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Elucidating pesticide sensitivity of two endogeic earthworm species through 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

Abstract

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

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

1. Introduction

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

Organophosphorus compounds (OP) are still commonly used insecticides in agriculture (Dalmolin et al., 2020) to control agricultural pests, and they display a high acute toxicity to wildlife (Köhler and Triebskorn, 2013). These insecticides are known neurotoxic chemicals targeting acetylcholinesterase (AChE, EC.3.1.1.7), a key enzyme in regulating the transmission of nervous impulse at the cholinergic synapses (Fairbrother et al., 1991; Nunes, 2011; Casida, 2017). Inhibition of AChE activity has been extensively reported as a sensitive and specific biomarker of exposure to OPs (Sanchez-Hernandez, 2011). In the particular case of earthworms, species-specific differences in OP sensitivity has been reported in previous 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

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

2.2. Soil and earthworms

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

Soil was sieved at <2 mm and the water content was adjusted to 20-21% (approximately 81% of the maximum water holding capacity) with distilled water. Afterwards, wet soil samples (1 kg) were spiked with ethyl-parathion or chlorpyrifos solutions (40 ml) to yield the final concentrations of 1 mg and 1.3 mg active ingredient (a.i.) kg^{-1} wet soil respectively. Both insecticide concentrations refer to the usual application rate and calculation of the Predicted Environmental Concentration (PEC). A control was prepared under the same conditions without pesticide application. The wet polluted or unpolluted soil was then split between Petri dishes (diameter 10 cm, height 3 cm) so that each contained 100 g soil. A single earthworm was placed in each Petri dish to limit interspecific interactions and to prevent cascade death (Sheppard and Evenden, 1992). Dishes were kept in a climate chamber (12±1°C, and permanent dark), and after 7 days of exposure to the pesticide, each earthworm was collected, 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^{-1}, 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^{-1}, antipain 1 µg mL^{-1}, and trypsin
143 inhibitor 1 mg mL^{-1}). 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 µL, final volume) contained 4 µL 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 µL final volume) containing 25 mM Tris-HCl (pH 7.6), 1 mM $CaCl_2$
171 and 2 µL 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 25ºC by the addition of 50 µL 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-naphtol]) 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 µL, final volume
178 including 2 µL 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 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 M^{-1} cm^{-1}). The reaction medium
184 (200 µL, final volume) contained 2 µL 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 (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 µl) were incubated individually with
197 5µl of serial concentrations of ethyl-paraoxon or chlorpyrifos-ethyl-oxon (10^{-12} to 10^{-3} mol.L^{-1}
198 final concentration) or with 5µl of solvent for the control, for 30 min at 25ºC. 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 5µL, 2.5µL 1/20, and
202 2.5µL 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 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 IC_{50} reference-value by the 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_{50} obtained for *A. caliginosa* by
215 the IC_{50} 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_{CbE} was calculated
218 by dividing IC_{50} value obtained from AChE (used as reference) to the IC_{50} value from CbE
219 ($SRcbe=IC_{50(AChE)}/ IC_{50(CbE)}$) 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_M and V_{max}, specific activity was plotted against substrate
224 concentration and data were fitted to the Michaelis-Menten equation:

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

226 where V_0 is the initial velocity and [S] is the substrate concentration.

227 The concentration of inhibitor that inhibited 50% of enzyme activity (IC_{50}) 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 $y=E_0 . e^{(-b \times [I])}+E_{res}$ (eq. 2)

231 where E_0 is the total activity of the control, E_{res} the percentage of residual enzyme activity
232 relative to controls, $b=k_i \times t$ (k_i inhibition kinetic constant, t the time in min) and $IC_{50}=\ln(2)/b$.

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 3 ± 0.1 %) and *A. chlorotica* (71.6 ± 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 ± 4.7 %) compare to *A. chlorotica* (65.4 ±
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 ± 1.8 % of membrane-bound versus 40.5 ± 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 ± 0.08 mM and 171.8 ± 3.49 mU mg^{-1} protein, respectively, when using *A.*
257 *caliginosa* species, whereas K_m and V_{max} were 2.90 ± 0.30 mM and 131.6 ± 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^{-1} (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^{-1} 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^{-1}.mg^{-1}) > 4-NPA (0.87-1.15
360 ml.min^{-1}.mg^{-1}) or 4-NPV(0.94-1.08 ml.min^{-1}.mg^{-1}), and they were in the same order of
361 magnitude as V_m/K_M ratio obtained for the snail *Xeropicta derbentina* CbE activity for 1-NA
362 (4.93 ml.min^{-1}.mg^{-1}) > 4-NPV (2.33 ml.min^{-1}.mg^{-1}) > 4-NPA (0.19 ml.min^{-1}.mg^{-1}), (Laguerre
363 et al., 2009) or those found in the gill (0.18 ml.min^{-1}.mg^{-1}) and serum (7.66 ml.min^{-1}.mg^{-1})
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_M values described in
379 zebrafish (Glisic et al., 2015), and basal activities (115 mU.mg^{-1}) are in the same order than
380 those previously measured in *A. caliginosa* and *A. chlorotica* (about 120 to 150 mU.mg^{-1})
381 (Givaudan et al., 2014; Sanchez-Hernandez et al., 2014) or in the wall muscle of *L. terrestris*
382 (323 mU.mg^{-1}) (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_{50} 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.

473 **6. References**

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664

665 Table 1. Kinetic parameters[a] for AChE, CbEs and GST activities measured in *A. caliginosa*

666 and *A. chlorotica* homogenate (mean ± SD).

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

667

668 [a] Kinetic parameters were obtained by fitting the experimental data points to the Michaelis-Menten equation

669 using XLSTAT software.

670

671

672 Table 2 : Molar concentrations of pesticides to yield *in vitro* 50% of enzyme inhibition (IC_{50})
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_{50} (mol.L^{-1})	[a]SRe	[b]SRs	[c]SRcbe
AChE	Paraoxon	*A. caliginosa*	*0.990*	(1.82 ± 0.13) 10^{-7}	-	-	-
		A. chlorotica	*0.968*	(1.34 ± 0.15) 10^{-6}	0.1	0.1	-
	Chlorpyrifos-oxon	*A. caliginosa*	*0.978*	(1.05 ± 0.12) 10^{-7}	1.7	-	-
		A. chlorotica	*0.943*	(2.13 ± 0.43) 10^{-8}	8,5	4.9	-
1-NA	Paraoxon	*A. caliginosa*	*0.934*	(6.22 ± 2.30) 10^{-7}	0.3	-	0.3
		A. chlorotica	*0.993*	(5.09 ± 0.72) 10^{-7}	0.4	1.2	2.6
	Chlorpyrifos-oxon	*A. caliginosa*	*0.926*	(4.79 ± 2.28) 10^{-7}	0.4	-	0.2
		A. chlorotica	*0.932*	(5.99 ± 2.55) 10^{-7}	0.3	0.8	0.0
4-NPA	Paraoxon	*A. caliginosa*	*0.951*	(4.87 ± 1.27) 10^{-7}	0.4	-	0.4
		A. chlorotica	*0.915*	(3.75 ± 2.05) 10^{-8}	4.9	13.0	35.8
	Chlorpyrifos-oxon	*A. caliginosa*	*0.969*	(7.50 ± 1.82) 10^{-7}	0.2	-	0.1
		A. chlorotica	*0.960*	(4.71 ± 1.38) 10^{-7}	0.4	1.6	0.0
4-NPV	Paraoxon	*A. caliginosa*	*0.981*	(5.08 ± 1.19) 10^{-8}	3.6	-	3.6
		A. chlorotica	*0.983*	(2.28 ± 0.71) 10^{-8}	8.0	2.2	58.8
	Chlorpyrifos-oxon	*A. caliginosa*	*0.976*	(4.43 ± 0.59) 10^{-8}	4.1	-	2.4
		A. chlorotica	*0.973*	(1.36 ± 0.23) 10^{-7}	1.3	0.3	0.2

676

677 [a] SRe corresponds to an esterase susceptibility ratio calculated by dividing IC_{50} value obtained for the
678 AChE from *A. caliginosa* inhibited by ethyl-paraoxon (used as a reference) to the IC_{50} value of other
679 esterase (from *A. caliginosa* or *A. chlorotica*) inhibited either by ethyl-paraoxon or chlorpyrifos-ethyl-
680 oxon (SRe= $IC_{50(AChE\ -A.caliginosa)}$/ $IC_{50(esterase)}$).

681 [b] SRs corresponds to a species susceptibility ratio calculated by dividing IC_{50} 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 [c] 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 Ration >1 denotes an increase in sensitivity compare to the reference.

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689

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 (± 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 (± 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 (± 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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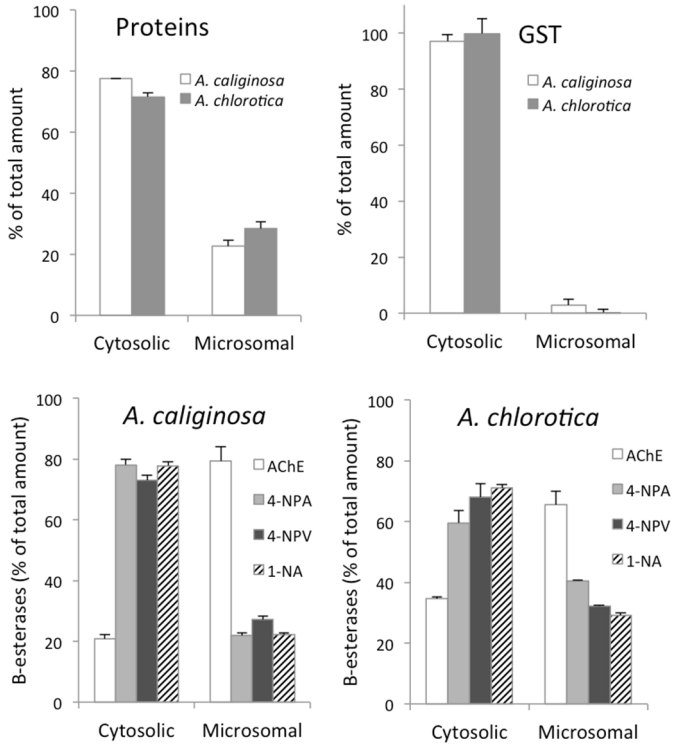

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728 Figure 1

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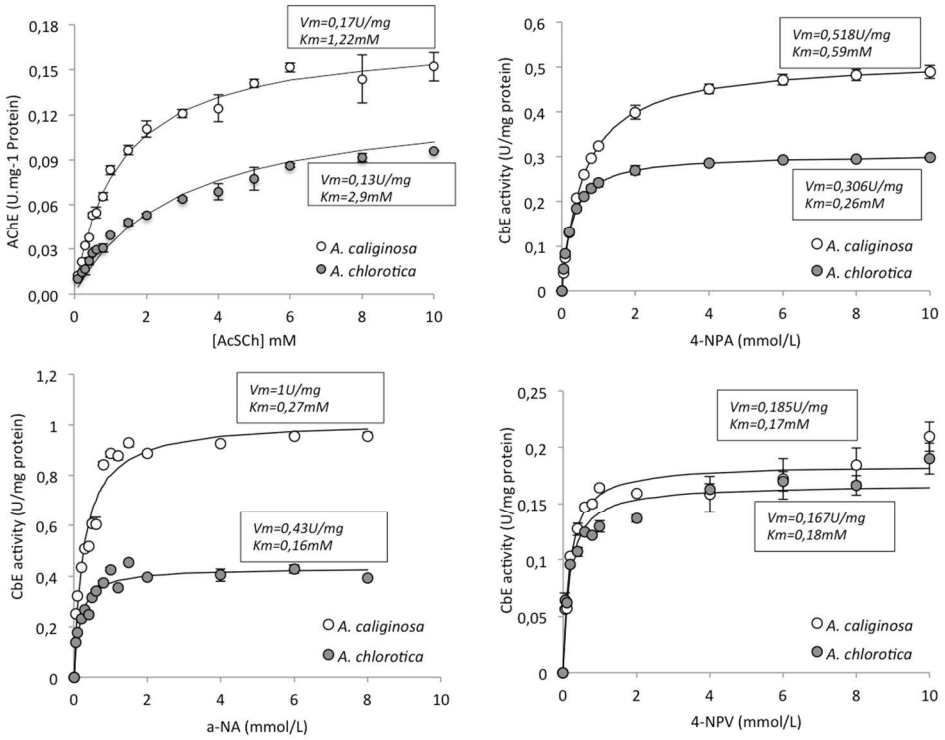
Vm=0,17U/mg
Km=1,22mM
Vm=0,13U/mg
Km=2,9mM
AChE (U.mg-1 Protein)
[AcSCh] mM
A. caliginosa
A. chlorotica
Vm=0,518U/mg
Km=0,59mM
Vm=0,306U/mg
Km=0,26mM
CbE activity (U/mg protein)
4-NPA (mmol/L)
Vm=1U/mg
Km=0,27mM
Vm=0,43U/mg
Km=0,16mM
a-NA (mmol/L)
Vm=0,185U/mg
Km=0,17mM
Vm=0,167U/mg
Km=0,18mM
4-NPV (mmol/L)

Figure 2

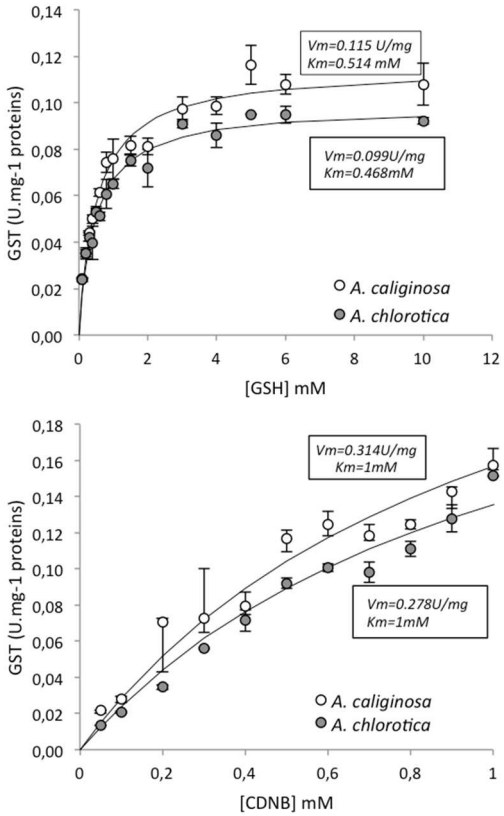
Vm=0.115 U/mg
Km=0.514 mM
Vm=0.099U/mg
Km=0.468mM
A. caliginosa
A. chlorotica
GST (U.mg-1 proteins)
[GSH] mM
Vm=0.314U/mg
Km=1mM
Vm=0.278U/mg
Km=1mM
A. caliginosa
A. chlorotica
GST (U.mg-1 proteins)
[CDNB] mM

Figure 3

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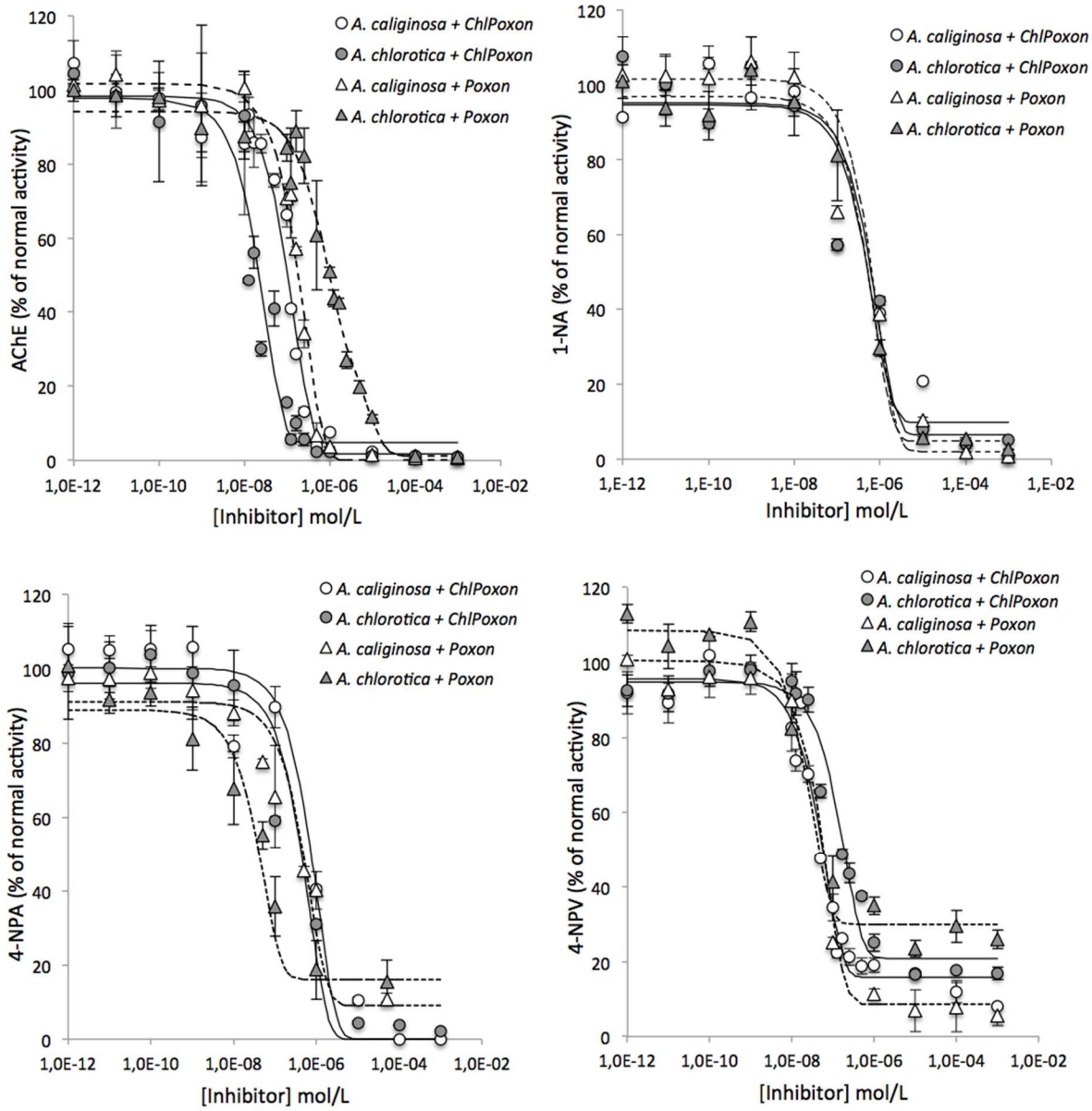

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789 Figure 4

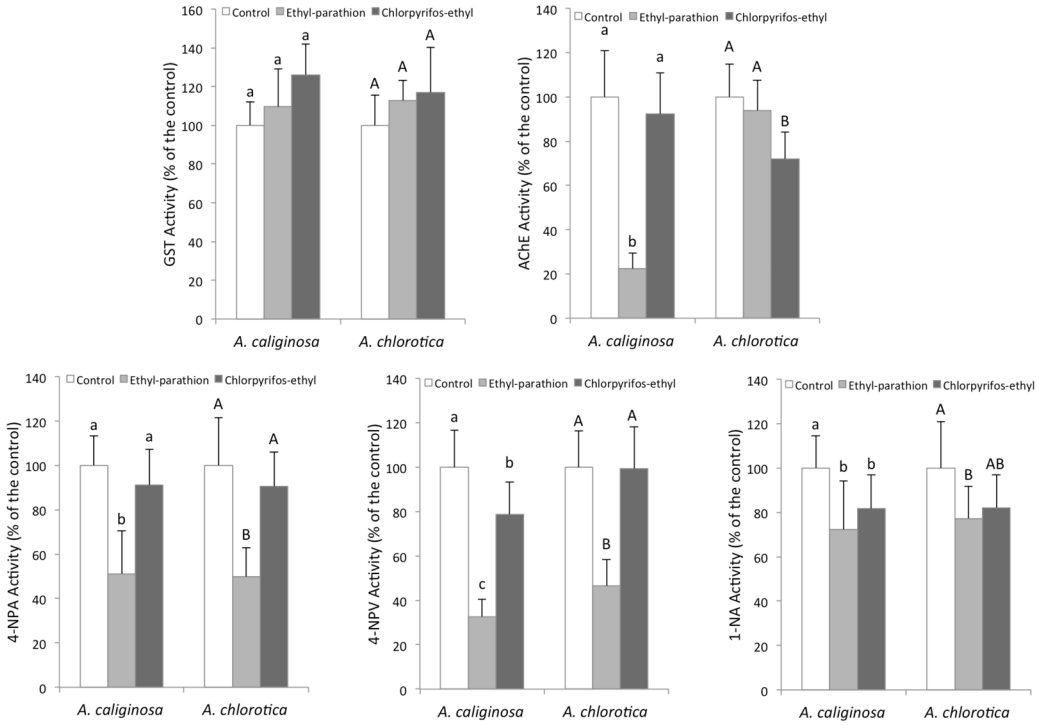
Control
Ethyl-parathion
Chlorpyrifos-ethyl
GST Activity (% of the control)
AChE Activity (% of the control)
4-NPA Activity (% of the control)
4-NPV Activity (% of the control)
1-NA Activity (% of the control)
A. caliginosa
A. chlorotica

Figure 5