves 2015).

After ChE characterization, inhibition of ChE activity by the CPF metabolite (chlorpyrifos-oxon – CPO) was validated for this species in an *in vitro* setup. The last part of the work included *in vivo* exposures, where effects of CPF were addressed both on ChE activities and behaviour alterations.

2. Materials and methods

2.1 Test organisms

Gibbula umbilicalis (Costa, 1778), of similar size (10 ± 1mm) were hand-collected from Carreiro de Joannes, a rocky beach in Peniche, central Portugal, with unknown sources of chemical contamination. The organisms were acclimated in the laboratory, for 7 days prior to each experiment, in aquaria with natural seawater at 20 ±1°C, with a 16h :8 h (light:dark) photoperiod. During this period, they were fed *ad libitum* with the green macroalgae *Ulva lactuca* (Linnaeus, 1753). Prior to testing, organisms were kept fasting for 24 hours.

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2.2 Characterization of cholinesterases activity

To characterize *G. umbilicalis* ChE, a total of 6 replicates were used, each containing a pool of 3 organisms. The snails were sacrificed on ice and then homogenized in 15 mL potassium phosphate buffer (0.1 M, pH 7.2). The tissue homogenate of each sample was centrifuged for 3 min at 3000 g (4ºC) and total protein concentration in the supernatant quantified according to the Bradford method (1976), adapted to microplate, using bovine γ-globulin as a standard. Samples were kept at −80 °C until further analysis.

Enzymatic activities were determined according to Ellman et al. (1961), using previously diluted samples to a final protein concentration of 0.8 mg L^{-1}, as described in Alves et al. (2015). In these assays, 250 µL of the reaction solution [30 ml potassium-phosphate buffer (0.1 M, pH=7.2), 1 mL of reagent 5,5'-dithiobis-(2-nitrobenzoic acid) 10 mM (DTNB) and 200 µL of substrate] were added to 50 µL of the diluted sample. The absorbance was measured at 414 nm (25 ºC) during 5 min. All spectrophotometric measurements were performed in triplicates using a microplate reader Synergy H1 Hybrid Multi-Mode (BioTek® Instruments, Vermont, USA).

2.2.1 Substrate affinity

The substrate preferences were investigated by determining the enzyme activity at 12 increasing concentrations (from 0.01 to 20.48 mM) of the substrates acetylthiocholine iodide (ATCh), propionylthiocholine iodide (PTCh), and S-butyrylthiocholine iodide (BTCh) (Sigma–Aldrich, USA). Cholinesterases activity was determined as described in section 2.2, with 200 µL of each substrate concentrations being dissolved in the reaction buffer. For each substrate concentration, blanks were prepared using potassium phosphate buffer (0.1 M, pH 7.2) instead of sample.

2.2.2 Specific inhibitions

Eserine hemisulfate, 1,5-bis(4-allyldimethylammoniumphenyl)pentan-3-one dibromide (BW284c51) and tetra-(monoisopropyl)pyrophosphortetramide (iso-OMPA) (Sigma–Aldrich, USA) were used as selective inhibitors of total ChEs, AChE, and BChE, respectively. ChE activities were measured as described above, using 200 µL of ATCh 0.075 M as substrate (0.4 mM final assay concentration), and testing 6 increasing concentrations of eserine hemisulphate (from 0.781 to 800 µM), BW284C51 (from 0.781 to 800 µM), and iso-OMPA (from 0.016 to 16 mM), which were dissolved in the reaction buffer. The choice for these final assay concentrations were based on ChEs kinetic

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knowledge for other species. For each inhibitor concentration, blank reactions were made using the same volume of potassium phosphate buffer instead of the sample. Control reactions without the inhibitors in the reaction buffer were also performed. The percentage of inhibition was calculated in relation to control, assuming ChE activity in control as 100% (0% inhibition).

2.3 *In vitro* effects of chlorpyrifos on cholinesterases activity

The organophosphate metabolite chlorpyrifos-oxon (CPO) was used to test for the *in vitro* inhibition of ChE (Greyhound Chromatography, UK). Stock solutions of CPO were prepared in ethanol to obtain the final tested concentrations ranging from 0.0365 to 2400 nM. The *in vitro* effect of the pesticide metabolite was evaluated using the method described for selective inhibitors (see Section 2.2.2). The percentage of inhibition was calculated in relation to control, assuming ChE activity in control as 100% (0% inhibition). An extra solvent control was performed using the same solvent concentration as in the maximum tested CPF concentration (0.1%).

2.4 *In vivo* effects of chlorpyrifos on cholinesterases activity and behaviour

To address *in vivo* effects of CPF on *G. umbilicalis*, the commercial formulation Dursban® was used, having chlorpyrifos as active ingredient (23.5 % of a.i.). Firstly, acute effects of this pesticide were assessed to determine lethal concentrations. Based on this information, two sublethal assays were then performed to evaluate: 1) effects on ChE activity; and 2) effects on behaviour (flipping test).

2.4.1 Exposure conditions

For acute assays, sea snails were exposed for 96 h to ten dilutions of the formulation (prepared in filtered seawater), according to the following concentrations of the active ingredient: 0.05, 0.07, 0.10, 0.14, 0.19, 0.26, 0.37, 0.51, 0.72, and 1.00 mg a.i.L^{-1}. Five replicates per treatment were used, including a control treatment with filtered seawater only, and mortality was the endpoint assessed after 96 h.

Based on the acute exposures, half of the LC_{10} was used as the highest concentration for the 96h sublethal bioassays, which were thus performed using the following range of pesticide concentrations: 7.95, 14.7, 27.18, 50.26, 92.95, and 171.9 μg of a.i. L^{-1}. Ten and eight replicates per treatment were used for ChE activity and behavioural parameters, respectively, including a control treatment with filtered seawater only.

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Tests were conducted in a climate-controlled room, at 20 ± 1°C, with a 16 h:8 h (light:dark) photoperiod, and experimental replicates consisted of 60 mL glass flasks, with one organism each, covered with a plastic mesh to prevent organisms from escaping and to ensure constant submersion. During exposure, no food was added, and media was renewed every 24 h to prevent excreta accumulation and any potential volatilization.

2.4.2 Cholinesterases activity

At the end of exposure (96 h), organisms were sacrificed, and after shell removal with the aid of a vise, they were weighed and stored at -80°C until ChE activity measurement. ChE was measured as described in section 2.2, using ATCh 0.4mM as substrate.

2.4.3 Snail behaviour - flipping test

After exposing the organisms for 96 h to the different CPF concentrations, a flipping test was performed using the method previously described by Cabecinhas et al. (2015). Briefly, the snails were withdrawn from the glass flasks, placed into 6-well plastic microplates with clean filtrated seawater (1 snail per well) and were intentionally left with the foot up. The time that each snail took to flip back to an upright position was recorded and considered the behaviour endpoint.

2.5 Statistical analysis

For the ChEs characterization and to assess enzyme affinity to each substrate, the following kinetic parameters were estimated by fitting experimental data to Michaelis–Menten equation: maximal velocity (V_{max}), Michaelis-Menten constant (Km) and their ratio (Vmax/Km), which indicates the catalytic efficiency of the enzyme.

In vitro and in *vivo* inhibition concentration values (IC_{50}) for CPO and CPF, respectively, were calculated using a nonlinear four parameter logistic curve. Differences between treatments in ChE activity with the specific inhibitors, as well as from the *in vitro, in vivo* and behavioural parameter were analysed using one-way analysis of variance (ANOVA). When the criteria of normality and equality were not satisfied, the non-parametric Kruskal-Wallis test was used. Normality and homoscedasticity were checked by Kolmogorov-Smirnov and Levene tests, respectively. To discriminate differences relative to the control group, either Dunnett's or Dunn’s multiple comparisons tests were used as

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post hoc analyses. All statistical analyses were performed with the software Sigmaplot for Windows, version 12 (Systat Software Inc., California, USA).

To estimate lethal concentration values in the acute test (LC_X), as well as median effect concentration (EC_{50}) for the behavioral endpoint, probit and logit regression models were fitted, using SPSS 25.0 for Windows (IBM-SPSS Inc, Armonk, NY).

3. Results and discussion

3.1 Characterization of cholinesterases activity

Enzymatic substrate affinity was tested in the marine snail using three different substrates (Fig. 1A). The substrate with the highest hydrolysis rate was ATCh (maximum ChE activity of 161.4 nmol/min/mg protein), followed by PTCh (99.2 nmol/min/mg protein) and BTCh (4.4 nmol/min/mg protein). Looking at the parameters from the Michaelis-Menten equation (Table 1), the higher ratio Vmax/Km obtained for ATCh (meaning greater catalytic efficiencies) further confirmed the preference for this substrate, followed by PTCh and BTCh.

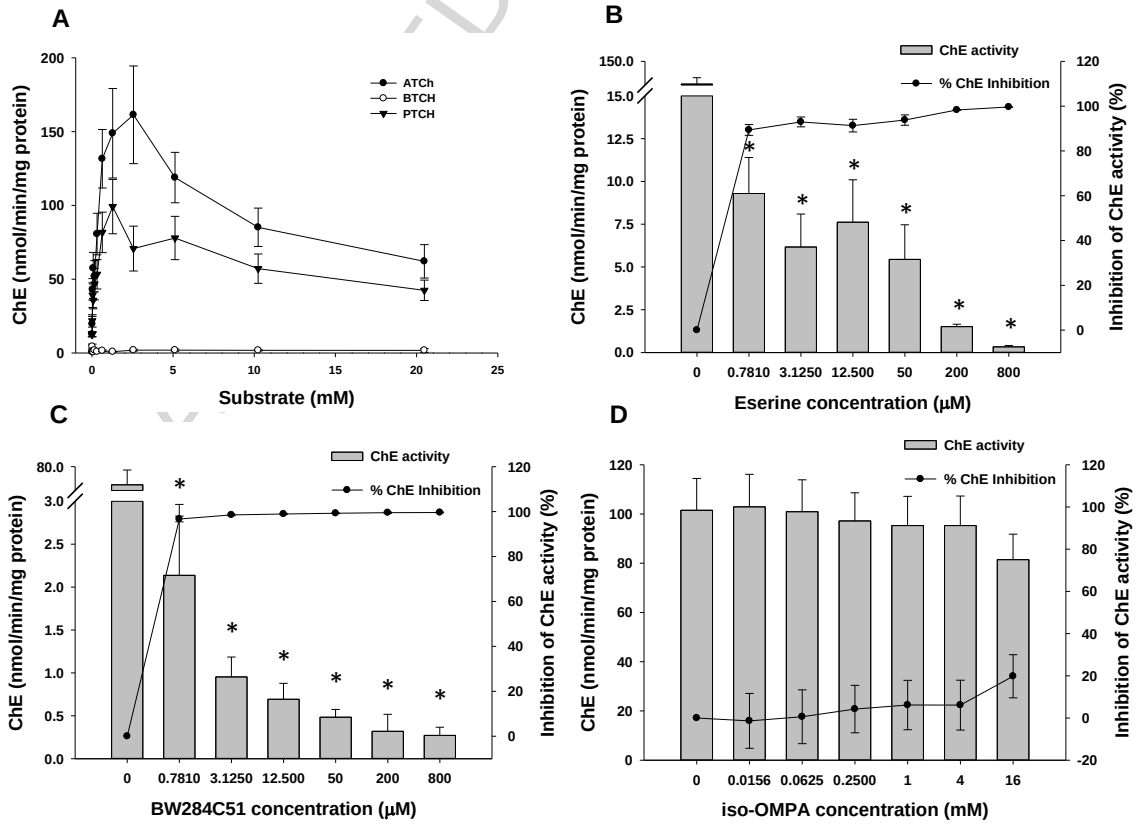

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Figure 1 – Characterization of cholinesterases (ChE) activity in *Gibbula umbilicalis* in terms of substrate affinity (A) and effects of the specific inhibitors: B) eserine, C) BW284C51, and D) iso-OMPA. Activity is expressed as mean values ± SD. ATCh = Acetylthiocholine iodide. BTCh = S-Butyrylthiocholine iodide. PTCh = Propionylthiocholine iodide. Asterisk indicate a significant difference from the control ($p < 0.05$).

Table 1 - Values of the Michaelis-Menten: constant (K_m), maximal velocity (V_{max}) and the catalytic efficiency of ChE (ratio V_{max}/ K_m) for the three substrates tested. Values of the Michaelis–Menten equation are expressed as the mean ± SE.

Substrate	K_m (mM)	V_{max} (nmol/min/mg protein)	V_{max}/K_m
ATCh	0.19 ± 0.038	168.69 ± 8.19	851.11
PTCh	0.13 ± 0.03	97.75 ± 6.16	739.99
BTCh	1.59e-11 ± 0.00	1.61 ± 0.17	1.01e-11

ATCh = Acetylthiocholine iodide; BTCh = S-Butyrylthiocholine iodide; PTCh = Propionylthiocholine iodide.

Additionally, a decrease on ChE activity with excess of substrate could be observed with ATCh and PTCh for concentrations higher than 2.56 and 1.28 mM, respectively (Fig. 1A). This inhibition by excess of substrate is a characteristic of true AChE (Toutant, 1989) and previously reported in gastropods species such as the sea snails *Monodonta lineata* and *Nucella lapillus* (Cunha et al., 2007), or the freshwater snails *Biomphalaia glabrata* (Kristoff et al. 2006) and *Valvata piscinalis* (Gagnaire et al. 2008).

The enzymatic inhibition by excess of substrate, along with substrate preference for ATCh (higher hydrolysis rates and greater catalytic efficiencies), suggests a higher presence of AChE in these organisms, which is also supported by the results on the specific inhibitors detailed below (Fig. 1C, D).

The incubation with eserine showed significant and almost complete inhibition of ChE activity along the tested concentrations, with a significant inhibition of 89% observed already at the lowest concentration tested (ANOVA $F_{6,35}$=224.179, Dunnett's p<0.001; Fig. 1B). This is a clear indication that the enzymatic activity measured was mainly due to cholinesterases and not to other non-specific esterases. Likewise, incubation with

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BW284C51, a specific inhibitor of AChE, also significantly inhibited ChE activity at all tested concentrations (Kruskal-Wallis H_6=37.471, Dunnett's p<0.001), with a complete inhibition of ChE activity (100%) at the highest concentration (800 μM; Fig. 1C). Regarding iso-OMPA, the specific inhibitor of BChE, no significant effects were observed with the concentrations tested (Kruskal-Wallis H_6=11.229, p=0.082; Fig. 1D), although there was a 20% inhibition at the maximum concentration (16mM).

The high sensitivity of these ChE to BW284C51 and the preference for the substrate ATCh, suggests that *G. umbilicalis* possess ChEs with characteristics of typical AChE, which should be the main form present (Kozlovskaya et al. 1993). For this reason, all further results are referred to as AChE activity.

Several studies in invertebrates showed the occurrence of only one or more ChE types with complex kinetic characteristics. Specifically concerning the class Gastropoda, there is a very species-specific variability in the types of ChEs present. For instance, Kristoff et al. (2006) determined AChE to be the main ChE present in *Biomphalaria glabrata*, a freshwater snail. On the other hand, the freshwater gastropod *Potamopyrgus antipodarum* possesses two isoforms of ChE, one with mixed properties of AChE and PChE, and another minor isoform corresponding to a BChE (Gagnaire et al. 2008). In this same study by Gagnaire et al. (2008), the authors also determined that *V. piscinalis* seems to possess only one isoform displaying typical properties of AChE. In the marine gastropods *M. lineata* and *N. lapillus* (Cunha et al. 2007), the ChE present in the soluble fraction of foot tissue homogenates could not be classified as true AChE or PChE since they showed properties typical of both enzymes. This very species-specific types of ChE in gastropods increases the relevance of the present ChE characterization in *G. umbilicalis* for future assessments of pollutant effects on this species.

3.2 *In vitro* effects of chlorpyrifos on acetylcholinesterases activity

In the *in vitro* assay of chlorpyrifos-oxon effects on AChE activity, no statistical differences were observed between the solvent control and control (Student t-test t_{10} = 0.329, p = 0.749; Fig. 2). All statistical comparisons were performed against solvent control.

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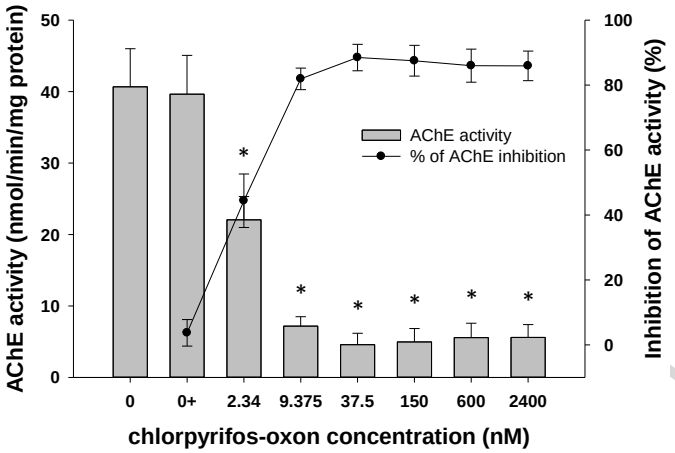

Figure 2 - Acetylcholinesterase (AChE) activity and percentage of activity inhibition (expressed as mean values ± SD) in *Gibbula umbilicalis* exposed *in vitro* to chlorpyrifos-oxon, using acetylthiocholine iodide (ATCh, (0.4 mM) as substrate. Asterisk indicate a significant difference from the solvent control (0+; Dunnett's, $p < 0.05$).

A dose-dependent inhibition of AChE activity with chlorpyrifos-oxon was observed over the range 2.34 - 37.5 nM (ANOVA $F_{7,40}$=139.149, Dunnett's p<0.001) with around 50% inhibition with respect to solvent control at the lowest concentrations (2.34 nM) and over 88% inhibition at 37.5 nM (Fig. 2). A median inhibitory concentration (IC_{50} ± SE) of CPO *in vitro* was estimated to be 2.29 ± 0.17 nM.

This chlorpyrifos oxon IC_{50} value is within the same range of the one reported by Xuereb et al. (2007) in the shrimp *Gammarus pulex* (0.99 nM) but much lower than the ones for the bivalves *M. galloprovincialis* and *Corbicula fluminea* with an IC_{50} of 0.62±0.04 μM and 6.15±0.73 μM, respectively (Mora et al. 1999). Although information concerning gastropods is scarce, these results point to a higher sensitivity of *G. umbilicalis* to the neuronal effects of this pesticide in comparison to bivalves.

3.3 *In vivo* effects of chlorpyrifos

3.3.1 Lethal effects

After the 96-h exposure, the obtained CPF LC_{50} [95% confidence interval] for this snail was 0.33 [0.24-0.44] mg a.i. L^{-1} and the LC_{10} was 0.26 [0.09-0.31] mg a.i. L^{-1}. No mortality was found in the controls.

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In the literature we can find a 96-h LC_{50} of 1.5 ug L^{-1} in the shrimp *Palaemonetes pugio* (Odenkirchen and Eisler 1988), which is lower than the 96-h LC_{50} of 0.247 mg L^{-1} for the bivalve *Donax faba* (JanakiDevi et al. 2013) and the 24-h LC_{50} of 3.19 mg L^{-1} (diferent time) for the brine shrimp *Artemia salina* (Varó et al. 2002), which are closer to the present results.

Larvae of other marine invertebrates have also been found to be more sensitive to this compound, for instance the ones of the spider crab *Maja squinado*, showing a 24-h and 48-h LC_{50} of 22.5 and 0.79 μg L^{-1}, respectively, and of sea urchin *Paracentrotus serratu*s, showing a 24-h and 48-h LC_{50} of 0.35 μg L^{-1} and 0.22 μg L^{-1}, respectively (Bellas et al. 2005).

3.3.2 Effects on AChE activity and behaviour

The *in vivo* exposure to a chlorpyrifos-based formulation showed that this pesticide was capable of exerting significant effects both on AChE activity and flipping behaviour (Fig. 3). During the 96 h of experiment, no mortality was observed for *G. umbilicalis*, neither in controls, nor in treatments.

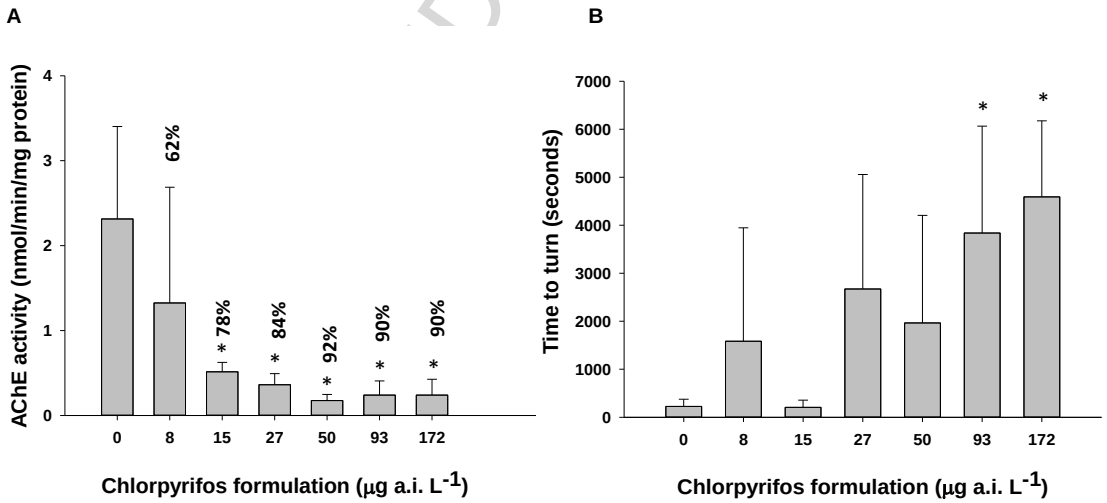

Figure 3 - Acetylcholinesterase (AChE) activity (A) and flipping (B) behavioural tests in *Gibbula umbilicalis* exposed *in vivo* to a chlorpyrifos-based formulation (μg a.i. L^{-1}). Values expressed as mean values ± SD. Asterisk indicate a significant difference from the control ($p < 0.05$). The percentage of AChE inhibition in relation to control is presented above each concentration bar in (A).

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Basal AChE activity of non-exposed *G. umbilicalis* was 2.31 ± 0.34 nmol/min/mg protein (Fig.3A). Similar activity values have been obtained in *A. salina* (2.65 ± 0.15 nmol/min/mg protein) and *Artemia parthenogenetica* (3.69 ± 0.17 nmol/min/mg protein) (Varó et al. 2002). Higher values have been reported for other gastropods. For example, in the gastropod *Hexaple trunculus* an activity of 58.79 ± 8.71 nmol/min/mg protein was measured in the digestive gland and 33.71 ± 5.35 in the muscle (Roméo et al. 2006), and in *M. lineata* and *N. lapillus* values of 110.6 ± 29.7 and 31.8 ± 11.9 nmol/min/mg protein were reported, respectively (Cunha et al. 2007). However, in this latter study different forms of ChEs were possibly being measured, and not exclusively AChE, since the characterization showed mixed properties between AChE and pseudocholinesterases.

Overall, the results showed that AChE activity of the sea snail decreased with increasing CPF concentrations, being significantly inhibited by concentrations equal or higher than 15 µg a.i. L^{-1} (Kruskal-Wallis H_6=51.320, Dunn's p<0.05; Fig. 3A). The chlorpyrifos concentration responsible for the inhibition of the 50% of AChE activity (IC_{50}) was estimated in 5.11 ± 1.84 µg a.i. L^{-1}. However, although the first concentration (8 µg a.i. L^{-1}) induced 62% inhibition in AChE activity, statistical differences to control were only detected after 15 µg a.i. L^{-1}. Strong AChE inhibitions (equal or higher than 90%) was found for concentrations higher than 50 µg a.i. L^{-1}. AChE inhibition in *G. umbilicalis* is in agreement with ChE activity observed in previous CPF exposure studies using other test species. For instance, acute testing with the oysters *Crassostrea corteziensis* showed inhibitory effects at 80 and 160 µg a.i. L^{-1} (Benitez-trinidad et al. 2014), while the effects with the clam *Donax faba* (JanakiDevi et al. 2013) were seen with increasing concentrations, for a 96h period, in the range 79 - 1265 µg CPF L^{-1}. From the described data, it is clear that CPF is also a potent inhibitor of AChE in *G. umbilicalis*, and the severe inhibition of this enzyme which can result neurotoxicity impacts mediated by cholinergic synapses.

Indeed, the flipping speed, a proxy for behaviour, showed significant differences after exposure to the higher concentrations of CPF tested (ANOVA $F_{6,48}$=6.314, Dunnett's p<0.05; Fig.3B). The increase in snails turnover time (the time needed for a snail to right itself after being turned onto its back) was evident at 93 µg a.i. L^{-1} (p<0.003) and 172 µg a.i. L^{-1} (p<0.001) of CPF exposures. In this assay, the snails that flip faster are considered to be in better condition (Cabecinhas et al. 2015), so it can be concluded that in the present study the behaviour of these organisms was affected by the increasing CPF

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concentrations. A slowed righting ability has been previously demonstrated in gastropods under other stressful circumstances (Cabecinhas et al. 2015; Fong et al. 2017; Fong and Hoy 2012; Fong and Molnar 2013; Ford et al. 2018). The EC_{50} [95% confidence interval] for flipping test was estimated as 45.64 [23.84 - 100.7] µg a.i. L^{-1}. When comparing concentrations of effect for these two *in vivo* exposure endpoints, it is possible to deduce that AChE was a more sensitive parameter than behaviour alterations measured through flipping test. These results are in accordance with the general knowledge that effects at a lower biological level precede and should be detectable earlier and at lower concentrations than effects at higher levels of organization (Lemos et al. 2010).

A correlation analysis between both measured endpoints also showed that AChE activity is significantly and negatively correlated with the snails' flipping ability (Pearson correlation, $r^2 = -0.376$, $p = 0.0046$). Given the present results, it can be hypothesised that the altered flipping behaviour of snails exposed to CPF may have been caused by the accumulation of the neurotransmitter acetylcholine in the synaptic junctions, which interferes with coordination between the nervous and muscular junctions (neurotoxicity). Previous studies have already shown the linkage between AChE inhibition with effects at higher levels of biological organization. Cabecinhas et al. (2015), for instance, also reported a negative correlation between AChE inhibition and flipping behaviour using this same marine snail species exposed to the metal mercury. Relationships between AChE inhibition and alteration in locomotor behaviour were reported in vertebrates exposed to pesticides, such as the juvenile coho salmon (*Oncorhynchus kisutch*) exposed to CPF (Sandahl et al. 2005) and *Oncorhynchus mykiss* exposed to diazinon and malathion (OPs) (Beauvais et al. 2000). Cooper and Bidwell (2006) observed a reduced capacity of *Corbicula fluminea* exposed to CPF to burrow into the substrate in parallel to AChE inhibition. Xuereb et al. (2007) also reported relations between AChE inhibition and changes in feeding and locomotor behaviours in amphipods *Gammarus fossarum* exposed to CPF. This mechanistic link is particularly straightforward in OP pesticides, which have a specific mode of action (AChE inhibition). Chlorpyrifos can disrupt the structure of the enzyme by attacking the active serine hydroxyl group of AChE, increasing acetylcholine levels. This can subsequently lead to the increase of catecholamines, which are involved in glicogenolysis and glycogen synthesis, thus interfering in the energy metabolism of nerve cells, and ultimately in behaviour (Üner et al., 2006).

By affecting normal behaviour, inhibition of AChE activity can result in alteration of chances to survive. For example, it can be expected that changes in *G. umbilicalis*

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capacity to turn over, lead to increased drift and disruption in their escape reaction from predators, feed, or even react in highly hydrodynamic areas, as observed in other studies (DeWhatley and Alexander, 2018; Ford et al., 2018) These disruptions at the individual level can ultimately impact responses at the population, community, and the ecosystem-levels.

5. Conclusions

In summary, results indicate that the main ChE form present in the marine snail *G. umbilicalis* is AChE. This is important knowledge for the successful application of this biomarker in future environmental biomonitoring surveys using this gastropod. Also, CPF as an organophosphate pesticide widely used for agricultural purposes and pest control, led to severe *in vivo* and *in vitro* AChE inhibition in this sea snail. Moreover, *in vivo* enzymatic inhibition was observed in the same pesticide treatments that induced alterations in the organisms' behavioural capacity to turn to their normal position, reflecting a negative correlation between both endpoints. This study provides the basis to interpret AChE inhibition in *G. umbilicalis* as a predictive endpoint of effects that might occur at higher ecologically relevant levels, such as behaviour, making it a sensitive representation of the organism´s neuro-physiological responses to environmental stressors.

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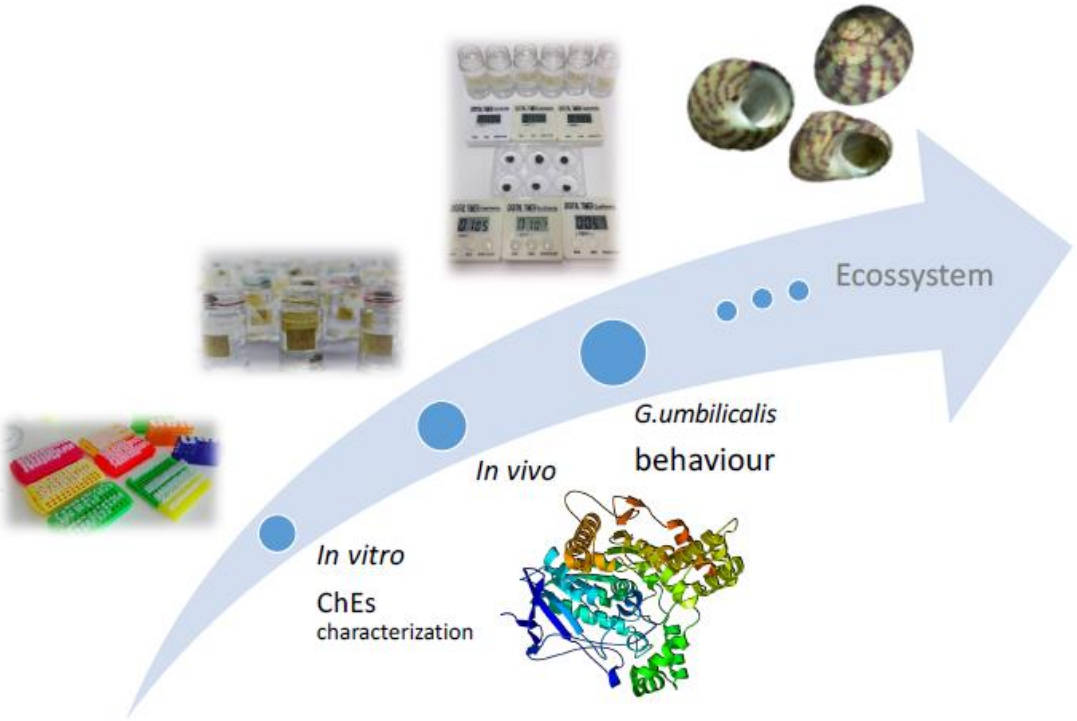
Graphical abstract



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