



**HAL**  
open science

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

B. Gagnaire, Olivier Geffard, B. Xuereb, C. Margoum, J. Garric

► **To cite this version:**

B. Gagnaire, Olivier Geffard, B. Xuereb, C. Margoum, J. Garric. Cholinesterase activities as potential biomarkers: characterization in two freshwater snails, *Potamopyrgus antipodarum* (Mollusca, Hydrobiidae, Smith 1889) and *Valvata piscinalis* (Mollusca, Valvatidae, Müller 1774). *Chemosphere*, 2008, 71 (3), p. 553 - p. 560. <10.1016/j.chemosphere.2007.09.048>. <hal-00453841>

**HAL Id: hal-00453841**

**<https://hal.science/hal-00453841v1>**

Submitted on 5 Feb 2010

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire HAL, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



HAL Authorization

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

4  
5  
6  
7 **Authors**

8 Beatrice GAGNAIRE<sup>1</sup>, Olivier GEFFARD<sup>1</sup>, Benoit XUEREB<sup>1</sup>, Christelle MARGOUM<sup>2</sup> and Jeanne  
9 GARRIC<sup>1\*</sup>

10

11 **Affiliations**

12 <sup>1</sup>CEMAGREF, Unité Biologie des Ecosystèmes Aquatiques, Laboratoire d'Ecotoxicologie, 3 bis  
13 quai Chauveau, CP 220, 69336 Lyon Cedex 09, France

14 <sup>2</sup>CEMAGREF, Unité Qualité des Eaux et Prévention des Pollutions, Laboratoire des  
15 Micropolluants Organiques, 3 bis quai Chauveau, CP 220, 69336 Lyon Cedex 09, France

16

17

18

19

20

---

\* Corresponding author: J. GARRIC, CEMAGREF, Lyon, France ; Tel: +33 4-72-20-89-05, Fax:  
+33 4-78-47-78-75, [garric@lyon.cemagref.fr](mailto:garric@lyon.cemagref.fr)

20 **Abstract**

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

41

42 **Keywords**

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

45

## 45 **Introduction**

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

52           Among anthropogenic contaminants, pesticides are widely detected in freshwater and  
53 estuarine ecosystems. These molecules are spread on terrestrial cultures and enter waterways  
54 from agricultural and urban run-off. Pesticides may have major ecological consequences  
55 (Ozretic and Krajnovic-Ozretic, 1992). The organophosphates (OPs) and carbamates (Cs) are  
56 modern synthetic insecticides and are potent neurotoxic molecules (Ashauer et al., 2006).  
57 They exert acute toxicity by blocking the breakdown of acetylcholine by the enzyme  
58 acetylcholinesterase (AChE: E.C.3.1.1.7) in vertebrate and invertebrate organisms (Fulton and  
59 Key., 2001). Acetylcholine is the primary neurotransmitter in the sensory and neuromuscular  
60 systems in most species. The activity of this system is vital to muscular function and  
61 represents a prime target on which OPs and Cs can exert a detrimental effect (Sarkar et al.,  
62 2006).

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

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

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

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

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

94 using different substrates (acetylthiocholine (ASCh), propionylthiocholine (PSCh) and  
95 butyrylthiocholine (BSCh)) and specific inhibitors (eserine for ChE, BW284c51 for AChE,  
96 *iso*-OMPA for BChE). Secondly, *in vivo* effects of a model insecticide, chlorpyrifos, on ChE  
97 activities were then studied in order to assess the value of *P. antipodarum* and *V. piscinalis* as  
98 sentinel species of freshwater insecticide contamination.  
99

99 **Material and Methods**

100 *Chemicals*

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

106

107 *Organisms*

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

113

114 *Cholinesterase activity*

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

119 The enzyme activity was measured following the Ellman method (1961). In a typical assay,  
120 330  $\mu$ L of 0.1 M phosphate buffer pH 7.8, 20  $\mu$ L of 0.0076 M the chromogenic agent DTNB  
121 and 20  $\mu$ L of sample were successively added in a 96 wells microtitre plate. Measurement of  
122 enzyme activity was initiated by the addition of ten  $\mu$ L of freshly prepared acetylthiocholine  
123 iodide solution in distilled water. Absorption of the 2-nitro-5-thiobenzoate anion, formed

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

130

### 131 Substrate affinity

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

138

### 139 Specific inhibitors

140 Eserine, *iso*-OMPA and BW284c51 were used as specific inhibitors of ChEs, BChEs and  
141 AChEs, respectively. Eserine and *iso*-OMPA were dissolved in ethanol and BW284c51 was  
142 dissolved in distilled water. Ten animals were individually homogenized and supernatants  
143 were pooled. Supernatants were then incubated 30 minutes at 20 °C with inhibitor or water or  
144 ethanol for eserine and *iso*-OMPA (1%). Final inhibitor concentrations ranged from 0.01 to  
145 100 µM for eserine and from 0.1 to 1000 µM for *iso*-OMPA and BW284c51. Effects of  
146 inhibitors on ChE activities were assessed using ASCh, BSCh and PSCh as substrates.

147

### 148 *Chlorpyrifos exposure*

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

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

165

#### 166 *Measurement of chlorpyrifos concentration in water*

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

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

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

186

### 187 *Data analysis*

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

197

## 197 **Results**

### 198 *In vitro* experiments

#### 199 Substrate affinity

200 For *P. antipodarum*, measured esterase activities depended on the substrate. The reaction rate  
201 increased with increasing substrate concentration, with ASCh > PSCh > BsCh ( $p < 0.05$ )  
202 (**Figure 1a**). However, for the highest concentrations ( $> 2$  mM), no differences were measured  
203 between activities with ASCh and PSCh (**Figure 1a**). On the same way, increased ChE  
204 activity were measured for *V. piscinalis*, according to the substrate, with ASCh > PSCh >  
205 BsCh ( $p < 0.05$ ) (**Figure 1b**). No inhibitory effect was observed whatever the substrate used  
206 for both species. At the highest substrate concentration (8 mM) enzymatic activities  
207 (expressed as  $\text{nmol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$  protein) for *P. antipodarum* were  $31.6 \pm 0.2$  for ASCh (100%),  
208  $30.2 \pm 1.2$  for PSCh (95.5%) and  $2.7 \pm 0.3$  for BSCh (8.5%). For *V. piscinalis*, enzymatic  
209 activities were  $17.3 \pm 0.5$  for ASCh (100%),  $9.1 \pm 0.7$  for PSCh (52.6%) and  $3.9 \pm 0.3$  for  
210 BSCh (22.5%). 4 mM was defined as the optimal concentration for ASCh and PSCh for both  
211 species .

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

216

#### 217 Specific inhibitors

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

222 values of eserine were lower for *P. antipodarum* (0.034, 5.29 and 0.024  $\mu\text{M}$ ) than for *V.*  
223 *piscinalis* (1.39, 8.68 and 1.40  $\mu\text{M}$ ) for ASCh, BSCh and PSCh, respectively.

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

227 BW284c51 significantly decreased the activities obtained for ASCh and PSCh for both  
228 gastropod species. For BSCh, the activity was significantly decreased only for *V. piscinalis*  
229 (**Figure 2 e&f**). The inhibition profiles of ASCh and PSCh and the  $\text{IC}_{50}$  values were similar  
230 for *P. antipodarum* (290.4 and 387.1  $\mu\text{M}$ ) and *V. piscinalis* (150.2 and 262.2  $\mu\text{M}$ ) for ASCh  
231 and PSCh, respectively.

232

233 *In vivo experiments*

234 Concentration of chlorpyrifos in water

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

242

243 *In vivo* effects of chlorpyrifos on ChE activity

244 During the seven days of experiment, no mortality was reported for *P. antipodarum*, neither  
245 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<sup>-1</sup>.mg<sup>-1</sup> 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<sup>-1</sup>.mg<sup>-1</sup> protein,  
254 respectively, which represented an activity of 40.5 and 20.2% of the control (100%). IC<sub>50</sub>  
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

259 **Discussion**

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

271 Vertebrate cholinesterases have been classified into two groups, acetyl ChE and  
272 butyryl ChE, depending on substrate hydrolysis and sensitivity to inhibitors. AChE  
273 hydrolyses ASCh much faster than other choline esters, like PSCh, and is inactive on BSCh,  
274 whereas BChE hydrolyses both BSCh and ASCh at an appreciable rate (Valbonesi et al.,  
275 2003). Several studies show that situation of invertebrate cholinesterases is more complex.  
276 ASCh has been reported as the preferential substrate for most bivalves including oysters  
277 (Bocquené et al., 1997; Valbonesi et al., 2003), marine and freshwater mussels (Mora et al.,  
278 1999; Romani et al., 2005; Binelli et al., 2006), as well as for *E. andrei* (Caselli et al., 2006),  
279 *B. glabrata* (Kristoff et al., 2006), and the common shredder, *Gammarus pulex* (Xuereb et al.,  
280 2007).

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

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

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

297  $V_{\text{max}}$  values were similar for both species. For *P. antipodarum*, the ASCh ratio  $V_{\text{max}}/K_m$   
298 was in the same range that those reported in *M. galloprovincialis* (0.24  $\text{mL}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$   
299 protein) (Valbonesi et al., 2003) and *E. andrei* (0.25  $\text{mL}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$  protein) (Caselli et al.,  
300 2006). ASCh  $V_{\text{max}}/K_m$  ratio for *V. piscinalis* was closer to *O. edulis* one ( $5.1\cdot 10^{-2}$   $\text{mL}\cdot\text{min}^{-1}$   
301  $\cdot\text{mg}^{-1}$  protein) (Valbonesi et al., 2003). These results indicated that *V. piscinalis* enzyme has a  
302 lower efficiency of hydrolysis than *P. antipodarum*, in agreement with the lower activity  
303 observed and with the lower substrate affinity, as discussed before.

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

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

316  
317  $IC_{50}$  reported for eserine in *P. antipodarum* were in the same range than the values  
318 reported for other invertebrates (0.01, 0.01, 0.14, 0.020 and 0.014  $\mu$ M for *B. glabrata*, *L.*  
319 *variegatus*, *O. edulis*, *M. galloprovincialis* and *E. andrei*, respectively) (Valbonesi et al.,  
320 2003; Caselli et al., 2006; Kristoff et al., 2006). However, values for *V. piscinalis* were  
321 higher. These results suggest that *V. piscinalis* ChEs are less sensitive than in *P. antipodarum*.

322 In our experiments, we measured effects of chlorpyrifos on *P. antipodarum* and *V.*  
323 *piscinalis*. Chlorpyrifos is a widely used organophosphate insecticide and is the active  
324 ingredient in a number of commonly used household and agricultural insecticide formulations  
325 (Fulton and Key, 2001). It is volatile and concentration decreases rapidly in water in constant  
326 exposure conditions. For these reasons, we performed the laboratory experiment using semi-  
327 static conditions. Nevertheless, an important decrease of chlorpyrifos level was measured  
328 after 24h. A similar 50% loss was reported in a contamination experiment of *G. pulex* to 0.3  
329 nM of chlorpyrifos: concentration reached 0.17 nM after 24 h of exposition (Ashauer et al.,  
330 2006).

331 Chlorpyrifos was tested in this study at concentrations ranging from 0.14 to 14.2 nM (0.05-5  
332  $\mu$ g.L<sup>-1</sup>). Measured concentrations of chlorpyrifos in surface waters often fall at concentrations

333 below the nM level (USEPA, 2002). However, studies showed that chlorpyrifos  
334 concentrations in small streams and wetlands adjacent to agricultural fields could range from  
335 0.2 to 2  $\mu\text{M}$  (Mazanti et al., 2003). Moreover, the relatively short-half life of chlorpyrifos in  
336 water may result in underestimate levels of exposure (Mazanti et al., 2003).

337 We demonstrated the dose-response and time-dependant effects of chlorpyrifos on *P.*  
338 *antipodarum*: for 14.2 nM, inhibition was 40% of the control after 24 h of contact and  
339 increased to 80% of the control after 168 h of contact, without significant mortality. However,  
340 an increase was shown on *V. piscinalis*. Chlorpyrifos have already been shown as a powerful  
341 AChE inhibitor in invertebrates. Chlorpyrifos decreased AChE activity after 96 h of contact in  
342 *D. polymorpha* (0.03 nM) (Binelli et al., 2006) and in *C. fluminea* (80% inhibition at 1.4 and  
343 2.8  $\mu\text{M}$ ) (Cooper and Bidwell, 2006). Chlorpyrifos also decreased *in vitro* AChE activity in  
344 *M. edulis* (Galloway et al., 2002) and in *in vivo* exposures in the midge, *Chironomus riparius*  
345 (Callaghan et al., 2001) and in rat brain (Hancock et al., 2007), which agrees with our results.  
346 On the contrary, this OP increased AChE activity in *S. inaequalvis* after 15 days of exposure  
347 to 0.3 nM (Romani et al., 2005), which comforts our results on *V. piscinalis*; however, the  
348 biological explanation remains unknown.  $\text{IC}_{50}$  of chlorpyrifos for AChE was 9.71 nM in *P.*  
349 *antipodarum* at 96 h, which was ten times higher that for *G. pulex* in the same experimental  
350 conditions (Xuereb et al., 2007).

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

355

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

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

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

375

## 376 **Conclusion**

377 The aim of our work was to characterize *P. antipodarum* and *V. piscinalis* ChEs. Our results  
378 show that *P. antipodarum* possesses several isoforms of ChEs, one undifferentiated between  
379 an AChE and a PChE, and another one which could be assimilated as a BChE. *V. piscinalis*  
380 seems to possess only one isoform close to the vertebrate AChE. Our results also illustrate the  
381 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

393 **References**

- 394 Ashauer, R., Boxall, A., Brown, C., 2006. Uptake and elimination of chlorpyrifos and  
395 pentachlorophenol into the freshwater amphipod *Gammarus pulex*. Arch. Environ. Con. Tox.  
396 51, 542-548.
- 397 Binelli, A., Ricciardi, F., Riva, C., Provini, A., 2006. New evidences for old biomarkers:  
398 Effects of several xenobiotics on EROD and AChE activities in Zebra mussel (*Dreissena*  
399 *polymorpha*). Chemosphere 62, 510-519.
- 400 Bocquené, G., Roig, A., Fournier, D., 1997. Cholinesterases from the common oyster  
401 (*Crassostrea gigas*). Evidence for the presence of a soluble acetylcholinesterase insensitive to  
402 organophosphate and carbamate inhibitors. FEBS Lett. 407, 261-266.
- 403 Callaghan, A., Hirthe, G., Fisher, T., Crane, M., 2001. Effect of short-term exposure to  
404 chlorpyrifos on developmental parameters and biochemical biomarkers in *Chironomus*  
405 *riparius* Meigen. Ecotox. Environ. Safe. 50, 19-24.
- 406 Caselli, F., Gastaldi, L., Gambi, N., Fabbri, E., 2006. *In vitro* characterization of  
407 cholinesterases in the earthworm *Eisenia andrei*. Comp. Biochem. Phys. C 143, 416-421.
- 408 Cooper, N.L., Bidwell, J.R., 2006. Cholinesterase inhibition and impacts on behavior of the  
409 Asian clam, *Corbicula fluminea*, after exposure to an organophosphate insecticide. Aquat.  
410 Toxicol. 76, 258-267.
- 411 Duft, M., Schmitt, C., Bachmann, J., Brandelik, C., Schulte-Oehlmann, U., Oehlmann, J.,  
412 2007. Prosobranch snails as test organisms for the assessment of endocrine active chemicals--  
413 an overview and a guideline proposal for a reproduction test with the freshwater mudsnail  
414 *Potamopyrgus antipodarum*. Ecotoxicology 16, 169-182.
- 415 Ellman, G.L., Courtney, K.D., Andres, V., Jr., Feather-Stone, R.M., 1961. A new and rapid  
416 colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7, 88-95.

- 417 Fulton, M.H., Key, P.B., 2001. Acetylcholinesterase inhibition in estuarine fish and  
418 invertebrates as an indicator of organophosphorus insecticide exposure and effects. *Environ.*  
419 *Toxicol. Chem.* 20, 37-45.
- 420 Galloway, T.S., Millward, N., Browne, M.A., Depledge, M.H., 2002. Rapid assessment of  
421 organophosphorous/carbamate exposure in the bivalve mollusc *Mytilus edulis* using combined  
422 esterase activities as biomarkers. *Aquat. Toxicol.* 61, 169-180.
- 423 Hancock, S., Ehrich, M., Hinckley, J., Pung, T., Jortner, B.S., 2007. The effect of stress on the  
424 acute neurotoxicity of the organophosphate insecticide chlorpyrifos. *Toxicol. Appl. Pharm.*  
425 219, 136-141.
- 426 Kristoff, G., Guerrero, N.V., de D'Angelo, A.M.P., Cochon, A.C., 2006. Inhibition of  
427 cholinesterase activity by azinphos-methyl in two freshwater invertebrates: *Biomphalaria*  
428 *glabrata* and *Lumbriculus variegatus*. *Toxicology* 222, 185-194.
- 429 Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the  
430 Folin phenol reagent. *J. Biol. Chem.* 193, 265-275.
- 431 Mazanti, L., Rice, C., Bialek, K., Sparling, D., Stevenson, C., Johnson, W.E., Kangas, P.,  
432 Rheinstein, J., 2003. Aqueous-phase disappearance of atrazine, metolachlor, and chlorpyrifos  
433 in laboratory aquaria and outdoor macrocosms. *Arch. Environ. Con. Tox.* 44, 67-76.
- 434 Mora, P., Michel, X., Narbonne, J.-F., 1999. Cholinesterase activity as potential biomarker in  
435 two bivalves. *Environ. Toxicol. Phar.* 7, 253-260.
- 436 Mouthon, J., Charvet, S., 1999. Compared sensitivity of species, genera and families of  
437 Molluscs to biodegradable pollution. *Ann. Limnol-Int. J. Lim.* 35, 31-39.
- 438 Najimi, S., Bouhaimi, A., Daubeze, M., Zekhnini, A., Pellerin, J., Narbonne, J.F., Moukrim,  
439 A., 1997. Use of acetylcholinesterase in *Perna perna* and *Mytilus galloprovincialis* as a  
440 biomarker of pollution in Agadir marine bay (South of Morocco). *B. Environ. Contam. Tox.*  
441 58, 901-908.

- 442 Oetken, M., Nentwig, G., Loffler, D., Ternes, T., Oehlmann, J., 2005. Effects of  
443 pharmaceuticals on aquatic invertebrates. Part I. The antiepileptic drug carbamazepine. Arch.  
444 Environ. Con. Tox. 49, 353-361.
- 445 Ozretic, B., Krajnovic-Ozretic, M., 1992. Esterase heterogeneity in mussel *Mytilus*  
446 *galloprovincialis*: effects of organophosphate and carbamate pesticides *in vitro*. Comp.  
447 Biochem. Phys. C 103, 221-225.
- 448 Rittschof, D., McClellan-Green, P., 2005. Molluscs as multidisciplinary models in  
449 environment toxicology. Mar. Pollut. Bull. 50, 369-373.
- 450 Romani, R., Isani, G., De Santis, A., Giovannini, E., Rosi, G., 2005. Effects of chlorpyrifos  
451 on the catalytic efficiency and expression level of acetylcholinesterases in the bivalve mollusk  
452 *Scapharca inaequivalvis*. Environ. Toxicol .Chem. 24, 2879-2886.
- 453 Romeo, M., Gharbi-Bouraoui, S., Gnassia-Barelli, M., Dellali, M., Aissa, P., 2006. Responses  
454 of *Hexaplex (Murex) trunculus* to selected pollutants. Sci. Total Environ. 359, 135-144.
- 455 Sarkar, A., Ray, D., Shrivastava, A.N., Sarker, S., 2006. Molecular biomarkers: Their  
456 significance and application in marine pollution monitoring. Ecotoxicology 15, 333-340.
- 457 Scaps, P., Demuyne, S., Descamps, M., Dhainaut, A., 1997. Effects of organophosphate and  
458 carbamate pesticides on acetylcholinesterase and choline acetyltransferase activities of the  
459 polychaete *Nereis diversicolor*. Arch. Environ. Con. Tox. 33, 203-208.
- 460 Scaps, P., Borot, O., 2000. Acetylcholinesterase activity of the polychaete *Nereis diversicolor*:  
461 effects of temperature and salinity. Comp. Biochem. Phys. C 125, 377-383.
- 462 U.S.E.P.A., 2002. Interim registration eligibility decision for chlorpyrifos. URL:  
463 [http://www.epa.gov/REDs/chlorpyrifos\\_ired.pdf](http://www.epa.gov/REDs/chlorpyrifos_ired.pdf). Final/Technical Report, U.S. Environmental  
464 Protection Agency, Washington D.C.

465 Valbonesi, P., Sartor, G., Fabbri, E., 2003. Characterization of cholinesterase activity in three  
466 bivalves inhabiting the North Adriatic sea and their possible use as sentinel organisms for  
467 biosurveillance programmes. *Sci. Total Environ.* 312, 79-88.

468 van der Oost, R., Beyer, J., Vermeulen, N.P.E., 2003. Fish bioaccumulation and biomarkers in  
469 environmental risk assessment: a review. *Environ. Toxicol. Phar.* 13, 57-149.

470 Xuereb, B., Noury, P., Felten, V., Garric, J., Geffard, O., 2007. Cholinesterase activity in  
471 *Gammarus pulex* (Crustacea, Amphipoda): characterization and effects of chlorpyrifos.  
472 *Toxicology* 236, 178-189.

473

474

475

476

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  $\pm$  SE of three  
493 replicates.