Cholinesterase activities as potential biomarkers: characterization in two freshwater snails, Potamopyrgus antipodarum (Mollusca, Hydrobiidae, Smith 1889) and Valvata piscinalis (Mollusca, Valvatidae, Müller 1774)

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Chemosphere, vol 71, n°3, p 553-560, doi:10.1016/j.chemosphere.2007.09.048

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Chemosphere, vol 71, n°3, p 553-560, doi:10.1016/j.chemosphere.2007.09.048

Abstract

Anticholinesterase insecticides constitute a major portion of modern synthetic pesticides and the assessment of cholinesterase (ChE) inhibition is widely used as a specific biomarker for evaluating the exposure of non-target organisms to these pollutants. However, most studies on this biomarker were developed on vertebrates and among invertebrates, gastropod mollusks are rarely used. However, gastropods are important members of aquatic habitats and therefore present a high ecological relevance for freshwater ecosystems. In this context, ChE activities were characterized in two freshwater gastropod mollusks, *Potamopyrgus antipodarum* and *Valvata piscinalis*, in order to ascertain their value as sentinel species. Firstly, characterization of ChE activities was performed using different substrates (acetylcholine iodide, butyrylcholine iodide and propionylcholine iodide) and specific inhibitors (eserine, *iso*-OMPA and BW284c51). Secondly, i*n vivo* effect of a widely used organophosphate insecticide, chlorpyrifos, was tested on ChE activity in both species. Results suggested that *P. antipodarum* possesses two isoforms of cholinesterases, one isoform which properties are intermediate between an acetyl and a propionyl ChE, and one minor isoform which correspond to a butyryl ChE, while *V. piscinalis* seems to possess only one isoform which displays typical properties of an acetyl ChE. Chlorpyrifos induced no effect on *V. piscinalis* ChE. In contrast, *P. antipodarum* activity was significantly decreased by environmental realistic chlorpyrifos concentrations (2.86 and 14.2 nM) after seven days of contact. The present study suggests that *P. antipodarum* may be employed as a biological indicator for assessing pesticide contamination.

Keywords

Cholinesterase activity; substrates; *Potamopyrgus antipodarum*; *Valvata piscinalis*; chlorpyrifos; biomarker.

Introduction

The measurement of the exposure to pollution and of the biological effects of toxicants has become of major importance for the assessment of the quality of the environment (van der Oost et al., 2003). The use of biological markers at the molecular or cellular level have been proposed as sensitive 'early warning' tools for biological effect measurement (van der Oost et al., 2003). This approach has been widely used both *in vivo* and *in vitro* for the evaluation of xenobiotic effects on animals (Binelli et al., 2006).

Among anthropogenic contaminants, pesticides are widely detected in freshwater and estuarine ecosystems. These molecules are spread on terrestrial cultures and enter waterways from agricultural and urban run-off. Pesticides may have major ecological consequences (Ozretic and Krajnovic-Ozretic, 1992). The organophosphates (OPs) and carbamates (Cs) are modern synthetic insecticides and are potent neurotoxic molecules (Ashauer et al., 2006). They exert acute toxicity by blocking the breakdown of acetylcholine by the enzyme acetylcholinesterase (AChE: E.C.3.1.1.7) in vertebrate and invertebrate organisms (Fulton and Key., 2001). Acetylcholine is the primary neurotransmitter in the sensory and neuromuscular systems in most species. The activity of this system is vital to muscular function and represents a prime target on which OPs and Cs can exert a detrimental effect (Sarkar et al., 2006).

Monitoring AChE activity in wildlife populations has been proposed as a general method for detecting environmental contamination from OPs and Cs, particularly since many of these chemicals have relatively short half-lives in the aquatic environment and are not water soluble. The World Health Organization (Paris) recognizes AChE biomonitoring as a preventive measure against OP overexposure in nontarget species (Romani et al., 2005). Its use as a specific biomarker to assess the exposure of aquatic organisms to these compounds is

widely applied in laboratory and field studies (Bocquené et al., 1997; Scaps et al., 1997; Galloway et al., 2002; Binelli et al., 2006).

In vertebrates two isoforms occur, acetylcholinesterase (AChE) which preferentially hydrolyses acetyl esters such as acetylcholine, and butyrylcholinestérases (BChE) which preferentially acts on butyrylcholine. The main function of AChE is the rapid hydrolysis of the neurotransmitter, whereas BChE has no known specific natural substrate, although it is able to hydrolyse acetylcholine (Fulton and Key, 2001; Valbonesi et al., 2003). Another isoform, propionylcholinesterase (PChE), has been characterized (Mora et al., 1999). Since the properties of ChE may differ between species, it is important to characterize the type of enzyme present in the species studied before its use as a biomarker (Kristoff et al., 2006).

Whilst ChEs have been extensively studied in vertebrates and insects, few data are available in molluscs (Mora et al., 1999). Molluscs, in particular bivalves, are often used as sentinel organisms: their world-wide distribution, their sedentary mode of life and their filter-feeding behaviour susceptible to induce pollutant bioaccumulation make them ideal species for the assessment of environmental pollution (Rittschof and McClellan-Green, 2005). Prosobranch snails including *Potamopyrgus antipodarum* (Hydrobiidae) and *Valvata piscinalis* (Valvatidae) are important members of aquatic habitats and possess a high ecological relevance for freshwater ecosystems (Mouthon and Charvet, 1999). They have proved to be sensitive test organisms in several studies (Oetken et al., 2005) and *P. antipodarum* has been recommended for toxicity tests by the Invertebrate testing group of OECD (Duft et al., 2007). Using these animals might facilitate the linking of laboratory data to field studies and field experiments could be undertaken on autochthonous or caged animals.

The aim of this study was to characterize the ChE of mudsnails and to investigate the relevance of ChE activities as early warning tools of neurotoxic stress in two freshwater mudsnails. Activities in *P. antipodarum* and *V. piscinalis* were firstly characterized *in vitro* by

using different substrates (acetylthiocholine (ASCh), propionylthiocholine (PSCh) and butyrylthiocholine (BSCh)) and specific inhibitors (eserine for ChE, BW284c51 for AChE, *iso*-OMPA for BChE). Secondly, *in vivo* effects of a model insecticide, chlorpyrifos, on ChE activities were then studied in order to assess the value of *P. antipodarum* and *V. piscinalis* as sentinel species of freshwater insecticide contamination.

Material and Methods

Chemicals

Acetylthiocholine iodide (ASCh), butyrylthiocholine iodide (BSCh), propionylthiocholine iodide (PSCh), 5,5-dithio-bis-2-nitrobenzoate (DTNB), eserine, BW284c51 (1,5-bis(4-allydimethylammoniumphenyl)-pentan-3-one dibromide), *iso*-OMPA (tetra-(monoisopropyl)pyrophosphor-tetra-mide) and chlorpyrifos were obtained from Sigma-Aldrich (Villefranche, France).

Organisms

P. antipodarum and *V. piscinalis* were obtained from the laboratory culture established in the laboratory (CEMAGREF, Lyon, France). Animals were reared under standard conditions in aerated glass aquariums (17-20 L), at a temperature of 22 ± 1 °C, and under a 16-8 h artificial light-dark photoperiod regime. For the cultures, animals were fed using Tetramin®. For all experiments, adult snails of similar size (4 mm) were used.

Cholinesterase activity

The whole animals with shell were weighed and homogenized with an Ultra-Turrax T25 basic® at 24 000 rpm for 40 seconds in 1:10 (W:V) for *V. piscinalis* and 1:20 for *P. antipodarum* 0.1 M phosphate buffer, pH 7.8, plus 0,1% Triton X-100. Homogenates were centrifuged at 9,000×*g* for 15 min at 4 °C. Supernatants were used as the enzyme source.

The enzyme activity was measured following the Ellman method (1961). In a typical assay, 330 µL of 0.1 M phosphate buffer pH 7.8, 20 µL of 0.0076 M the chromogenic agent DTNB and 20 µL of sample were successively added in a 96 wells microtitre plate. Measurement of enzyme activity was initiated by the addition of ten µL of freshly prepared acetylthiocholine iodide solution in distilled water. Absorption of the 2-nitro-5-thiobenzoate anion, formed

from the reaction, was then recorded at 405 nm every 60 s for 9 min at room temperature using a TECAN® *Safire*® spectrofluorimeter. Spontaneous substrate hydrolysis was assessed using a blank without sample. Kinetic was calculated in the linear range. Each sample was analyzed in triplicates. Total protein was determined according to the Lowry method (1951), using bovine serum albumin as standard. Enzyme activity was expressed as nmol ASCh hydrolysed min^{-1} mg^{-1} of protein.

Substrate affinity

Substrate preference in supernatants obtained from control organisms was assessed using ASCh, BSCh and PSCh as substrates. Fifteen animals sampled in our laboratory culture were individually homogenized and supernatants were pooled and used as samples. The effects of increased substrate concentration on supernatant ChE activity were determined with concentrations of ASCh, BSCh and PSCh ranging from 0.0625 to 8 mM. Three replicates of each substrate were performed.

Specific inhibitors

Eserine, *iso*-OMPA and BW284c51 were used as specific inhibitors of ChEs, BChEs and AChEs, respectively. Eserine and *iso*-OMPA were dissolved in ethanol and BW284c51 was dissolved in distilled water. Ten animals were individually homogenized and supernatants were pooled. Supernatants were then incubated 30 minutes at 20 °C with inhibitor or water or ethanol for eserine and *iso*-OMPA (1%). Final inhibitor concentrations ranged from 0.01 to 100 µM for eserine and from 0.1 to 1000 µM for *iso*-OMPA and BW284c51. Effects of inhibitors on ChE activities were assessed using ASCh, BSCh and PSCh as substrates.

Chlorpyrifos exposure

A seven days semi-static bioassay was performed, and the survival and the ChE activity of the snails were followed in the course of the experiment. Snails were placed in glass beakers filled with 300 mL of drilled ground water. Snails of both species were placed in the same beakers, one day before the beginning of the contamination experiment for acclimatization. Stock solutions of chlorpyrifos were prepared daily by dissolving chlorpyrifos in acetone used as solvent, and diluted in an appropriate amount of drilled ground water, using serial dilution. The concentration of acetone was kept at 0.05 ‰ in all pesticide solutions used. Solvent (acetone) and solvent-free (drilled ground water) controls were included in the test design. Water in beakers was renewed daily. For each concentration, five replicates with ten animals of each species were carried out. One beaker of each nominal chlorpyrifos concentration was added for chemical analyses. No food was added during the experiment. Tests were performed as in rearing conditions.

Snails were exposed to three nominal chlorpyrifos concentrations (0.14, 2.86 and 14.2 nM, which correspond to 0.05, 1 and 5 $\mu g.L^{-1}$). For each concentration, one individual of each species were sampled on each of the five beaker per condition at 0, 24, 96 and 168 h and immediately frozen at –80 °C until analysis.

Measurement of chlorpyrifos concentration in water

Samples for chlorpyrifos analyses were collected every day, 10 minutes and 24 h after the water renewal in two beakers of each contamination levels (0.14, 2.86 and 14.2 nM). Chlorpyrifos was quantified after direct injection in LC-MS-MS. Chlorpyrifos ethyl standards were purchased from Riedel De Haën (Sigma Aldrich, France). Standard stock solutions were prepared by dissolving 5 mg of accurately weighed reference standard in 50 mL acetone. The stock solutions were diluted with ultrapure water (Milli-Q, Millipore) for LC-MS-MS analysis standards.

Water samples were collected in glass bottles and then filtered on 0.20 µm polyester filters (Chromafil PET 20/15 MS, Macherey-Nagel, Hoerdt, France). 990 µL of filtered water was added to 10 µL of deutered diuron (D6) used as injection standard.

Liquid chromatography was performed on an Agilent Series 1100 HPLC system (Agilent Technologies, Les Ulis, France). Chromatographic separation was achieved using a Synergi Fusion-RP 80A analytical column (4 µm particle size, 2 mm x 50 mm) from Phenomenex (Le Pecq, France), at a flow rate of 200 µL min^{-1} with mobile phase consisting of acetonitrile and water (80/20, v/v), both with 0.1% v/v formic acid. Injection volume was 100 µL. The HPLC system was interfaced to a triple quadripole mass spectrometer (API 4000, Applied Biosystems, Les Ulis, France). The following transitions 352→200 and 350→198 m/z were used respectively for quantification and confirmation of chlorpyriphos ethyl. Quantification was performed by internal calibration using diuron D6.

Data analysis

Results were expressed as means ± standard error. Values were transformed (log X) to achieve normality when necessary. Data were analysed using ANOVA on Statgraphics® Centurion version XV.II software. Significance was set at $p \leq 0.05$. In the case of rejection of H_0, an *a posteriori* LSD (Least Significant Difference) test was applied. When data showed a concentration-dependant relationship, the median inhibitory concentration (IC_{50}) was calculated by logistic curve-fitting procedure using REGTOX® (http://eric.vindimian.9online.fr). The Michaelis-Menten constant (K_m) and the maximum velocity of substrate hydrolysis (V_{max}) were calculated using GOSA® software (http://www.bio-log.biz).

Results

In vitro experiments

Substrate affinity

For *P. antipodarum*, measured esterase activities depended on the substrate. The reaction rate increased with increasing substrate concentration, with ASCh > PSCh > BsCh ($p<0.05$) (**Figure 1a**). However, for the highest concentrations (>2 mM), no differences were measured between activities with ASCh and PSCh (**Figure 1a**). On the same way, increased ChE activity were measured for *V. piscinalis,* according to the substrate, with ASCh > PSCh > BsCh ($p<0.05$) (**Figure 1b**). No inhibitory effect was observed whatever the substrate used for both species. At the highest substrate concentration (8 mM) enzymatic activities (expressed as $nmol.min^{-1}.mg^{-1}$ protein) for *P. antipodarum* were 31.6 ± 0.2 for ASCh (100%), 30.2 ± 1.2 for PSCh (95.5%) and 2.7 ± 0.3 for BSCh (8.5%). For *V. piscinalis,* enzymatic activities were 17.3 ± 0.5 for ASCh (100%), 9.1 ± 0.7 for PSCh (52.6%) and 3.9 ± 0.3 for BSCh (22.5%). 4 mM was defined as the optimal concentration for ASCh and PSCh for both species .

ChE activities followed the Michaelis-Menten kinetic when ASCh and PSCh were used as substrates for both species (**Figure 1**). Kinetics parameters (K_m, V_{max}, and V_{max}/K_m) are reported in **Table 1**. K_m values were ten times higher for *V. piscinalis* than for *P. antipodarum*, whatever the substrate used.

Specific inhibitors

Eserine decreased significantly ChE activities measured with the three substrates for the two species (**Figure 2 a&b**). However, at 100 µM of eserine, inhibition was lower for BSCh than for ASCh and PSCh for both snails. The inhibition profiles of ASCh and PSCh were similar for *P. antipodarum* and *V. piscinalis*. Nevertheless, whatever the substrate used, the IC_{50}

values of eserine were lower for *P. antipodarum* (0.034, 5.29 and 0.024 µM) than for *V. piscinalis* (1.39, 8.68 and 1.40 µM) for ASCh, BSCh and PSCh, respectively.

Iso-OMPA only induced a significant decrease butyrylcholinesterase activity of *P. antipodarum* (**Figure 2 c&d**). However, 50% of inhibition was not reached in our experiment).

BW284c51 significantly decreased the activities obtained for ASCh and PSCh for both gastropod species. For BSCh, the activity was significantly decreased only for *V. piscinalis* (**Figure 2 e&f**). The inhibition profiles of ASCh and PSCh and the IC_{50} values were similar for *P. antipodarum* (290.4 and 387.1 µM) and *V. piscinalis* (150.2 and 262.2 µM) for ASCh and PSCh, respectively.

In vivo *experiments*

Concentration of chlorpyrifos in water

Chlorpyrifos concentrations in water were analyzed after 10 minutes and 24 h in the experimental conditions. Measured concentrations of chlorpyrifos for the three nominal concentrations (0.14, 2.86 and 14.2 nM) were 0.28 ± 0.02, 2.65 ± 0.2 and 13.12 ± 0.8 nM 10 minutes after water renewal, respectively, and 0.22 ± 0.02, 1.48 ± 0.1, 5.98 ± 0.4 nM 24 h after water renewal, respectively (data not shown). After 24 h of contamination, a decrease of concentration was observed: chlorpyrifos measured concentrations were 58.1, 39.5 and 27.2% of the concentrations measured at 10 minutes (data not shown).

In vivo effects of chlorpyrifos on ChE activity

During the seven days of experiment, no mortality was reported for *P. antipodarum*, neither in controls, nor in contaminated beakers. A slight mortality was registered for *V. piscinalis* (5-

246 6% of cumulative mortality), but this was not significantly different between controls and
247 contaminated animals (data not shown).
248 *In vivo* exposure to chlorpyrifos led to inhibition of ChE activity in *P. antipodarum* (**Figure**
249 **3a**). Decrease of activity was time and dose-dependent. After 24 h and 96 h of exposure,
250 activity was significantly decreased for 14.2 nM compared to control. Values were 11.4 and
251 4.9 nmol ASCh.min^{-1}.mg^{-1} protein, respectively, which represented an activity of 63.3 and
252 31.5% of the control (100%). At 168 h, activity was significantly decreased for 2.86 and 14.2
253 nM compared to control. Values were 5.9 and 2.9 nmol ASCh.min^{-1}.mg^{-1} protein,
254 respectively, which represented an activity of 40.5 and 20.2% of the control (100%). IC_{50}
255 values of chlorpyrifos were 16.34, 9.71 and 3.15 nM at 24, 96 and 168 h, respectively.
256 No significant inhibition occurred for *V. piscinalis*, however a slight significant increase
257 occurred for nominal concentration of 0.14 nM after seven days of contamination ($p<0.05$,
258 **Figure 3b**).
259

Discussion

The level of ChE activity obtained for our species (31.6 for *P. antipodarum* and 17.3 nmol.min^{-1}.mg^{-1} protein for *V. piscinalis*) was quite similar to those reported in literature for several bivalve species (between 3 and 20 nmol.min^{-1}.mg^{-1} protein) (Bocquené et al., 1997; Najimi et al., 1997; Mora et al., 1999; Valbonesi et al., 2003; Binelli et al., 2006). Literature reports AChE activities values of 20-45 nmol.min^{-1}.mg^{-1} protein for annelids (*Eisena andrei* (Caselli et al., 2006), *Nereis diversicolor* (Scaps and Borot, 2000)). However, an AChE activity of 320 nmol.min^{-1}.mg^{-1} protein was reported in *Lumbriculus variegatus* (Kristoff et al., 2006). A few studies reported AChE level for other gastropods. The basal AChE activity of the bloodfluke planorb, *Biomphalaria glabrata* was 45 nmol.min^{-1}.mg^{-1} protein (Kristoff et al., 2006) and was 60 nmol.min^{-1}.mg^{-1} protein for the murex, *Hexaplex trunculus* (Romeo et al., 2006).

Vertebrate cholinesterases have been classified into two groups, acetyl ChE and butyryl ChE, depending on substrate hydrolysis and sensitivity to inhibitors. AChE hydrolyses ASCh much faster than other choline esters, like PSCh, and is inactive on BSCh, whereas BChE hydrolyses both BSCh and ASCh at an appreciable rate (Valbonesi et al., 2003). Several studies show that situation of invertebrate cholinesterases is more complex. ASCh has been reported as the preferential substrate for most bivalves including oysters (Bocquené et al., 1997; Valbonesi et al., 2003), marine and freshwater mussels (Mora et al., 1999; Romani et al., 2005; Binelli et al., 2006), as well as for *E. andrei* (Caselli et al., 2006), *B. glabrata* (Kristoff et al., 2006), and the common shredder, *Gammarus pulex* (Xuereb et al., 2007).

Both species of gastropods studied here showed different affinities toward the three substrates used (ASCh, PSCh and BSCh). At high concentrations of substrates, *P. antipodarum* cholinesterase presented the same affinity for ASCh and PSCh. *V. piscinalis*

presented a higher affinity for ASCh. However, the enzymatic activity level was lower for *V. piscinalis*. In our study, gastropods revealed a low BSCh hydrolysis.

Comparative analysis of the *K*m values in *P. antipodarum* indicated that ChE affinity for ASCh and PSCh were in the same range of that reported for *E. andrei* (0.18 and 0.14 mM for ASCh and PSCh, respectively) (Caselli et al., 2006) and the Pacific oyster, *Crassostrea gigas* (0.124 mM for PSCh) (Bocquené et al., 1997), while *V. piscinalis* showed higher values, closer to those reported in the blue mussel, *Mytilus edulis* (1.3 mM for ASCh) (Galloway et al., 2002). Moreover, these values appeared one order of magnitude higher than that generally reported for bivalves: 50-93 µM for *Ostrea edulis*, *Mytilus galloprovincialis*, *Crassostrea. gigas* (ASCh), *Corbicula fluminea* (PSCh), *Perna perna* (Bocquené et al., 1997; Najimi et al., 1997; Mora et al., 1999; Valbonesi et al., 2003). High *K*m values represent lower ChE affinity by substrate. Therefore, ChE activities of *V. piscinalis* presented lower affinity to substrates than *P. antipodarum* ones.

*V*max values were similar for both species. For *P. antipodarum,* the ASCh ratio *V*max/*K*m was in the same range that those reported in *M. galloprovincialis* (0.24 $mL.min^{-1}.mg^{-1}$ protein) (Valbonesi et al., 2003) and *E. andrei* (0.25 $mL.min^{-1}.mg^{-1}$ protein) (Caselli et al., 2006). ASCh *V*max/*K*m ratio for *V. piscinalis* was closer to *O. edulis* one ($5.1.10^{-2}$ $mL.min^{-1}.mg^{-1}$ protein) (Valbonesi et al., 2003). These results indicated that *V. piscinalis* enzyme has a lower efficiency of hydrolysis than *P. antipodarum*, in agreement with the lower activity observed and with the lower substrate affinity, as discussed before.

Enzymatic activity observed with BSCh was inhibited for both species by eserine, a cholinesterase inhibitor. Enzymatic activities measured using ASCh and PSCh were almost totally inhibited by eserine in both species. I*so*-OMPA, a specific inhibitor of BChE in vertebrates (Bocquené et al., 1997), did not modified this activity in *V. piscinalis*. However, a

significant inhibition occurred in *P. antipodarum*. An important decrease was observed in both species with BW284c51, specific inhibitor of AChEs in vertebrates (Caselli et al., 2006). The whole results suggest that *V. piscinalis* possesses a single ChE isoform, which presents all the properties of a vertebrate AChE: high preference for ASCh and low for BSCh; high sensitivity to eserine and BW284c51, but not to *iso*-OMPA. On the contrary, more complex isoforms of ChE seem to coexist in *P. antipodarum*, one major isoform presenting properties intermediate between an AChE and a PChE, and another minor isoform presenting properties of a BChE.

IC_{50} reported for eserine in *P. antipodarum* were in the same range than the values reported for other invertebrates (0.01, 0.01, 0.14, 0.020 and 0.014 µM for *B. glabrata, L. variegatus, O. edulis, M. galloprovincialis* and *E. andrei*, respectively) (Valbonesi et al., 2003; Caselli et al., 2006; Kristoff et al., 2006). However, values for *V. piscinalis* were higher. These results suggest that *V. piscinalis* ChEs are less sensitive that in *P. antipodarum.*

In our experiments, we measured effects of chlorpyrifos on *P. antipodarum* and *V. piscinalis.* Chlorpyrifos is a widely used organophosphate insecticide and is the active ingredient in a number of commonly used household and agricultural insecticide formulations (Fulton and Key, 2001). It is volatile and concentration decreases rapidly in water in constant exposure conditions. For these reasons, we performed the laboratory experiment using semi-static conditions. Nevertheless, an important decrease of chlorpyrifos level was measured after 24h. A similar 50% loss was reported in a contamination experiment of *G. pulex* to 0.3 nM of chlorpyrifos: concentration reached 0.17 nM after 24 h of exposition (Ashauer et al., 2006).

Chlorpyrifos was tested in this study at concentrations ranging from 0.14 to 14.2 nM (0.05-5 $\mu g.L^{-1}$). Measured concentrations of chlorpyrifos in surface waters often fall at concentrations

below the nM level (USEPA, 2002). However, studies showed that chlorpyrifos concentrations in small streams and wetlands adjacent to agricultural fields could range from 0.2 to 2 µM (Mazanti et al., 2003). Moreover, the relatively short-half life of chlorpyrifos in water may result in underestimate levels of exposure (Mazanti et al., 2003).

We demonstrated the dose-response and time-dependant effects of chlorpyrifos on *P. antipodarum*: for 14.2 nM, inhibition was 40% of the control after 24 h of contact and increased to 80% of the control after 168 h of contact, without significant mortality. However, an increase was shown on *V. piscinalis*. Chlorpyrifos have already been shown as a powerful AChE inhibitor in invertebrates. Chlorpyrifos decreased AChE activity after 96 h of contact in *D. polymorpha* (0.03 nM) (Binelli et al., 2006) and in *C. fluminea* (80% inhibition at 1.4 and 2.8 µM) (Cooper and Bidwell, 2006). Chlorpyrifos also decreased *in vitro* AChE activity in *M. edulis* (Galloway et al., 2002) and in *in vivo* exposures in the midge, *Chironomius riparius* (Callaghan et al., 2001) and in rat brain (Hancock et al., 2007), which agrees with our results. On the contrary, this OP increased AChE activity in *S. inaequivalvis* after 15 days of exposure to 0.3 nM (Romani et al., 2005), which comforts our results on *V. piscinalis*; however, the biological explanation remains unknown. IC_{50} of chlorpyrifos for AChE was 9.71 nM in *P. antipodarum* at 96 h, which was ten times higher that for *G. pulex* in the same experimental conditions (Xuereb et al., 2007).

High resistance of *V. piscinalis* to eserine *in vitro* and to chlorpyrifos *in vivo* may be related to the lower ChE affinity to the substrate. On the contrary, high substrate specificity and high sensitivity to eserine *in vitro* and chlorpyrifos *in vivo* would suggest that *P. antipodarum* ChEs could be very sensitive to anticholinesterase agents.

In our study, we showed that inhibition of AChE happened for *P. antipodarum* without any mortality. The relationship between AChE inhibition and mortality in

invertebrates is generally less well established than in vertebrates. No mortality was observed after an environmental contamination with azinphos-methyl in *B. glabrata* et *L. variegatus* (Kristoff et al., 2006), even though inhibition of AChE reached between 35 and 99% of control. However, a high mortality was observed in *N. diversicolor* contaminated with parathion and malathion when 55% of AChE inhibition occurred (Scaps et al., 1997). A high mortality and 70% of AChE inhibition also occurred in *G. pulex* exposed to chlorpyrifos (Xuereb et al., 2007). More research is needed to clarify the relationships between OP exposure, AChE inhibition and mortality.

P. antipodarum AChE could be an useful biomarker of pesticide contamination as its inhibition occurred at low concentration without mortality. Therefore, *P. antipodarum* could be used in field contamination assessment. However, ChE activities may be differentially modulated depending on the pollutant tested (Ozretic and Krajnovic-Ozretic, 1992). To better assess the interest of *P. antipodarum* as field sentinel species, we need to confirm its sensitivity to several anti-cholinesterase compounds and in different exposure conditions. Moreover, as pesticide contamination in the field is a discontinuous phenomenon, it will be necessary to test recovery of AChE activity after a contamination and to assess the effects of successive contamination exposure.

Conclusion

The aim of our work was to characterize *P. antipodarum* and *V. piscinalis* ChEs. Our results show that *P. antipodarum* possesses several isoforms of ChEs, one undifferentiated between an AChE and a PChE, and another one which could be assimilated as a BChE. *V. piscinalis* seems to possess only one isoform close to the vertebrate AChE. Our results also illustrate the relative insensitivity of ChE activity following *V. piscinalis* exposure to environmental

382 concentrations of chlorpyrifos. On the contrary, the present study gives valuable indications
383 for selecting *P. antipodarum* in biomonitoring programs.
384 Laboratory studies generally do not take into account natural stressors, including fluctuations
385 in biotic and abiotic factors, which could have effects on AChE activity (Bocquené et al.,
386 1997). Some complementary experiments are needed in order to identify the factors inducing
387 ChE variability (age, season) in order to make the difference between effects due to chemical
388 exposure and the natural variability.
389

390 **Acknowledgments**

391 R. Mons and H. Queau are acknowledged for their technical assistance during chlorpyrifos
392 contamination. C. Guillemain is acknowledged for the chlorpyrifos analyses.
393

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476 **Figure captions**

477 Figure 1: substrate affinity of *P. antipodarum* (a) and *V. piscinalis* (b) ChEs measured at
478 increasing concentrations of ASCh, PSCh and BSCh. Values are means of three replicates.
479 Standard error is presented. a, b, and c represent significant differences between substrates at
480 $p<0.05$ (a>b>c).

481

482 Figure 2: effects of eserine (a & b), *iso*-OMPA (c & d) and BW284c51 (e & f) on ChE
483 activity in *P. antipodarum* (a, c & e) and *V. piscinalis* (b, d & f). Standard error is presented.
484 *: $p<0.05$; ***: $p<0.001$.

485

486 Figure 3: inhibition percentages of ChE activities for *P. antipodarum* (a) and *V. piscinalis* (b)
487 during *in vivo* contamination with chlorpyrifos. Values are means of five replicates. Standard
488 error is presented. ***: $p<0.001$.

489

490

491 Table 1. Michaelis-Menten constant (K_m) and maximum rate of substrate hydrolysis (V_{max}) of
492 ChEs of *P. antipodarum* and *V. piscinalis*. Results are expressed as the mean ± SE of three
493 replicates.