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1 **Elucidating pesticide sensitivity of two endogeic earthworm species through**  
2 **the interplay between esterases and glutathione *S*-transferases**

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11 **Highlights**

- 12 - Species-specific tissue distribution of B-esterases was assessed
- 13 - Glutathione-S-transferase is exclusively cytosolic in both earthworm species
- 14 - Similar efficiency and OP sensitivity of carboxylesterase was found in both species
- 15 - Species differences in acetylcholinesterase inhibition depend on OP structure
- 16 - *A. chlorotica* was less sensitive to OP after in vivo compared to in vitro exposure

17 **Abstract**

18 Earthworms are common organisms in soil toxicity-testing framework, and endogeic species  
19 are currently recommended due to their ecological role in agroecosystem. However, little is  
20 known on their pesticide metabolic capacities. We firstly compared the baseline activity of B-  
21 esterases and glutathione-S-transferase in *Allolobophora chlorotica* and *Aporrectodea*  
22 *caliginosa*. Secondly, vulnerability of these species to pesticide exposure was assessed by *in*  
23 *vitro* trials using the organophosphate (OP) chlorpyrifos-ethyl-oxon (CPOx) and ethyl-  
24 paraoxon (POx), and by short-term (7 days) *in vivo* metabolic responses in soil contaminated  
25 with pesticides. Among B-esterases, acetylcholinesterase (AChE) activity was abundant in the  
26 microsomal fraction (80% and 70% of total activity for *A. caliginosa* and *A. chlorotica*,  
27 respectively). Carboxylesterase (CbE) activities were measured using three substrates to  
28 examine species differences in isoenzyme and sensitivity to both *in vitro* and *in vivo*  
29 exposure. CbEs were mainly found in the cytosolic fraction (80% and 60% for *A. caliginosa*  
30 and *A. chlorotica* respectively). GST was exclusively found in the soluble fraction for both  
31 species. Both OPs inhibited B-esterases in a concentration-dependent manner. *In vitro* trials  
32 revealed a pesticide-specific response, being *A. chlorotica* AChE more sensitive to CPOx  
33 compared to POx. CbE activity was inhibited at the same extent in both species. The 7-d  
34 exposure showed *A. chlorotica* less sensitive to both OPs, which contrasted with outcomes  
35 from *in vitro* experiments. This non-related functional between both approaches for assessing  
36 pesticide toxicity suggests that other mechanisms linked with *in vivo* OP bioactivation and  
37 excretion could have a significant role in the OP toxicity in endogeic earthworms.

38 **Keywords** : B-esterases; glutathione-S-transferase ; *Aporrectodea caliginosa*; *Allolobophora*  
39 *chlorotica*; organophosphorus; pesticide metabolism

## 40 **1. Introduction**

41 Conventional agriculture is characterized by a permanent pesticide and fertilizer use to crop  
42 protection and improve crop yields and productivity. However, the excessive use of  
43 agrochemicals may lead to soil degradation (Tilman et al., 2002; Stavi et al., 2016) and the  
44 pesticide accumulation may trigger toxic effects on non-target organisms of beneficial interest  
45 in agriculture such as earthworms (Pelosi et al., 2013a; Plaas et al., 2019). Earthworms are  
46 considered as ecosystem engineers due to their ability to modify physical, chemical and  
47 biological properties of soil (Jones et al., 1994; Edwards, 2004; Lavelle et al., 2006). Through  
48 their burrowing activity, they are strongly affected by various soil contaminants and they  
49 represent relevant indicators to evaluate environmental stresses, soil quality and pollution  
50 (Paoletti, 1999; Pelosi et al., 2013a; Stanley and Preetha, 2016). Among earthworm's species  
51 used for ecotoxicological studies, there is a growing concern in the inclusion of soil-dwelling  
52 species because of their high abundance in temperate agroecosystems, their higher sensitivity  
53 to agrochemicals compared with *Eisenia* sps (Pelosi et al., 2013b; Jouni et al., 2018), and their  
54 feeding habits (geophagus) that means a high exposure level to organic pollutants bound to soil  
55 organic matter.

56 Organophosphorus compounds (OP) are still commonly used insecticides in agriculture  
57 (Dalmolin et al., 2020) to control agricultural pests, and they display a high acute toxicity to  
58 wildlife (Köhler and Triebkorn, 2013). These insecticides are known neurotoxic chemicals  
59 targeting acetylcholinesterase (AChE, EC.3.1.1.7), a key enzyme in regulating the  
60 transmission of nervous impulse at the cholinergic synapses (Fairbrother et al., 1991; Nunes,  
61 2011; Casida, 2017). Inhibition of AChE activity has been extensively reported as a sensitive  
62 and specific biomarker of exposure to OPs (Sanchez-Hernandez, 2011). In the particular case  
63 of earthworms, species-specific differences in OP sensitivity has been reported in previous  
64 works by our research group (Rault et al., 2008; Jouni et al., 2018) and others (Velki and

65 Hackenberger, 2012). Although the species-specific sensitivity to OPs could be explained by  
66 the affinity of the OP to the AChE active site, the number of available OP molecules able to  
67 interact with AChE is also a determinant variable (Maxwell, 1992; Küster, 2005).  
68 Nevertheless, the OP availability depends of a bioactivation step (i.e., oxidative desulfuration  
69 reaction mainly catalyzed by cytochrome P450-dependent monooxygenases) whereby the  
70 formed OP metabolite (named “oxon” metabolite) display a much higher affinity by the active  
71 site of AChE (and other esterases) than the parent OP (Chambers et al., 2010). Additionally,  
72 the OPs react with the serine hydroxyl group of AChE to form a stable phosphorylated  
73 enzyme, which can undergo two main processes: oxime-induced reactivation and aging.  
74 Chemical-induced reactivation using oximes (nucleophilic reagents able to attack the  
75 phosphoryl group bound to the active site of the esterase) is a mechanism of AChE activity  
76 restoration which is effective as long as the elapsed time since enzyme inhibition be short  
77 (hours to a few days) (Thompson & Richardson, 2004). If the enzyme remains long time  
78 inhibited, one alkyl group of the OP pesticide can release from the phosphyl-esterase complex  
79 (dealkylation), thus leading the enzyme to a permanent inactivation. This stage of the enzyme  
80 is known as aging and using oximes cannot reactivate the enzyme activity. The only pathway  
81 to recover the normal variation of esterase activity is the synthesis of new enzyme. In the OP  
82 pesticide toxicology, there are other two enzymes that modulate the toxicity of these  
83 chemicals: carboxylesterases (CbEs, EC.3.1.1.1) and glutathione-S-transferase (GST EC  
84 2.5.1.18) (Jokanovic, 2002; Sogorb and Vilanova, 2002). The interplay between bioactivation  
85 and metabolic pathways of OP insecticides influence both the availability and distribution of  
86 OPs in animal tissue, and the sensitivity of organisms to these compounds.

87 In earthworms, the role of CbEs and GST as biochemical modulators has not been  
88 investigated with the scope of establishing species-specific differences in OP toxic impact.  
89 Such information would be of interest in predictive models (i.e., adverse outcome pathways

90 framework) to identify species at risk of agricultural OP applications. Alternatively, such  
91 approach would help to select the most suitable earthworm species for bioremediation of OP-  
92 contaminated soils. Recently, we have reported marked differences in ethyl-parathion  
93 sensitivity in the two earthworm species used in the current study, which indirectly impacted  
94 the animal behavior as measured by cast production and burrowing activity (Jouni et al.,  
95 2018). However, some studies have shown that sensitivity of B-type esterases to OP exposure  
96 and bioactivation rate are highly dependent on the OP type. Therefore, with the scope of  
97 increasing the understanding on the biochemical mechanisms that explain of species-species  
98 in earthworms, we hypothesized that levels of B-type esterase and GST activities as well as  
99 the sensitivity of B-type esterases to OPs define the *in vivo* sensitivity of earthworms to these  
100 pesticides. The aims of this study were: 1) to perform an enzymological characterization of  
101 AChE, CbEs and GST activities in term of cellular location, abundance and kinetic  
102 parameters in the endogeic earthworms *Aporrectodea caliginosa* and *Allolobophora*  
103 *chlorotica*; and 2) to compare *in vitro* and *in vivo* responses of B-type esterases to two model  
104 OP parathion-ethyl and the chlorpyrifos-ethyl. *In vivo* response of both *Aporrectodea*  
105 *caliginosa* and *Allolobophora chlorotica* species was investigated using the corresponding  
106 commercial formulation of the OP compounds, in order to assess the potential role of these  
107 two metabolic pathways in the species-specific sensitivity in earthworms.

## 108 **2. Materials and methods**

### 109 *2.1. Chemicals*

110 The following chemicals were purchased from Sigma–Aldrich (Saint Quentin-Fallavier,  
111 France): acetylthiocholine iodide (AcSCh), 4-nitrophenyl acetate (4-NPA), 4-nitrophenyl  
112 valerate (4-NPV), 1-naphtyl acetate (1-NA), 1-naphtol, DTNB, Fast Garnet, 1-chloro-2,4-  
113 dinitro-benzene (CDNB), Reduced Glutathione (GSH), protease inhibitors, bovine serum

114 albumin (BSA). The OP pure molecule ethyl-paraoxon (O,O-Diethyl O-(4-nitrophenyl)  
115 phosphate ; PESTANAL<sup>®</sup>) was purchased from Sigma-Aldrich and chlorpyrifos-oxon (O,O-  
116 diethyl O-3,5,6-trichloro-2-pyridyl phosphate) was purchased from Cluzeau Info Lab  
117 (France). Commercial formulations of parathion (Oleobladan<sup>®</sup>; 93g.L<sup>-1</sup>) and chlorpyrifos-  
118 ethyl (Pyrinex<sup>®</sup>; 250g.L<sup>-1</sup>), two OP insecticides, were supplied by Bayer and Philagro  
119 respectively.

## 120 2.2. Soil and earthworms

121 Soil (23.4% clay, 57% silt, 19.6% sand, 28.3 g kg<sup>-1</sup> organic matter, pH 8.3) and healthy  
122 adults' specimens of *A. caliginosa* and *A. chlorotica* were collected from an apple orchard in  
123 Montfavet near Avignon, France. This orchard has received no pesticide treatments for the  
124 last 10 years. Worms were collected manually and were placed into plastic pots filled with  
125 fresh soil collected from the same orchard.

126 Soil was sieved at <2 mm and the water content was adjusted to 20-21% (approximately 81%  
127 of the maximum water holding capacity) with distilled water. Afterwards, wet soil samples (1  
128 kg) were spiked with ethyl-parathion or chlorpyrifos solutions (40 ml) to yield the final  
129 concentrations of 1 mg and 1.3 mg active ingredient (a.i.) kg<sup>-1</sup> wet soil respectively. Both  
130 insecticide concentrations refer to the usual application rate and calculation of the Predicted  
131 Environmental Concentration (PEC). A control was prepared under the same conditions  
132 without pesticide application. The wet polluted or unpolluted soil was then split between Petri  
133 dishes (diameter 10 cm, height 3 cm) so that each contained 100 g soil. A single earthworm  
134 was placed in each Petri dish to limit interspecific interactions and to prevent cascade death  
135 (Sheppard and Evenden, 1992). Dishes were kept in a climate chamber (12±1°C, and  
136 permanent dark), and after 7 days of exposure to the pesticide, each earthworm was collected,  
137 weighed and frozen at -80°C until biochemical measurements (n=10 per treatment).



138 *2.3. Homogenate preparation*

139 Frozen worms were sliced in 1 cm length pieces and homogenized individually on ice with  
140 Ultra Turrax IKA T18 basic at 14000 rev min<sup>-1</sup>, 4 times 10s with 1 min interval, in 20% (w/v)  
141 low-salt buffer containing 10mM Tris-HCl pH 7.3 10mM NaCl, supplemented with protease  
142 inhibitors (aprotinin, leupeptin and pepstatin, 5 µg mL<sup>-1</sup>, antipain 1 µg mL<sup>-1</sup>, and trypsin  
143 inhibitor 1 mg mL<sup>-1</sup>). The homogenate was centrifuged (3000 × g, 10min, 4°C) and the  
144 supernatant obtained, named crude extract, was used for determining enzyme activities. In  
145 order to separate cytosolic or soluble fraction and microsomal fraction, the crude extract was  
146 centrifuged at 100,000g for 1h, at 4°C. All the different fractions (crude extract, cytosolic and  
147 microsomal ones) were kept at -80°C, supplemented with 10% glycerol as stabilizing agent,  
148 until biochemical analysis. Total protein content was measured by the method of Lowry  
149 modified according to Markwell et al. (1978), using bovine serum albumin as a standard.

150 *2.4. Enzyme characterization*

151 We used the crude extract to assess the appropriate assay conditions for ensuring maximal  
152 activity of each enzyme. The effect of substrate concentration (0-10 mM) on the enzyme  
153 activity was measured to ensure substrate-saturating conditions. Modulation of GST activity  
154 by CDNB was examined at a range of substrate concentrations between 0 and 1 mM because  
155 of CDNB precipitation in the reaction medium at higher concentrations. The general  
156 conditions for activity measurements were carried out as specified below.

157 *2.5. Enzyme activity assays*

158 Enzyme activities were monitored spectrophotometrically at 25 °C using a microplate reader  
159 (Synergy HT, Bio-Tek®), using the selected concentrations of substrates as obtained in the  
160 characterization step

161 The activity AChE was monitored at 412 nm and 25°C following the method by Ellman et al.  
162 (1961), and adapted by Rault et al. (2008), for a microplate reader. The reaction medium (200

163  $\mu\text{L}$ , final volume) contained 4  $\mu\text{L}$  of homogenate, 0.375 mM DTNB and 3 mM of AcSCh in  
164 0.1 M sodium phosphate buffer (pH 7).

165 Because the sensitivity of carboxylesterase activity to OP is highly dependent on the  
166 substrate, this activity was assayed using three different substrates, which differ in their  
167 carbon-ester chains: 1-naphtyl-acetate (1-NA), 4-nitrophenyl acetate (4-NPA) and 4-  
168 nitrophenyl valerate (4-NPV) to characterize multiple CbE isoenzymes following the  
169 microplate-scale assay by Thompson (1999). The hydrolysis of 1-NA was determined in a  
170 reaction medium (200  $\mu\text{L}$  final volume) containing 25 mM Tris-HCl (pH 7.6), 1 mM  $\text{CaCl}_2$   
171 and 2  $\mu\text{L}$  of diluted (1/20) homogenate. The reaction was initiated by adding 2 mM 1-Na  
172 (final conc.), and stopped after 10 min of incubation at 25°C by the addition of 50  $\mu\text{L}$  2.5%  
173 SDS containing 0.1% Fast Garnet in 2.5% Triton X-100. The solutions were left to stand for  
174 10 min at room temperature and dark, and the absorbance of the 1-naphthol-Fast Garnet  
175 complex was read at 530 nm. Enzyme activity was calculated from a standard curve  
176 ( $A_{530}=f([1\text{-naphthol}])$ ) containing 1-naphthol in the same reaction mixture free of substrate.  
177 Hydrolysis of 4-NPV and 4-NPA was determined in a reaction mixture (250  $\mu\text{L}$ , final volume  
178 including 2  $\mu\text{L}$  of diluted (1/5) enzyme extract) contained 1 mM 4-NPV and 50 mM Tris-HCl  
179 (pH 7.5), or 5 mM 4-NPA in 20 mM Tris-HCl (pH 8.0) and 1mM EDTA (to inhibit  $\text{Ca}^{2+}$   
180 dependent phosphotriesterases). In both assays, the formation of 4-nitrophenol was monitored  
181 for 5 min at 405 nm, 25°C and quantified using a 4-nitrophenol standard curve.

182 GST activity was monitored at 340 nm and 25°C following the method described by Habig et  
183 al. (1974) using a millimolar extinction coefficient ( $9,600 \text{ M}^{-1} \text{ cm}^{-1}$ ). The reaction medium  
184 (200  $\mu\text{L}$ , final volume) contained 2  $\mu\text{L}$  of homogenate, 1 mM CDNB and 5 mM of GSH in  
185 0.1 M potassium phosphate buffer (pH 7.4).

186 Reagent blanks (reaction mixture free of sample) were checked for non-enzymatic hydrolysis  
187 of the substrates. All enzyme assays were run at least in triplicate and the activities were

188 expressed as units per milligram of total protein ( $\text{U mg}^{-1}$  protein). One unit of enzyme activity  
189 was defined as one micromole of formed product per minute under the experimental  
190 conditions described above.

#### 191 2.6. *In-vitro B-esterase inhibition by pesticides*

192 Sensitivity of both *A. caliginosa* and *A. chlorotica* AChE and CbE activities to chlorpyrifos-  
193 ethyl-oxon and ethyl-paraoxon (the pure OP molecules) were evaluated *in vitro*. The oxon  
194 forms of the OP were used for *in vitro* inhibition of B-esterases instead of its commercial  
195 formulation because *in vivo* OP pesticides are only activated after being metabolized.

196 Tissue homogenates from unexposed earthworms (20  $\mu\text{l}$ ) were incubated individually with  
197 5 $\mu\text{l}$  of serial concentrations of ethyl-paraoxon or chlorpyrifos-ethyl-oxon ( $10^{-12}$  to  $10^{-3}$   $\text{mol.L}^{-1}$   
198 final concentration) or with 5 $\mu\text{l}$  of solvent for the control, for 30 min at 25°C. An aliquot of  
199 these mixtures (corresponding to a 4/5 dilution of the homogenate in the incubation mixture)  
200 was used to measure the residual enzyme activity (in triplicates) according to the methods  
201 described above for allowing to reach the maximal velocity. We used 5 $\mu\text{L}$ , 2.5 $\mu\text{L}$  1/20, and  
202 2.5 $\mu\text{L}$  1/5 of the mixture for AChE, 1-NA, 4-NPA/4-NPV activities respectively. For each  
203 assay the total volume of the reaction medium was adjusted with the corresponding buffer.  
204 Inhibition assay was terminated by addition of the corresponding substrate, and the residual  
205 B-esterase activity was measured as described above. Controls (no pesticides) were run in the  
206 same conditions for initial activity measurements. Because (1) the target of OP pesticides is  
207 the AChE activity, (2) the paraoxon was a well-known toxic compound, and (3) *A. caliginosa*  
208 has been pointed out as a sensitive earthworm species, we used the  $\text{IC}_{50}$  value obtained for *A.*  
209 *caliginosa* AChE in the presence of ethyl-paraoxon, as a reference. An esterase Susceptibility  
210 Ratio (SRe) was then calculated by dividing this  $\text{IC}_{50}$  reference-value by the  $\text{IC}_{50}$  value  
211 obtained for the other esterase activities either in the presence of ethyl-paraoxon or  
212 chlorpyrifos-ethyl-oxon, for both earthworm species. Besides, in order to compare the

213 sensitivity of a given esterase activity between *A. caliginosa* and *A. chlorotica*, a species  
214 Susceptibility Ratio (SRs) was calculated by dividing the IC<sub>50</sub> obtained for *A. caliginosa* by  
215 the IC<sub>50</sub> obtained for *A. chlorotica*, for a considered enzyme activity and a given pesticide.  
216 Finally, a carboxylesterase susceptibility ration (SRcbe) was used in order to assess the  
217 potential bioscavenger function of CbE to protect AChE activity. This SR<sub>CbE</sub> was calculated  
218 by dividing IC<sub>50</sub> value obtained from AChE (used as reference) to the IC<sub>50</sub> value from CbE  
219 (SRcbe=IC<sub>50(AChE)</sub>/ IC<sub>50(CbE)</sub>) for a given species and a given pesticide.  
220 The increase or decrease in sensitivity is indicated by SR values greater or lesser than one,  
221 respectively.

## 222 2.7. Data analysis

223 To estimate the apparent K<sub>M</sub> and V<sub>max</sub>, specific activity was plotted against substrate  
224 concentration and data were fitted to the Michaelis-Menten equation:

$$225 \quad V_0 = (V_{\max} \cdot [S]) / (K_M + [S]) \quad (\text{eq. 1})$$

226 where V<sub>0</sub> is the initial velocity and [S] is the substrate concentration.

227 The concentration of inhibitor that inhibited 50% of enzyme activity (IC<sub>50</sub>) under our specific  
228 experimental conditions (pH, temperature, time, protein concentration) was calculated using  
229 the logistic curve from the library of non-linear regressions of the XLSTAT software:

$$230 \quad y = E_0 \cdot e^{(-b \times [I])} + E_{\text{res}} \quad (\text{eq. 2})$$

231 where E<sub>0</sub> is the total activity of the control, E<sub>res</sub> the percentage of residual enzyme activity  
232 relative to controls, b=k<sub>i</sub> x t (k<sub>i</sub> inhibition kinetic constant, t the time in min) and IC<sub>50</sub>=ln(2)/b.

233 Comparisons of concentrations, differences between species, and differences between  
234 cytosolic and microsomal fractions were analyzed with one-way ANOVA followed by Tukey  
235 HSD post-hoc comparison test using XLSTAT software (version 2013.3.01).

### 236 3. Results

#### 237 3.1. Cellular localization of enzymes

238 Taking into account the whole protein contents, we observed a higher part located in the  
239 cytosolic fraction for both *A. caliginosa* ( $77.4 \pm 0.1 \%$ ) and *A. chlorotica* ( $71.6 \pm 1.4 \%$ ) and  
240 only about 22% and 28% in the microsomal fraction respectively. For both earthworm  
241 species, AChE activity was mainly located in the microsomal fraction with a higher amount  
242 of membrane-bound enzyme for *A. caliginosa* ( $79.3 \pm 4.7 \%$ ) compare to *A. chlorotica* ( $65.4 \pm$   
243  $4.4 \%$ ). In contrast, CbE activities were mainly found in the cytosolic fractions. For both  
244 species the repartition of CbEs follows the general distribution of the whole protein contents  
245 with more than 75% of soluble CbE for *A. caliginosa* and less than 70% of soluble CbE  
246 activities for *A. chlorotica* (Fig. 1). Among the different substrates, the CbE activity related to  
247 4-NPA substrate was found to be almost equally distributed between both fractions with  $59.5$   
248  $\pm 1.8 \%$  of membrane-bound versus  $40.5 \pm 0.9 \%$  as a soluble enzyme. The two other  
249 substrates 4-NPV and 1-Na exhibited about 68% and 71% of soluble form respectively.  
250 Concerning the GST activity, only one major soluble enzymatic form was detected in the  
251 cytosolic fraction with more than 97% of the total activity for both *A. caliginosa* and *A.*  
252 *chlorotica* (Fig.1).

#### 253 3.2. Characterization of enzymes using specific substrates

254 Acetylcholinesterase activity displayed a Michaëlis–Menten kinetics within the selected range  
255 of substrate concentrations for both earthworm species (Fig. 2). The apparent  $K_m$  and  $V_{max}$   
256 were  $1.22 \pm 0.08$  mM and  $171.8 \pm 3.49$  mU  $mg^{-1}$  protein, respectively, when using *A.*  
257 *caliginosa* species, whereas  $K_m$  and  $V_{max}$  were  $2.90 \pm 0.30$  mM and  $131.6 \pm 4.38$  mU mg  
258 protein, respectively, for AChE activity using *A. chlorotica*. No AChE inhibition by high  
259 substrate concentrations (up to 40 mM) was observed for *A. chlorotica*, while a 30% decrease

260 in AChE activity was obtained for *A. caliginosa* species (data not shown) as previously  
261 described by Rault et al. (2007). CbE activity showed a Michaelis-Menten kinetic regardless  
262 of substrate (Fig. 2). The substrates 4-NPA and 1-NA were more efficiently hydrolyzed by  
263 esterases using *A. caliginosa* extracts compared to *A. chlorotica* ( $V_{\max}$  *A. caliginosa* higher  
264 than  $V_{\max}$  *A. chlorotica*). Concerning the affinity towards the different substrates, the  $K_M$   
265 showed a close relationship with both species and for these three CbE substrates we observed  
266 a higher affinity than for AcSCh substrate ( $K_M$  for 4-NPA, 4-NPV and 1-NA lower than  $K_M$   
267 for AcSCh). Thus, the best catalytic efficiency estimated as the  $V_{\max}/K_M$  ratio was obtained  
268 for CbE activity with 1-NA as substrate (Table 1). For both species, AChE exhibited a weak  
269 catalytic efficiency compare to CbE activity with ten times and twenty times lower values for  
270 *A. caliginosa* and *A. chlorotica* respectively, compare to the catalytic efficiency obtained with  
271 4-NPA and 4-NPV substrates. All the kinetic parameters for AChE and CbEs enzymes with  
272 the three substrates tested, are shown in Table 1.

273 Glutathione-S-Transferase activity displayed a Michaelis–Menten kinetics as well, within the  
274 selected range of substrate concentrations for both substrates and both earthworm species  
275 (Fig. 3). No significant differences were observed between species either concerning the  
276 affinity towards substrates than the efficiency of hydrolysis. Then, the catalytic specificities of  
277 the GST enzyme are in the same range for both earthworm species (Table 1).

### 278 3.3. *In vitro* inhibition of B-esterases

279 Inhibition of AChE and CbE activities of both earthworm species by OP pesticides followed a  
280 sigmoidal concentration response model (Fig. 4). All pesticides caused a strong inhibition on  
281 both enzyme activities with  $IC_{50}$  varying from  $10^{-6}$  to  $10^{-8}$  mol.L<sup>-1</sup> (Table 2). We observed that  
282 the inhibition strongly depends on species and OP structure. *A. caliginosa* AChE was strongly  
283 inhibited by POx compare to *A. chlorotica* as previously observed (Jouni et al., 2018).  
284 However, we here observed that CPOx exerted a higher toxicity on AChE, with the strongest

285 inhibitory effect on *A. chlorotica*. Then, it appears that inhibition of AChE depends on both  
286 OP structure and earthworm's species. *A. chlorotica* AChE exhibited a 4.9 to 8.5 times higher  
287 sensitivity than *A. caliginosa* towards CPOx and POX respectively (SRs and SRe, Table 2).  
288 When comparing AChE from *A. chlorotica* species, we found a 63 times higher sensitivity  
289 towards CPOx compared to POx. The CbE 1-NA-dependent activity exhibited similar  
290 sensitivity towards either POx or CPOx pesticides for both earthworm's species. It is  
291 noteworthy that this isoform which is the most efficient one (Table 1) is less sensitive to *in*  
292 *vitro* inhibition than 4-NPV and 4-NPA activities. Considering the effect of POx, *A.*  
293 *chlorotica* 1-NA, 4-NPA and 4-NPV CbEs appeared highly susceptible compared to AChE  
294 with a ratio of 2.6, 35.8 and 58.8 respectively. Moreover, CbEs from *A. chlorotica* appeared  
295 more sensitive than *A. caliginosa* ones (SRs from 1.2 to 13 depending on CbE's substrate). In  
296 *A. caliginosa* species, the B-esterases exhibited very close related sensitivities towards POx.  
297 Incubation using chlorpyrifos-oxon induced a higher susceptibility of AChE activity in both  
298 species. Except for *A. chlorotica* AChE, and *A. caliginosa* 4-NPV, the inhibition induced by  
299 chlorpyrifos-ethyl-oxon on the others B-esterases was about the same order of magnitude than  
300 the inhibition effect of ethyl-paraoxon.

#### 301 *3.4. In vivo response of B-esterases and GST after OP exposure*

302 Seven-days exposure of earthworms, either to ethyl-parathion or chlorpyrifos-ethyl using 1  
303 mg and 1.3 mg active ingredient (a.i.) kg<sup>-1</sup> wet soil respectively (corresponding to the usual  
304 application rate), did not induce significantly GST activity (Fig. 5). The response was similar  
305 for both species, and a slight but not significant increase in GST activity was observed with a  
306 stronger effect for *A. caliginosa* species. Concerning B-esterase activities, a dose of 1mg.kg-1  
307 of ethyl-parathion-contaminated soils caused a significant species-specific response of these  
308 enzymes (Fig. 5) that is in agreement with our previous study (Jouni et al., 2018). Whereas  
309 the mean AChE activity strongly decreased in *A. caliginosa* (78% loss; p<0.0001), this

310 esterase activity remained unchanged in *A. chlorotica* exposed to ethyl-parathion.  
311 Carboxylesterase activities dependent to 4-NPA and 4-NPV were inhibited in both  
312 earthworms' species compared to the control. A decrease in 4-NPA activity of 49% and 50%  
313 for *A. caliginosa* and *A. chlorotica* respectively, and a decrease in 4-NPV activities of 68%  
314 and 54% for *A. caliginosa* and *A. chlorotica* respectively ( $p < 0.0001$ ) were observed after  
315 exposure to ethyl-parathion. Chlorpyrifos-ethyl appeared less toxic to earthworms than ethyl-  
316 parathion. Except for the CbE activity towards 1-NA (19% loss,  $p < 0.001$ ), no significant  
317 inhibition could be observed after exposure to this OP in *A. caliginosa* species. Concerning *A.*  
318 *chlorotica* species, we observed a 30% loss of AChE activity ( $p < 0.001$ ). CbE activities from  
319 *A. chlorotica* were not significantly inhibited after chlorpyrifos-ethyl exposure.

## 320 **4. Discussion**

### 321 *4.1. Characterization of B-esterases and GST activities*

322 Exposure to OP insecticides and use of B-esterase activities as biochemical biomarkers has  
323 been extensively studied in different earthworm species (Booth and O'Hollaran, 2001;  
324 Denoyelle et al., 2007; Rault et al., 2007; Schreck et al., 2008; Sanchez-Hernandez and  
325 Wheelock, 2009; Collange et al., 2010; Jordaan et al., 2012). Studies conducted in laboratory  
326 conditions led to the conclusion that *A. chlorotica* species was generally less sensitive than *A.*  
327 *caliginosa*, especially after parathion-ethyl exposure (Rault et al., 2008; Jouni et al., 2018).  
328 However, the role of metabolic pathways in species-specific sensitivity of earthworms  
329 remains poorly described. Soluble AChE had been shown to be involved in stress regulation  
330 in the honeybee *Apis mellifera* (Kim et al., 2017). In the fruit fly having a single ace locus, the  
331 soluble form of AChE, was generated by alternative splicing and plays non-neuronal roles,  
332 such as chemical defense (Kim et al., 2014). Using earthworm species, previous studies  
333 reported that *A. chlorotica* exhibited multiple cholinesterase forms with an equal amount of



334 AChE and BuChE activities (Rault et al., 2007). Native PAGE exhibited a higher part of  
335 soluble AChE in *A. chlorotica* than *A. caliginosa* (Jouni et al., 2018) that was confirmed  
336 herein where 35% of the *A. chlorotica* activity appeared in the cytosolic fraction. Either the  
337 soluble form of AChE in *A. chlorotica* species is involved in defense mechanisms remains to  
338 be elucidated. However, these species-differential forms of AChE could account for a better  
339 capacity of *A. chlorotica* to cope with OP insecticide, and deeper analysis of such a potential  
340 role of soluble cholinesterases need to be completed. Jouni et al. (2018) clearly revealed that  
341 the activity of *A. caliginosa* AChE was more sensitive to ethyl-paraoxon than that of *A.*  
342 *chlorotica*. Comparing the AChE  $K_M$  values for both earthworm species, *A. chlorotica* exhibit  
343 a lower affinity for its substrate. These different outcomes strongly suggest that the intrinsic  
344 enzymatic properties of *A. chlorotica* AChE could be a significant cause for the lower  
345 sensitivity of this species towards ethyl-paraoxon insecticides. The relationship between  
346 species-specific variations and change in AChE structure remain to be explored. The  
347 possibility for AChE forms to possess different physiological functions in this species could  
348 act as bioscavenger that can sequester OP insecticides, as described above (Kim and Lee,  
349 2018).

350 Despite the fact that a biochemical analysis of cholinesterase forms was investigated in  
351 different earthworm species (Rault et al., 2007), no enzyme characterization was conducted  
352 on carboxylesterases either in *A. caliginosa* or *A. chlorotica* species. CbEs could play an  
353 important role as bioscavengers because of their higher sensitivity to OP compare to AChE  
354 (Küster, 2005; Wheelock et al., 2008; Kristoff et al., 2010). In the present study, for both *A.*  
355 *caliginosa* and *A. chlorotica*, AChE catalytic efficiency are about 10 to 60 times below the  
356 catalytic efficiency obtained for the CbE activities, depending on the substrate used and  
357 earthworm species (Table 1). The high  $V_m/K_M$  ratio obtained for CbEs support their important  
358 role in pesticide metabolism. Both earthworm species exhibited the same range of catalytic

359 efficiency for CbEs, following the order 1-NA (2.64-3.78 ml.min<sup>-1</sup>.mg<sup>-1</sup>) > 4-NPA (0.87-1.15  
360 ml.min<sup>-1</sup>.mg<sup>-1</sup>) or 4-NPV(0.94-1.08 ml.min<sup>-1</sup>.mg<sup>-1</sup>), and they were in the same order of  
361 magnitude as V<sub>m</sub>/K<sub>M</sub> ratio obtained for the snail *Xeropicta derbentina* CbE activity for 1-NA  
362 (4.93 ml.min<sup>-1</sup>.mg<sup>-1</sup>) > 4-NPV (2.33 ml.min<sup>-1</sup>.mg<sup>-1</sup>) > 4-NPA (0.19 ml.min<sup>-1</sup>.mg<sup>-1</sup>), (Laguerre  
363 et al., 2009) or those found in the gill (0.18 ml.min<sup>-1</sup>.mg<sup>-1</sup>) and serum (7.66 ml.min<sup>-1</sup>.mg<sup>-1</sup>)  
364 CbEs of the rainbow trout *Oncorhynchus mykiss* (Barron et al., 1999). The main location of  
365 CbEs in the cytosolic fraction in addition with multiple isoenzymes shown by Jouni et al  
366 (2018) occurred also in other species such as the earwig *F. auricularia* (Malagnoux et al.,  
367 2014) or the crayfish *Procambarus clarkii* (Vioque-Fernández et al., 2007). However, in *A.*  
368 *chlorotica* the cellular distribution of B-esterases appeared more evenly distributed between  
369 cytosolic and microsomal fractions, with a decrease in the cytosolic part to the benefit of the  
370 membrane bound counterpart compare to *A. caliginosa*. Such cellular distribution could  
371 enhance the role of CbE activities in the metabolic defense of the organism, in particular  
372 being close to the membrane-bound form of AChE. Beside the fact that the total CbE  
373 activities measured in *A. chlorotica* did not appear high enough to support an efficient  
374 mechanism against OP toxicity on their own, tissue-specific, cellular expression and catalytic  
375 efficiency of CbE could play an important role depending on their specificity.

376 GST activity was almost exclusively found in the cytosolic fraction for *A. chlorotica* and *A.*  
377 *caliginosa*, and kinetic parameters are very close related in both species. Michaëlis constant  
378 toward CDNB (1mM) or GSH (about 0.5mM) were similar to K<sub>M</sub> values described in  
379 zebrafish (Glisic et al., 2015), and basal activities (115 mU.mg<sup>-1</sup>) are in the same order than  
380 those previously measured in *A. caliginosa* and *A. chlorotica* (about 120 to 150 mU.mg<sup>-1</sup>)  
381 (Givaudan et al., 2014; Sanchez-Hernandez et al., 2014) or in the wall muscle of *L. terrestris*  
382 (323 mU.mg<sup>-1</sup>) (Sanchez-Hernandez and Wheelock, 2009).

383 *4.2. Sensitivity of B-esterase to OP pesticides*

384 From the different susceptibility ratios (Table 2) we found that (1) CbEs appeared to be more  
385 sensitive in *A. chlorotica* compared to *A. caliginosa* to POx inhibition *in vitro* and (2) CbEs  
386 appeared even more sensitive than AChE to POx inhibition in *A. chlorotica* species. However,  
387 these differences between IC<sub>50</sub> for CbEs inhibition are lower than those found in the earwig *F.*  
388 *auricularia* (45 to 400-times higher depending on substrate) (Malagnoux et al., 2014). It is  
389 generally assumed that CbEs have the potential to sequester available OP pesticides, hence  
390 protecting AChE from being inhibited (Barata et al., 2004). Based on this hypothesis, the  
391 stronger inhibitory effect of POx on CbE activities from *A. chlorotica* species would enhance  
392 their buffering capacity and could provide an efficient scavenger protecting effect of AChE  
393 from an irreversible inhibition. Our results support the irreversible inhibition of CbE activity  
394 by POx as an efficient detoxification pathway to better protect the molecular target AChE in  
395 *A. chlorotica* species. It is well known that OP binds stoichiometrically to CbE, thereby  
396 reducing its impact on nervous system AChE activity, the main target for acute OP toxicity  
397 (Maxwell and Brecht, 2002; Sogorb and Vilanova, 2002). However, in this study we  
398 highlighted that the susceptibility of B-esterases depends on OP structure. Considering the *in*  
399 *vitro* inhibitory effect of CPOx, another OP molecule, we observed that *A. chlorotica* AChE  
400 activity was strongly inhibited and displayed the highest sensitivity among the B-esterases  
401 investigated in this study. In contrast, CbE activities exhibited similar sensitivity to CPOx for  
402 both species, while previous studies have shown that the abundance of multiple CbE isozymes  
403 was higher in *A. caliginosa* compared to *A. chlorotica* (Jouni et al., 2018). As a consequence,  
404 considering together the above outcomes (lower sensitivity to OP, and fewer isoforms of  
405 CbEs) the availability of CbE molecules in *A. chlorotica* species could be not efficient enough  
406 to afford their protective role.

#### 407 4.3. *In-vivo* responses

408 The *in vivo* response of B-esterases after chlorpyrifos exposure for *A. chlorotica* was under  
409 the expected inhibition taking into account the high sensitivity of the AChE after *in vitro*  
410 experiments. Only a 30% loss of AChE activity, and no significant decrease in CbE activities  
411 were observed for *A. chlorotica* species. This finding suggests that other mechanisms are  
412 involved in the protection of AChE activity for *A. chlorotica* following *in vivo* exposure.  
413 GSTs are the most important enzyme group involved in the conjugation of pollutants  
414 (Kostaropoulos et al., 2001; Enayati et al., 2005). These enzymes are implied in the  
415 detoxification of various endogenous and xenobiotic compounds such as drugs, insecticides,  
416 organic pollutants, secondary metabolites and other toxins (Hayes et al., 2005). The cellular  
417 localization of GSTs as well as their activity have been linked to insecticide resistance in  
418 some insect species (Hemingway and Ranson, 2000; Li et al., 2007; Lumjuan et al., 2011).  
419 Likewise, previous studies have demonstrated the role of both CbEs and GSTs in the  
420 metabolism of OP compounds in earthworms (Stenersen and Øien., 1981; Stenersen, 1984).  
421 GST activity has been shown to give good responses to environmental disturbances for *E.*  
422 *fetida* and *Lumbricus rubellus* in previous studies (Brown et al., 2004; Ribera et al., 2001).  
423 Schreck et al. (2008) showed that a short-term exposure (3 days) of *A. caliginosa* to the  
424 insecticides chlorpyrifos increased GST activity, while long-term exposure induced a  
425 decrease in GST activity. Those reports indicated that GST could participate in the  
426 detoxification of selected insecticides in a time-dependent manner. Velki and Hackenberger  
427 (2013) showed that duration of exposure significantly changed the effects of pirimiphos-  
428 methyl (OP) and deltamethrin (pyrethroid) on *Eisenia Andrei* GST activity, with an increase  
429 on the first 3 days of exposure. Time-dependent response was also observed after chlorpyrifos  
430 exposure, with a significant increase of GST activity (33–35% of controls) in *A. caliginosa*  
431 short-term exposed to the OP (3 days exposure) while 21 days exposure did not induce any  
432 increase of GST activity (Sanchez-Hernandez et al., 2014). In our study, 7 days exposure to

433 OP insecticides did not induced significant GST activity. However, we cannot exclude that an  
434 efficient increase of GST activity in the first days of exposure could occur, helping  
435 earthworm's species to better face impact of OP. Assessing time responses depending on  
436 species to better cope with one or another OP molecule needs to be completed. To date, the  
437 absence of GST response following 7 days exposure is consistent with previous observations  
438 made on *A. caliginosa* and *A. chlorotica* species after exposure either to a fungicide, an  
439 herbicide or their mixture (Givaudan et al., 2014). These authors suggested that a pre-  
440 exposure to the pesticides in the fields was required for activation of the GST detoxication  
441 system. In our study, earthworms were collected in apple orchards free from pesticides since  
442 at least 10 years, which could impact the ability to quickly induce their metabolic defenses.  
443 Mechanisms linked with *in vivo* OP bioactivation could have a significant role in the OP  
444 toxicity and in addition to GST, cytochrome P450-dependent monooxygenases are of prime  
445 importance. Organophosphorus compounds could induce differential toxicity depending on  
446 the CYP450 family involved in their metabolization, as mentioned in honey bees and  
447 bumblebees (Mao et al., 2011; Johnson, 2015; Reid et al., 2020), in rats (Karanth et al., 2004)  
448 or in human liver microsomes (Buratti et al., 2003). This finding highlights the need to  
449 consider each compound independently, within an insecticide class sharing the same mode of  
450 action. The full understanding of the toxicity of a variety of OP pesticides is still unclear,  
451 because of the extreme complex mechanism of action. Some investigations used a  
452 quantitative structure-toxicity relationship to assess OP toxicity (Can, 2014), while others  
453 investigated the toxic effects of pesticides by combining absorption, distribution, metabolism,  
454 excretion and toxicity properties (Zhu et al., 2018).

## 455 **5. Conclusion**

456 The present study is the first characterization of both CbE and GST activities in two endogeic  
457 species of earthworms. Moreover, the sensitivity of both *A. chlorotica* and *A. caliginosa*  
458 species was assessed either *in vitro* and *in vivo*. We highlighted differential toxicity of two OP  
459 pesticides, depending on earthworms' species. Firstly, we confirmed a better tolerance to  
460 parathion-ethyl of *A. chlorotica* compare to *A. caliginosa*, and secondly, we pointed out  
461 higher toxicity of chlorpyrifos-ethyl in *A. chlorotica* compare to *A. caliginosa* AChE, after *in*  
462 *vitro* experiments. Still, *A. chlorotica* was the more tolerant species after *in vivo* exposure.  
463 The main biochemical differences observed lie in the homogeneous distribution of AChE  
464 forms, depending on species. Potential bioscavenger capabilities of OP from soluble  
465 cholinesterase forms could be responsible for an efficient mechanism in the species-specific  
466 differences observed towards pesticides. Considering metabolic pathways, the apparent  
467 constitutive tolerance of *A. chlorotica* to OP pesticides did not seem to depend on catalytic  
468 properties of either *A. chlorotica* CbEs or GST activities. Other toxicodynamic factors should  
469 contribute to the inter-specific toxicity of OP insecticides in earthworm species. Different  
470 biological processes must be investigated to further disentangle the mechanisms involved in  
471 OP bioactivation and in the modification of this chemical compound while inside the  
472 organisms, compare to *in vitro* responses.

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662 Zhu J., Wang J., Ding Y., Liu B., Xiao W. 2018. A systems-level approach for investigating  
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665 Table 1. Kinetic parameters<sup>a</sup> for AChE, CbEs and GST activities measured in *A. caliginosa*  
 666 and *A. chlorotica* homogenate (mean  $\pm$  SD).

Enzyme	Earthworm	$V_m$ (U.mg <sup>-1</sup> )	$K_M$ (mM)	$V_m/K_M$
	species			(mL.min <sup>-1</sup> .mg <sup>-1</sup> )
AChE	<i>A. caliginosa</i>	0.172 $\pm$ 0.003	1.216 $\pm$ 0.077	0.141 $\pm$ 0.003
	<i>A. chlorotica</i>	0.132 $\pm$ 0.004	2.904 $\pm$ 0.307	0.045 $\pm$ 0.001
1-NA	<i>A. caliginosa</i>	1.019 $\pm$ 0.035	0.270 $\pm$ 0.039	3.776 $\pm$ 0.131
	<i>A. chlorotica</i>	0.433 $\pm$ 0.016	0.164 $\pm$ 0.030	2.636 $\pm$ 0.095
4-NPA	<i>A. caliginosa</i>	0.518 $\pm$ 0.011	0.597 $\pm$ 0.051	0.869 $\pm$ 0.019
	<i>A. chlorotica</i>	0.306 $\pm$ 0.008	0.265 $\pm$ 0.035	1.153 $\pm$ 0.031
4-NPV	<i>A. caliginosa</i>	0.185 $\pm$ 0.006	0.172 $\pm$ 0.030	1.076 $\pm$ 0.035
	<i>A. chlorotica</i>	0.167 $\pm$ 0.007	0.178 $\pm$ 0.040	0.939 $\pm$ 0.039
GST-GSH	<i>A. caliginosa</i>	0.115 $\pm$ 0.000	0.514 $\pm$ 0.000	0.224 $\pm$ 0.000
	<i>A. chlorotica</i>	0.099 $\pm$ 0.000	0.468 $\pm$ 0.000	0.211 $\pm$ 0.000
GST-CDNB	<i>A. caliginosa</i>	0.314 $\pm$ 0.000	1 $\pm$ 0	0.314 $\pm$ 0.000
	<i>A. chlorotica</i>	0.278 $\pm$ 0.000	1 $\pm$ 0	0.278 $\pm$ 0.000

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668 <sup>a</sup> Kinetic parameters were obtained by fitting the experimental data points to the Michaelis-Menten equation  
 669 using XLSTAT software.

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671

672 Table 2 : Molar concentrations of pesticides to yield *in vitro* 50% of enzyme inhibition (IC<sub>50</sub>)  
 673 of acetylcholinesterase (AChE) and carboxylesterase (CbE) activities of *A. caliginosa* and *A.*  
 674 *chlorotica* and correlation coefficient ( $R^2$ ) for nonlinear regressions.  
 675

Esterase	Pesticides	Eartworm species	$R^2$	IC <sub>50</sub> (mol.L <sup>-1</sup> )	<sup>a</sup> SRe	<sup>b</sup> SRs	<sup>c</sup> SRcbe
AChE	Paraoxon	<i>A. caliginosa</i>	0.990	(1.82 ± 0.13) 10 <sup>-7</sup>	-	-	-
		<i>A. chlorotica</i>	0.968	(1.34 ± 0.15) 10 <sup>-6</sup>	0.1	0.1	-
	Chlorpyrifos-oxon	<i>A. caliginosa</i>	0.978	(1.05 ± 0.12) 10 <sup>-7</sup>	1.7	-	-
		<i>A. chlorotica</i>	0.943	(2.13 ± 0.43) 10 <sup>-8</sup>	8,5	4.9	-
1-NA	Paraoxon	<i>A. caliginosa</i>	0.934	(6.22 ± 2.30) 10 <sup>-7</sup>	0.3	-	0.3
		<i>A. chlorotica</i>	0.993	(5.09 ± 0.72) 10 <sup>-7</sup>	0.4	1.2	2.6
	Chlorpyrifos-oxon	<i>A. caliginosa</i>	0.926	(4.79 ± 2.28) 10 <sup>-7</sup>	0.4	-	0.2
		<i>A. chlorotica</i>	0.932	(5.99 ± 2.55) 10 <sup>-7</sup>	0.3	0.8	0.0
4-NPA	Paraoxon	<i>A. caliginosa</i>	0.951	(4.87 ± 1.27) 10 <sup>-7</sup>	0.4	-	0.4
		<i>A. chlorotica</i>	0.915	(3.75 ± 2.05) 10 <sup>-8</sup>	4.9	13.0	35.8
	Chlorpyrifos-oxon	<i>A. caliginosa</i>	0.969	(7.50 ± 1.82) 10 <sup>-7</sup>	0.2	-	0.1
		<i>A. chlorotica</i>	0.960	(4.71 ± 1.38) 10 <sup>-7</sup>	0.4	1.6	0.0
4-NPV	Paraoxon	<i>A. caliginosa</i>	0.981	(5.08 ± 1.19) 10 <sup>-8</sup>	3.6	-	3.6
		<i>A. chlorotica</i>	0.983	(2.28 ± 0.71) 10 <sup>-8</sup>	8.0	2.2	58.8
	Chlorpyrifos-oxon	<i>A. caliginosa</i>	0.976	(4.43 ± 0.59) 10 <sup>-8</sup>	4.1	-	2.4
		<i>A. chlorotica</i>	0.973	(1.36 ± 0.23) 10 <sup>-7</sup>	1.3	0.3	0.2

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 677 <sup>a</sup> SRe corresponds to an esterase susceptibility ratio calculated by dividing IC<sub>50</sub> value obtained for the  
 678 AChE from *A. caliginosa* inhibited by ethyl-paraoxon (used as a reference) to the IC<sub>50</sub> value of other  
 679 esterase (from *A. caliginosa* or *A. chlorotica*) inhibited either by ethyl-paraoxon or chlorpyrifos-ethyl-  
 680 oxon (SRe= IC<sub>50</sub>(AChE -*A. caliginosa*)/ IC<sub>50</sub>(esterase) ).

681 <sup>b</sup> SRs corresponds to a species susceptibility ratio calculated by dividing IC<sub>50</sub> value obtained from *A.*



682 caliginosa (used as reference) to the  $IC_{50}$  value from *A. chlorotica* ( $SRs = IC_{50(A. caliginosa)} / IC_{50(A. chlorotica)}$ )  
683 for a given esterase.

684 <sup>c</sup> SRcbe corresponds to a carboxylesterase susceptibility ratio calculated by dividing  $IC_{50}$  value  
685 obtained from AChE (used as reference) to the  $IC_{50}$  value from CbE ( $SRcbe = IC_{50(AChE)} / IC_{50(CbE)}$ ) for a  
686 given species and a given pesticide.

687 A Susceptibility Ratio  $>1$  denotes an increase in sensitivity compare to the reference.

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690 **Figure Legends**

691 Figure n°1: Cellular distribution of proteins, GST and B-esterases activities in *A. caliginosa*  
692 and *A. chlorotica* species. Protein and enzyme contents were measured in the cytosolic and  
693 microsomal fractions of the crude extract.

694 Figure n°2: Determination of B-esterase kinetic parameters in both *A. caliginosa* and *A.*  
695 *chlorotica* species. Kinetic parameters (apparent  $V_m$  and  $KM$ ) were estimated by fitting the  
696 data to the Michaëlis-Menten equation. Each point corresponds to the mean of three  
697 independent assays ( $\pm$  standard deviation). Kinetic parameters are shown in Table 1.

698 Figure n°3: Determination of GST kinetic parameters in both *A. caliginosa* and *A. chlorotica*  
699 species. Kinetic parameters (apparent  $V_m$  and  $KM$ ) were estimated by fitting the data to the  
700 Michaelis-Menten equation. Each point corresponds to the mean of three independent assays  
701 ( $\pm$  standard deviation). Kinetic parameters are shown in Table 1.

702 Figure n°4 : Effect of the organophosphate insecticides ethyl-paraoxon and chlorpyrifos-oxon  
703 on *A. caliginosa* and *A. chlorotica* B-esterases. Each point corresponds to the mean of three  
704 independent assays ( $\pm$  standard deviation).  $IC_{50}$  values are shown in Table 2.

705 Figure n°5: Response of GST and B-esterases (AChE and CbE) activities in earthworms  
706 exposed to ethyl-parathion or chlorpyrifos-ethyl contaminated soils for 7 days. The  
707 carboxylesterase activities were measured using the substrates 4-nitrophenyl acetate (4-NPA),  
708 4-nitrophenyl valerate (4-NPV), and 1-naphtyl acetate (1-NA). Different letters indicate  
709 significant differences between treatments ( $p < 0.001$ ).

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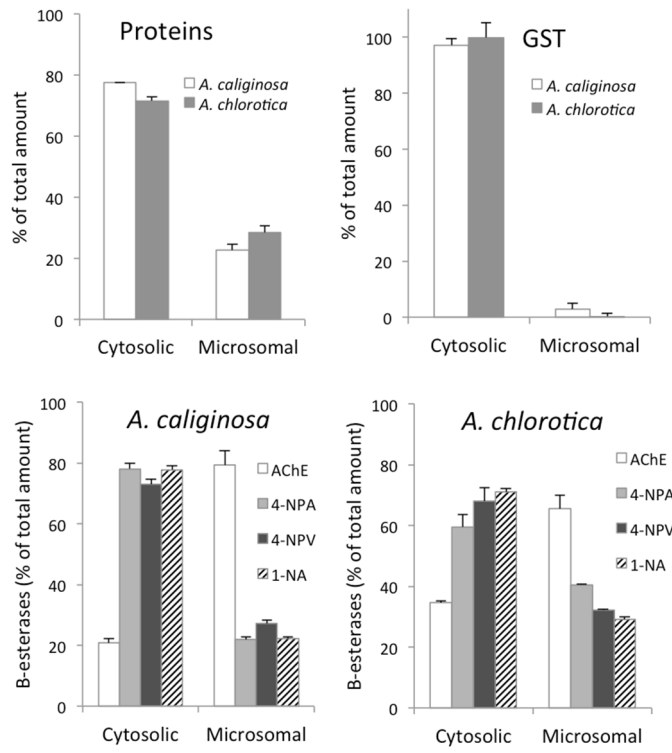
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Figure 1

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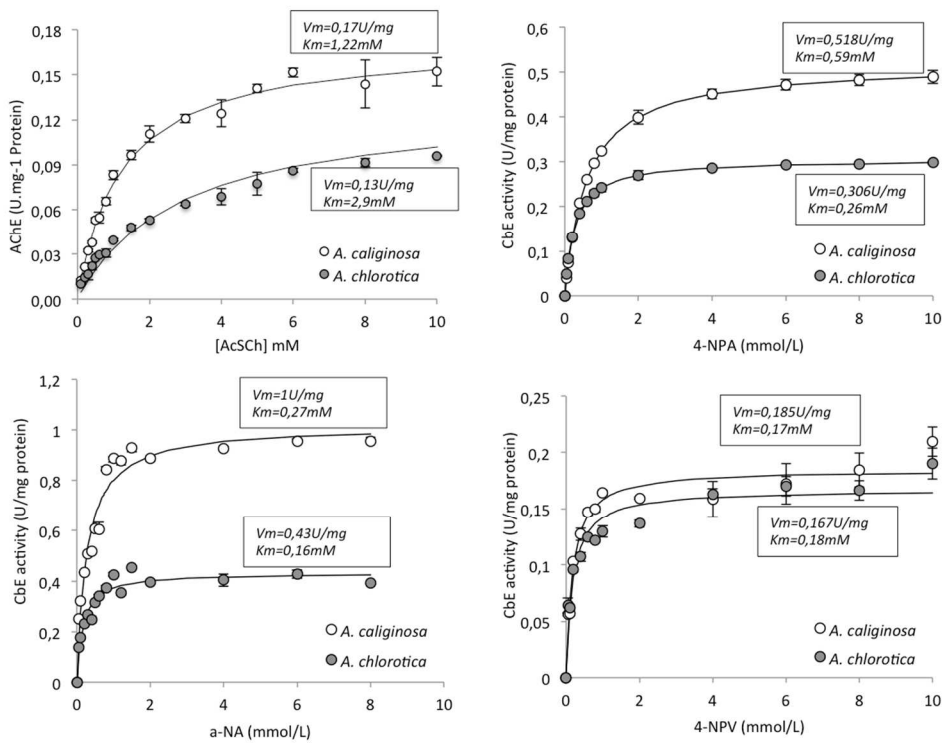


Figure 2

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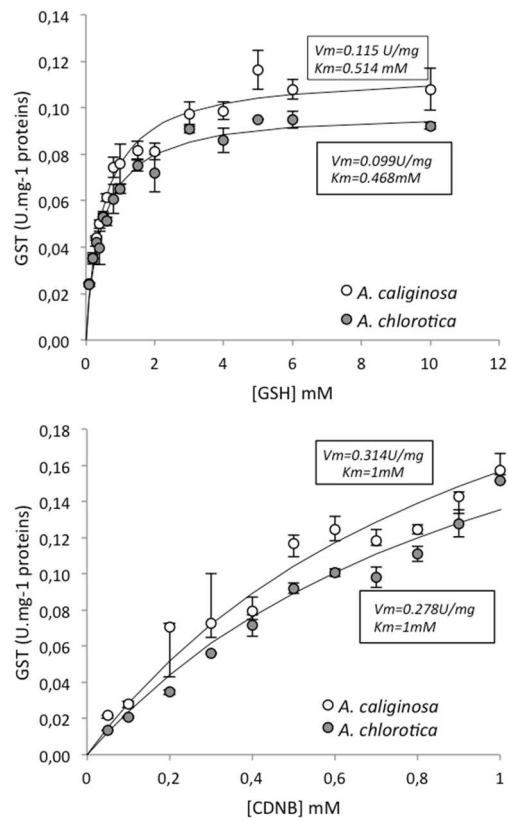


Figure 3

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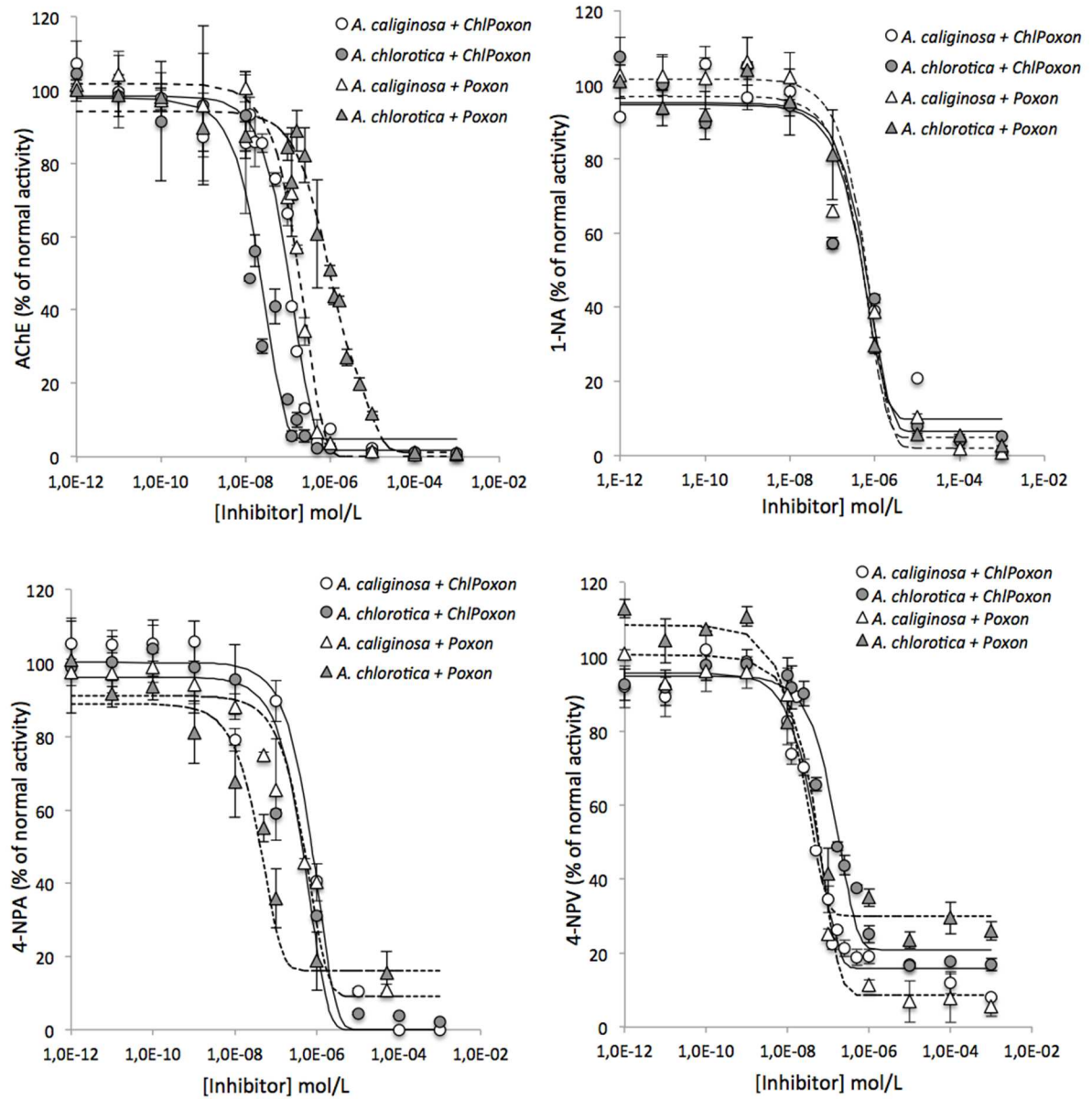


Figure 4

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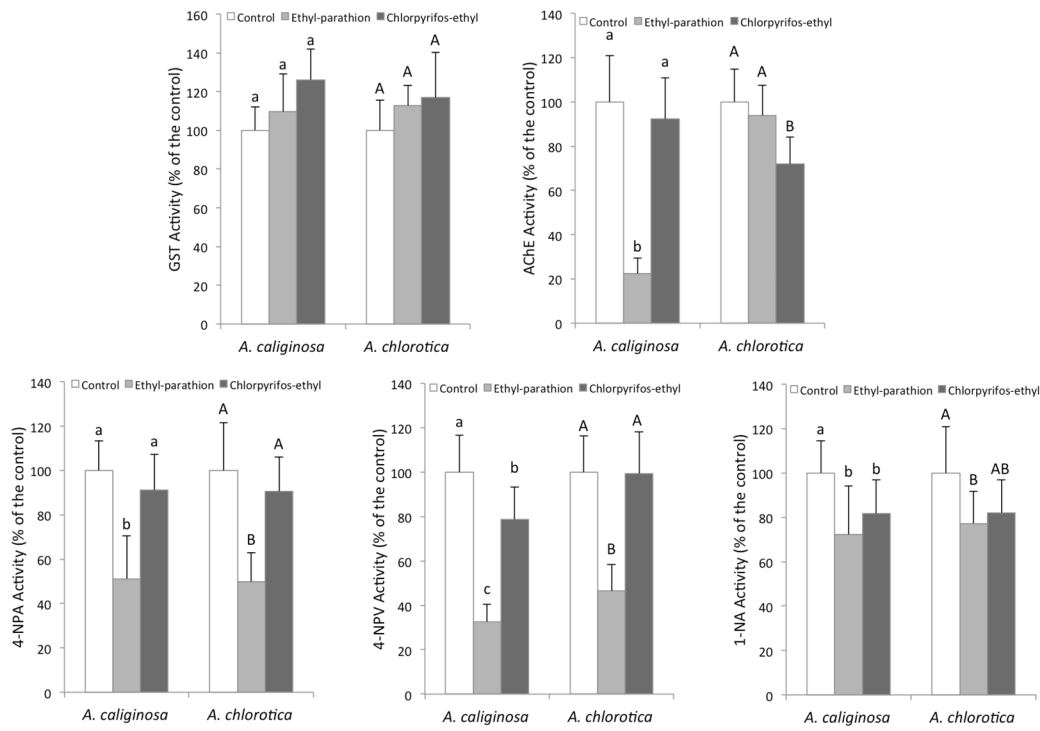


Figure 5