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1 Interspecific differences in biochemical and behavioral biomarkers in endogeic  
2 earthworms exposed to ethyl-parathion

3

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20

21 Abstract

22 Earthworms are common organisms in the soil toxicity-testing framework, and the epigeic  
23 *Eisenia andrei* and *E. fetida* are the recommended species. However, *Eisenia* species are  
24 rarely found in agricultural soils and recent studies have pointed out endogeic species are  
25 more sensitive to pesticide than *Eisenia*. *Allolobophora chlorotica* and *Aporrectodea*  
26 *caliginosa* are two endogeic soil-dwelling species that are abundant in the agroecosystem.  
27 However, knowledge on pesticide impact on this ecological group of earthworms is still  
28 incipient. Herein, we compared the biochemical (acetylcholinesterase [AChE] and  
29 carboxylesterase [CbE] activities) behavioral (burrowing, casting and feeding) biomarker  
30 responses of these two endogeic earthworm species exposed for 7 days to soils contaminated  
31 with 0.1, 1 and 10 mg kg<sup>-1</sup> ethyl-parathion. The results showed marked species-specific  
32 differences in both groups of biomarkers, suggesting *A. caliginosa* the most sensitive species  
33 to this organophosphorus pesticide under the exposure conditions in this study. Moreover, an  
34 *in vitro* inhibition trial with ethyl-paraoxon evidenced a higher sensitivity of *A. caliginosa*  
35 AChE activity compared with that of *A. chlorotica*. This finding suggested that this molecular  
36 target endpoint could contribute to the interspecific differences of behavioral responses rather  
37 than CbE activity; this latter considered a potent mechanism of OP removal. Our results  
38 suggest the inclusion of more than one endogeic earthworm species to assess toxicity from  
39 organophosphorus insecticides. However, the use of *A. caliginosa* in the environmental risk  
40 assessment framework of organophosphorus contamination is highly recommended because  
41 of its higher sensibility to this class of pesticides, in addition to its abundance in the  
42 agroecosystem.

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45 Keywords: behavioral biomarkers; esterases; *Aporrectodea caliginosa*; *Allolobophora*  
46 *chlorotica*; organophosphorus

## 47 **1. Introduction**

48 Cholinesterase-inhibiting pesticides such as organophosphates (OP) continue to be an  
49 important group of agrochemicals used in the chemical control of agricultural pests. Although  
50 these pesticides show a relatively low environmental persistence, they lack target specificity  
51 and generally display a high acute toxicity towards non-target organisms such as earthworms  
52 (Pelosi et al., 2013a). The mechanism of acute toxicity of these chemicals is one of the best  
53 described in the toxicology literature and involves the inhibition of acetylcholinesterase,  
54 (AChE, EC 3.1.1.7); a key enzyme in the nervous system (Fukuto, 1990; Thompson and  
55 Richardson, 2004; Casida, 2017). This enzyme hydrolyses the neurotransmitter acetylcholine  
56 at the synaptic cleft, thus ending the transmission of the nerve impulse. Species-specific  
57 differences in OP sensitivity and resistance is due mainly not only to the affinity of the OP by  
58 the active site of AChE, but also by the number of OP molecules that interact with AChE  
59 (Maxwell, 1992; Küster, 2005). In this context, the enzymes carboxylesterases (CbE, EC  
60 3.1.1.1.) play an important role in the detoxification of OPs. This serine hydrolase is often  
61 more sensitive to inhibition by OPs than AChE (Wheelock et al. 2008), so this chemical  
62 interaction is considered an efficient mechanism of OP detoxification by which fewer OP  
63 molecules may reach the active site of AChE (Chanda et al. 1997). Indeed, CbE activity  
64 participates in the tolerance of some insect pests to OP pesticides (Farnsworth et al. 2010).  
65 Despite this potential role of CbE activity in the interspecific differences in OP susceptibility,  
66 its implication in the OP toxicity in earthworms is still very limited.

67 The classical use of AChE and CbE activities in ecotoxicology is to be biomarkers of OP  
68 exposure, using a wide range of methodological approaches that involve both laboratory  
69 toxicity bioassays and field monitoring surveys (Otero and Kristoff, 2016). However, it is  
70 necessary to know what consequences for the organism and its population will come from the  
71 inhibition of AChE activity (Scott and Sloman, 2004). Because this enzyme is implied in

72 animal locomotion and sensorial functions, pesticide-exposed organisms would manifest  
73 some kind of neurobehavioral disruption. However, this functional link between biochemical  
74 and behavioral toxicological endpoints has not been well established for earthworms. Indeed,  
75 many investigations have linked behavioral changes with inhibition of AChE activity in OP-  
76 exposed birds (Walker, 2003), fish (Tilton et al. 2011; Khalil et al., 2017), and invertebrates  
77 (Jensen et al., 1997; García-de la Parra et al. 2006; Malagnoux et al. 2015). In the case of  
78 earthworms, which are recommended organisms in terrestrial ecotoxicity testing (EC, 2002),  
79 some studies have developed simple and low cost procedures to assess behavioral  
80 perturbations in polluted environments, but they do not include the measurement of exposure  
81 biomarkers as initiating molecular events of toxicity. For example, the capability of  
82 earthworms to avoid unfavourable or contaminated soils has been implemented in a  
83 standardized test named avoidance behavior response test (ISO, 2008). Similarly, Capowiez et  
84 al. (2010) developed a simple and quick procedure to measure the cast production (egested  
85 soil after gut transfer) by soil-dwelling earthworms (i.e., endogeic and anecic species), which  
86 was a sensitive behavioral endpoint to soil contamination. Despite these methodological  
87 advances in earthworm behavioral toxicology, little is known on species-specific differences  
88 of earthworm behavior related to chemical stressors such as pesticides. This is probably  
89 because *Eisenia* sps. are the recommended organisms in soil toxicity testing so far.  
90 Nonetheless, there is a growing concern in the inclusion of soil dwelling species because of  
91 their significant impact on physicochemical and biological properties of soil, their population  
92 density in the agroecosystems, and their higher sensitivity to agrochemicals compared with  
93 *Eisenia* sps. (Pelosi et al., 2013b)

94 Our study is focused on endogeic earthworms because of a set of environmental and  
95 biological features. These earthworms change the soil structure, nutrient dynamics and  
96 microbial communities by their continuous burrowing, feeding and casting activities (Jégou et

97 al., 2001; Lipiec et al., 2016). They represent the largest soil biota biomass, although their  
98 abundance and biodiversity are often negatively impacted by conventional agricultural  
99 practices that are characterized by a high pesticide input (Pelosi et al. 2013a). However,  
100 reservations about pesticide toxicity in earthworms should be taken into account because  
101 marked species-specific differences in pesticide exposure level (digestive and/or dermal  
102 uptake), earthworm ecology (endogeic, anecic and epigeic species), life traits (e.g.,  
103 reproduction rate, growth), behavior (e.g., feeding mineral soil to obtain organic matter or  
104 feeding on decay organic matter), and basal levels of ecotoxicological biomarkers (Rault et al.  
105 2007).

106 This study sets out to establish a functional link between initiating molecular events of  
107 OP toxicity (i.e, esterase inhibition) with changes at individual level (i.e., behavior).  
108 Considering the role of these soil-dwelling earthworm species in soil quality, we selected the  
109 burrowing activity and cast production as behavioral measurements with direct implications  
110 in promoting soil fertility. Therefore, the aims of this study were: 1) to compare the response  
111 of AChE and CbE activities between both earthworm species, and to evidence whether a  
112 higher percentage of AChE inhibition corresponded to a lower level of CbE activity or less  
113 sensibility of CbE activity to the OP, 2) to assess whether OP-induced changes in the  
114 earthworm behavior are linked to inhibition of AChE activity. This research will bring insight  
115 in ecologically consequences of adverse effect on behavioral implications from toxic effects  
116 at molecular initiating events.

## 117 2. Materials and methods

### 118 2.1. Chemicals

119 The substrates for esterase activity assay (acetylthiocholine iodide [AcSCh], 4-nitrophenyl  
120 acetate [4-NPA], 4-nitrophenyl valerate [4-NPV], 1-naphthyl acetate [1-NA]), the product of

121 carboxylesterase-mediated hydrolysis (1-naphthol), the chromogens (5,5'-dithiobis-2-  
122 nitrobenzoic acid [DTNB] and Fast Garnet), the OP pesticide ethyl-paraoxon, and a protease  
123 inhibitor cocktail were purchased from Sigma–Aldrich (Saint Quentin-Fallavier, France). For  
124 in vivo tests, we used a commercial liquid formulation of the OP insecticide ethyl-parathion  
125 (Oleobladan<sup>®</sup>, active ingredient 93g L<sup>-1</sup>, Bayer).

## 126 2.2. Soil and earthworms

127 A silt loamy soil (23.4% clay, 57% silt, 19.6% sand, 28.3 g kg<sup>-1</sup> organic matter, pH 8.3), and  
128 healthy adults of *A. caliginosa* and *A. chlorotica* were collected from an apple orchard in  
129 Montfavet near Avignon (France). This orchard has received no pesticide treatments for the  
130 last 10 years. Earthworms were collected manually, weighed and kept in the laboratory into  
131 plastic pots filled with fresh soil collected from the same orchard.

132 Soil was sieved at <2 mm, and the water content was adjusted to 20-21%  
133 (approximately 81% of the maximum water holding capacity) with distilled water.  
134 Afterwards, wet soil samples (1 kg) were spiked with ethyl-parathion solutions (40 ml) to  
135 yield the final concentrations of 0.1, 1 and 10 mg active ingredient (a.i.) kg<sup>-1</sup> wet soil. These  
136 ethyl-parathion concentrations were chosen according to the recommended application rates  
137 by the supplier, and the calculations of the Predicted Environmental Concentration (PEC)  
138 using the equation (FOCUS, 2006):  $PEC_{S,O} = A \times (1 - fint) / (100 \times depth \times bd)$ , where  $PEC_{S,O}$  is  
139 the initial soil concentration (mg kg<sup>-1</sup>) of the insecticide immediately following a single  
140 application,  $A$  is the application rate (g ha<sup>-1</sup>),  $fint$  is the fraction of pesticide intercepted by the  
141 crop canopy,  $depth$  is the mixing depth (cm), and  $bd$  is the dry bulk soil density (g cm<sup>-3</sup>). The  
142  $PEC_{S,O}$  was calculated according to a bulk soil density of 1.5 g cm<sup>-3</sup>, a depth soil layer of 5  
143 cm of pesticide penetration, and no crop interception. Therefore, recommended application  
144 rates of ethyl-parathion corresponded to an initial PEC of 0.7 mg a.i. kg<sup>-1</sup> soil, which is close  
145 to the nominal concentration of 1 mg kg<sup>-1</sup> we used. A control soil was prepared under the

146 same conditions but free of pesticide. All treated soils were split into 100-g subsamples,  
147 which were placed into plastic pots (10-cm d × 3-cm h), to yield 20 replicates per treatment.  
148 The earthworms were washed in tap water, blotted dried on filter paper, weighed (without gut  
149 voiding) and individually placed into the test containers (control and ethyl-parathion-treated).  
150 For each treatment (control, 0.1, 1 and 10 mg ethyl-parathion) 20 earthworms were placed  
151 individually into the containers to prevent cascade death (Sheppard et al., 1992), and they  
152 were kept in a dark cold chamber (12±1°C) for 7 days. After this incubation time, earthworms  
153 were weighed and either frozen at -20°C until biochemical measurements (n=10 per  
154 treatment) or placed in a two-dimensional terrarium for recording behavioral responses (n=10  
155 per treatment). Earthworm-free containers (n=10) containing 100 g of soil were used as  
156 controls to assess cast production. Body weight variations were expressed as a percentage of  
157 the initial average weight.

### 158 *2.3. Earthworm behavior*

159 Two behavior responses were assessed: the burrowing activity and the cast production. The  
160 former behavior was monitored using two-dimensional (2D) terraria (Evans 1947), which  
161 consisted of two glass sheets (30×42 cm) separated by a 3-mm apart and filled with 550 g of 2  
162 mm sieved uncontaminated soil, allowing thereby the recording of earthworm burrowing  
163 (Capowiez, 2000). Forty 2D terraria were used taken into account the four treatments: control,  
164 0.1, 1 and 10 mg a.i. kg<sup>-1</sup> of the insecticide. At the beginning of the assay, one earthworm was  
165 introduced into each 2D terrarium. The location of the earthworm and the burrows  
166 architecture was recorded daily on a transparent sheet for 6 days. Once the assay was  
167 completed, information gathered on each 2D terrarium was digitized (Capowiez, 2000).  
168 Burrow lengths in each terrarium were computed using ImageJ. After a calibration step, the  
169 area was transformed in burrow lengths for the 6 days of continuous monitoring.



170 Cast production was determined according to Capowiez et al. (2010). The casts were  
171 separated using a set of four 15-cm sieves (mesh sizes = 5.6, 4, 3.15, 2.5 mm), because  
172 earthworm activity strongly modifies the soil texture by increasing aggregate fractions. The  
173 soils from Petri dishes, including that adhered to the dish wall which was removed using a  
174 knife, was carefully sieved avoiding the fragmentation of casts. The set of sieves was  
175 manually shaken for 10 s. The soil fractions retained in each sieve was weighed. The effect of  
176 earthworm bioturbation was then examined by calculating the changes in the particle size  
177 distribution, i.e. weight of fresh soil in each sieve minus the corresponding weight of soil for  
178 the control soil (earthworm free). The cast production was expressed as wet cast weight per  
179 earthworm fresh body mass per day.

#### 180 *2.4. Homogenate preparation*

181 Frozen individuals were sliced in 1 cm length pieces and homogenized individually on ice  
182 using an Ultra Turrax IKA T18 basic apparatus at 14000 rpm, 4 times of 10s duration with 1-  
183 min interval, in 20% (w/v) low-salt buffer containing 10 mM Tris-HCl pH=7.3, 10 mM NaCl,  
184 supplemented with protease inhibitors (aprotinin, leupeptin and pepstatin=5  $\mu\text{g mL}^{-1}$ ,  
185 antipain=1  $\mu\text{g mL}^{-1}$ , trypsin inhibitor=1  $\text{mg mL}^{-1}$ ). The homogenates were centrifuged (3000  
186 g, 10 min, 4°C) to obtain a post-mitochondrial fraction (supernatants) which was  
187 supplemented with glycerol (10% v/v, final concentration) as an enzyme stabilizing agent,  
188 and frozen at -20°C.

#### 189 *2.5. Esterase activity assays*

190 Enzyme activities were measured by microplate-scale spectrophotometric procedures using  
191 the tissue homogenates, and the products of the hydrolysis reaction were measured using a  
192 microplate reader (Synergy HT, Bio-Tek). The activity AChE was monitored at 412 nm and  
193 25°C following the method by Ellman et al. (1961), and adapted by Rault et al. (2008), using

194 a millimolar extinction coefficient ( $6,800 \text{ M}^{-1} \text{ cm}^{-1}$ ) calculated according to a dithiotreitol-  
195 DTNB external calibration curve. The reaction medium (200  $\mu\text{L}$ , final volume) contained 4  
196  $\mu\text{L}$  of homogenate, 0.375 mM DTNB and 3 mM of AcSCh in 0.1 M sodium phosphate buffer  
197 (pH 7).

198 Because the sensitivity of carboxylesterase activity to OP is highly dependent on the  
199 substrate, this activity was assayed using three different substrates, which differ in their  
200 carbon-ester chains: 1-naphtyl-acetate (1-NA), 4-nitrophenyl acetate (4-NPA) and 4-  
201 nitrophenyl valerate (4-NPV) to characterize multiple CbE isoenzymes following the  
202 microplate-scale assay by Thompson (1999). The hydrolysis of 1-NA was determined in a  
203 reaction medium (200  $\mu\text{L}$  final volume) contained 25 mM Tris-HCl (pH 7.6), 1 mM  $\text{CaCl}_2$   
204 and 2  $\mu\text{L}$  of diluted (1/20) homogenate. The reaction was initiated by adding 2 mM 1-Na  
205 (final conc.), and stopped after 10 min of incubation at  $25^\circ\text{C}$  by the addition of 50  $\mu\text{L}$  2.5%  
206 SDS containing 0.1% Fast Garnet in 2.5% Triton X-100. The solutions were left to stand for  
207 10 min at room temperature and dark, and the absorbance of the 1-naphthol-Fast Garnet  
208 complex was read at 530 nm. Enzyme activity was calculated from a standard curve  
209 ( $A_{530} = f([1\text{-naphthol}])$ ) containing  $\alpha$ -naphthol in the same reaction mixture free of substrate.  
210 Hydrolysis of 4-NPV and 4-NPA was determined in a reaction mixture (250  $\mu\text{L}$ , final volume  
211 including 2  $\mu\text{L}$  of enzyme extract) contained 1 mM 4-NPV and 50 mM Tris-HCl (pH 7.5), or  
212 5 mM 4-NPA in 20 mM Tris-HCl (pH 8.0) and 1mM EDTA (to inhibit  $\text{Ca}^{2+}$  dependent  
213 phosphotriesterases). In both assays, the formation of 4-nitrophenol was monitored for 5 min  
214 at 405 nm,  $25^\circ\text{C}$  and quantified using a 4-nitrophenol standard curve.

215 Blanks (reaction mixture free of sample) were checked for non-enzymatic hydrolysis  
216 of the substrates. All enzyme assays were run at least in triplicate and expressed as units per  
217 milligram of total protein ( $\text{U mg}^{-1}$  protein). One unit of enzyme activity was defined as one  
218 micromole of formed product per minute under the experimental conditions described above.

219 Total protein content was determined by the Lowry method modified by Markwell et al.  
220 (1978), and using bovine serum albumin as the standard.

#### 221 *2.6. Acetylcholinesterase inhibition kinetics*

222 *In vitro* inhibition kinetics were conducted to test for a direct interaction between ethyl-  
223 paraoxon and AChE activity. Tissue homogenates from unexposed earthworms (20  $\mu$ l) were  
224 incubated individually with serial concentrations of ethyl-paraoxon ( $10^{-12}$  to  $10^{-3}$  M final  
225 concentration) for 30 min at 25°C. Inhibition assay was terminated by addition of the  
226 substrate (AcSCh), and the residual AChE activity was measured as described above.

#### 227 *2.7. Native polyacrylamide electrophoresis for esterase isoenzymes*

228 Non-denaturing polyacrylamide gel electrophoresis (native PAGE) was performed on a Bio-  
229 Rad Tetracell Electrophoresis Unit (Bio-Rad, USA). Homogenates (20  $\mu$ L) were loaded on  
230 4% stacking gel and 12.5% resolving gel for CbE analysis, or 8% resolving gel for AChE  
231 analysis, 0.75 mm polyacrylamide gel (25 mM Tris, 192 mM glycine, pH 8.3 as running  
232 buffer), and electrophoresed at an initial constant voltage of 30 V for 30 min, and afterwards  
233 150 V until bromophenol blue tracking dye reached the bottom of the gel. The in-gel staining  
234 esterase activities were performed according to Manchenko (2002). Protein bands of CbE  
235 were visualized by incubation (2–3 min at room temperature) of the gels with a staining  
236 solution containing 100 mM Na-phosphate buffer (pH 6.4), 0.5 mg mL<sup>-1</sup> 1-NA and 0.025g of  
237 Fast Blue RR salt, which was prepared and filtered immediately before use. Protein bands of  
238 AChE were visualized by incubation (12 hours at room temperature) of the gels with a  
239 staining solution containing 200 mM maleate buffer (pH 6.0), trisodium citrate 100 mM,  
240 copper sulphate 30 mM, 5 mM potassium ferricyanide and 150 mg AcSCh.

#### 241 *2.8. Statistical analysis*

242 The Kruskal-Wallis test and the *post hoc* Mann-Whitney test were used to compare ranges of  
243 the esterase activities between treatments. Furthermore, we used the Jonckheere–Terpstra  
244 trend test (J-T trend test) to assess significant relationships between the median values of  
245 enzyme activities and the concentration of ethyl-parathion in soil. *In vitro* inhibition curves  
246 were fitted to the non-linear model (library of GraphPad Prism software, ver. 7.00, La Jolla  
247 California USA):

$$248 \quad E = min + \frac{(max-min)}{1 + \left(\frac{I}{IC_{50}}\right)^{-Hillslope}}$$

249 where  $E$  is the percentage of residual enzyme activity relative to controls,  $min$  is the  $E$   
250 response to the highest ethyl-paraoxon concentration,  $max$  is the  $E$  response to the lowest  
251 insecticide concentration,  $I$  is the logarithmic-transformed concentration of insecticide, the  
252 *Hillslope* coefficient describes the steepness of the dose–response curve, and  $IC_{50}$  is the  
253 median insecticide concentration that leads to a 50% reduction in the initial enzyme activity.  
254 Burrow length and cast production (both log-transformed) for each pesticide concentration  
255 were compared using a one-way ANOVA for each species separately.

### 256 **3. Results**

#### 257 *3.1. Effect of ethyl-parathion on earthworm body weight and behavior*

258 The insecticide had a significant impact on earthworm body weight, which was species-  
259 specific (Fig. 1A). A significant decrease ( $12.6 \pm 0.8\%$  to  $21.58 \pm 1.04\%$  compared with control  
260 values) was found in the body mass of *A. caliginosa* at 1 and 10 mg kg<sup>-1</sup> ( $p < 0.001$ ), whereas  
261 *A. chlorotica* experienced an increase (8–10%) of body weight at the concentrations of 0.1  
262 and 1 mg kg<sup>-1</sup>, but such a weight gain was inhibited at the highest ethyl-parathion  
263 concentration ( $p = 0.017$ ). No dead were recorded during this toxicity bioassay.

264 The length of burrows produced by non-exposed individuals from both species was  
265 comparable (between 140 and 160 cm length) (Fig. 1B). Moreover, 0.1 and 1 mg kg<sup>-1</sup> ethyl-  
266 parathion did not change in the burrow length formed by both species, although a significant  
267 shortening of burrow length was observed at the highest insecticide concentrations for *A.*  
268 *caliginosa* compared with its respective controls. However, cast production showed evidence  
269 of an insecticide effect on this behavior that was species-specific, but not dose-dependent  
270 (Fig. 1C). Interestingly, both earthworm species exhibited the same cast production in  
271 pesticide-free soils (about 3.6 g of wet casts g<sup>-1</sup> earthworm day<sup>-1</sup>). Soils contaminated with 0.1  
272 to 10 mg ethyl-parathion kg<sup>-1</sup> caused a significant reduction in the cast production of *A.*  
273 *caliginosa*, whereas this behavioral response remained unchanged for *A. chlorotica*, except in  
274 the highest insecticide concentration.

### 275 3.2. Effect of ethyl-parathion on esterase activities

276 Basal levels of AChE and CbE activities for both earthworm species are summarized in Table  
277 1. The activity of both esterases was two times higher in *A. caliginosa* than in *A. chlorotica*,  
278 and CbE activity was higher than AChE activity for both species. Exposure of earthworms to  
279 ethyl-parathion-contaminated soils caused a significant response of these esterases that was  
280 species-specific ( $H_3=52.6$ ,  $p<0.001$  for *A. caliginosa*,  $H_3=25.1$ ,  $p<0.001$  for *A. chlorotica*)  
281 (Fig. 2A). Whereas the mean AChE activity decreased in a dose-dependent manner ( $p<0.001$ ,  
282 J-T trend test) in *A. caliginosa* (34%, 78% and 87% inhibition at the concentrations of 0.1, 1  
283 and 10 mg kg<sup>-1</sup>), this esterase activity remained unchanged in *A. chlorotica* exposed to 0.1  
284 and 1 mg kg<sup>-1</sup> ethyl-parathion, although was drastically inhibited (85% of controls) at the  
285 highest insecticide concentration ( $z=-4.36$ ,  $p<0.0001$ , Mann-Whitney test). Native PAGE  
286 corroborated these enzyme activity outcomes (Fig. 2A). In-gel staining AChE activity  
287 revealed two isoforms in the homogenates of both species. These protein bands may  
288 correspond to a membrane-isoform (the heaviest molecular weight one) and the soluble

289 isoform of AChE. The staining intensity of these protein bands decreased in the ethyl-  
290 parathion-exposed earthworms accordingly with the inhibition of AChE activity found in the  
291 corresponding homogenates.

292 *In vitro* inhibition kinetics of homogenates incubated in the presence of ethyl-paraoxon  
293 generated typical sigmoid dose-dependent curves (Fig. 2B). The estimated  $IC_{50}$  values for the  
294 *A. caliginosa* AChE response were  $1.81 \times 10^{-7}$  M ( $1.63 \times 10^{-7}$ – $2.03 \times 10^{-7}$ , 95% confidential  
295 limits,  $r^2=0.99$ ) and  $1.12 \times 10^{-6}$  M ( $8.16 \times 10^{-7}$ – $1.54 \times 10^{-6}$ ,  $r^2=0.98$ ) for the *A. chlorotica* AChE  
296 response.

297 The response of CbE activity to ethyl-parathion was also species-specific, and depended on  
298 the substrate used in the enzyme assay. The hydrolysis rate of 1-NA was equally inhibited  
299 (24.5-38.5% of controls) in both earthworm species at the doses of 1 and 10 mg kg<sup>-1</sup> ethyl-  
300 parathion (Fig. 3). However, the impact of ethyl-parathion exposure on the hydrolysis of  
301 nitrophenyl esters was markedly dependent on the species as evidenced by the CbE response  
302 at the lowest ethyl-parathion dose. The enzyme activity towards 4-NPV and 4-NPA was  
303 significantly depressed in *A. caliginosa* exposed to 0.1 mg kg<sup>-1</sup> ethyl-parathion, whereas the  
304 hydrolysis rates of these substrates remained unchanged in *A. chlorotica* compared with its  
305 controls. Despite these interspecific differences, the J-T trend test showed a significant dose-  
306 dependent inhibition of CbE activity with ethyl-parathion concentration ( $p<0.0001$ ).

307 We also performed in-gel staining for CbE activity using the substrate 1-NA on post-  
308 electroforesed gels. These results showed multiple CbE isozymes, whose abundance was  
309 higher in *A. caliginosa* compared to *A. chlorotica* (Fig. 3). Moreover, the OP caused a  
310 reduction of staining intensity in those homogenates from the individuals exposed to the  
311 highest concentrations. These zymographic outcomes also confirmed the impact of ethyl-  
312 parathion exposure on the CbE activity of both earthworm species.

313 Correlations between AChE inhibition and behavioral biomarkers were tested. Although there  
314 was a linear relationship between the mean values of AChE inhibition and changes in body  
315 weight for both species ( $r^2 > 0.94$ ,  $p < 0.05$ ) (Fig. 4A), the relationship between AChE inhibition  
316 and cast production was species-specific (Fig. 4B). A non-linear relationship was observed  
317 for *A. caliginosa*, by which low concentrations of ethyl-parathion caused a drastic inhibition  
318 of both biomarkers.

#### 319 4. Discussion

320 Short-term exposure to ethyl-parathion caused a marked interspecific difference in the  
321 response of both biochemical and behavioral biomarkers. According to these sublethal  
322 responses, the earthworm *A. chlorotica* was a more tolerant species to OP-contaminated soils  
323 than *A. caliginosa*. This assumption is supported by previous results that evidenced a  
324 progressive recovery of *A. chlorotica* AChE activity after 14-d exposure to ethyl-parathion (1  
325 mg kg<sup>-1</sup>), whereas this enzyme remained fully inhibited in *A. caliginosa* during more than 2  
326 months of a recovery period in uncontaminated soil (Rault et al., 2008). On the other hand, if  
327 we assume that both species display a similar behavior in soil because they are endogeic and,  
328 therefore, exposure to the pesticide should occur at the same extent, then factors related to  
329 pesticide bioactivation and metabolism could contribute significantly to these species-specific  
330 differences in pesticide sublethal toxicity. In this line, many authors have suggested that CbEs  
331 play an important role as pesticide bioscavengers because of their higher sensitivity to OP  
332 inhibition compared with AChE sensitivity (Küster, 2005; Wheelock et al., 2008; Kristoff et  
333 al., 2010). Moreover, the number of CbE molecules, and therefore of binding sites, seems also  
334 an important factor in the OP toxicity, at least in mammals (Chanda et al., 1997). However,  
335 our results with this esterase activity indicated the following: i) basal CbE activity of un-  
336 exposed individuals of *A. caliginosa* was two-times higher than that of *A. chlorotica*, ii) the *in*  
337 *vivo* exposure trial revealed that CbE activity of *A. caliginosa* was more sensitive to inhibition

338 by the insecticide than *A. chlorotica* CbE activity, and iii) the abundance of CbE isozymes  
339 was higher in *A. caliginosa* compared with that in *A. chlorotica*. According to these  
340 observations, the species *A. caliginosa* would be able to reduce the impact of an acute  
341 exposure to ethyl-parathion through its higher levels and higher sensitivity of CbE activity  
342 compared to *A. chlorotica*. However, changes in behavior biomarkers, particularly cast  
343 production, suggest the contrary. Clearly, other potential toxicodynamic and toxicokinetic  
344 factors contribute to the acute toxicity of OP pesticides. For example, enzymatic complexes  
345 such as the cytochrome P450-dependent monooxygenases are responsible for increasing OP  
346 toxicity through oxidative desulfuration of the parent compound to yield the oxon metabolite  
347 (Lee, 1998; Chambers et al., 2010; Hodgson, 2012; Dzul-Caamal et al., 2014). Other  
348 detoxifying enzymes such as phosphotriesterases (Vilanova and Sogorb, 1999) or glutathione  
349 *S*-transferases (Jokanović, 2001) participate equally in ethyl-parathion (and metabolites)  
350 detoxification.

351 However, interspecific differences in OP susceptibility may also be explained by differences  
352 in the intrinsic AChE sensitivity to oxon metabolites of OPs (Chambers and Carr, 1995).  
353 Likewise, it is well known that gut motility of annelids is under cholinergic control (Fieber,  
354 2017), so inhibition of AChE activity is expected to alter gastrointestinal transit of food with  
355 changes in cast production rate. Therefore, we performed *in vitro* inhibition trial with ethyl-  
356 paraoxon to elucidate whether the higher sensibility of *A. caliginosa* to the pesticide, in terms  
357 of behavioral responses, can be explained by ethyl-parathion-related disruption of cholinergic  
358 pathways. These results clearly revealed that the activity of *A. caliginosa* AChE was more  
359 sensitive to ethyl-paraoxon than that of *A. chlorotica*. Therefore, the intrinsic sensibility of  
360 AChE to this OP insecticide could be a significant cause for the interspecific differences in  
361 sublethal toxicity between these species. Nevertheless, we are aware that these results are still  
362 premature to make solid prediction about impact of OP exposure in natural populations of



363 earthworms. Indeed, some studies show that applications of pesticides to agricultural lands  
364 cause a similar response in the abundance of *A. caliginosa* and *A. chlorotica* in France (Dinter  
365 et al., 2012), highlighting the importance of field monitoring studies in the predictions of  
366 pesticide impact at an earthworm population level.

367 One of the main scopes in the use of ecotoxicological biomarkers is to predict population-  
368 level adverse effects from sub-individual responses. Indeed, some studies have tried to link  
369 earthworm behavioral responses such as avoidance (Pereira et al., 2010; Jordaan et al., 2012;  
370 Martínez-Morcillo et al., 2013), burrowing activity (Capowiez et al., 2003) or cast production  
371 (Capowiez et al., 2010) to AChE inhibition. Current data support this cause-effect relationship  
372 with *A. caliginosa*, as the inhibition of AChE activity lead to either a decrease in the body  
373 weight, a strong threshold effect in the cast production, but no effect in the burrowing  
374 activity. It has long been demonstrated that the continue activity of feeding, burrowing and  
375 casting of endogeic and anecic earthworms has a strong influence on soil structure and  
376 function (Lavelle et al., 2006). Particularly, earthworm casts are significant hot spots of  
377 microbial proliferation and enzyme activities, that may contribute to mitigate the impact of  
378 environmental contaminants in soil (Lipiec et al., 2016; Sanchez-Hernandez et al., 2014).  
379 Therefore, cast production is a behavioral biomarker of ecological relevance as early  
380 suggested by Capowiez et al. (2010), which has resulted very sensitive in soils contaminated  
381 by neonicotinoid insecticides (Pisa et al., 2015) or herbicides (Gaupp-Berghausen et al.,  
382 2015). In our study, the cast production and weight changes (an indirect indicator of feeding  
383 activity) were more sensitive markers to discriminate species-specific differences in pesticide  
384 toxicity compare with the burrowing activity.

## 385 **5. Conclusions**

386 Sublethal exposure to ethyl-parathion caused interspecific changes in the activity of target and  
387 detoxifying enzymes, as well as in the behavior of endogeic earthworms. The species *A.*

388 *caliginosa* was more impacted by the OP insecticide than *A. chlorotica* was. The dose-  
389 dependent inhibition of both AChE and CbE activities of *A. caliginosa* was reflected in a  
390 significant decrease of body weight (as an indicator of feeding activity) and cast production.  
391 Despite a lower number of CbEs isoenzymes and a lower total specific activity observed in *A.*  
392 *chlorotica* species, we confirmed that CbE activity could act as a detoxifying enzyme able to  
393 reduce the impact of ethyl-parathion on the target enzyme (AChE). This suggested that *A.*  
394 *chlorotica* CbE should be highly efficient in scavenging OP. Moreover, the *in vitro* outcomes  
395 revealed that the sensitivity of AChE to ethyl-paraoxon could be an additional mechanism in  
396 the species-specific differences observed in the selected toxicity endpoints. Our results  
397 suggest that these two species are highly recommended in the field monitoring of OP  
398 contamination, not only because they are among the most abundant species in the agricultural  
399 lands, but also because the high sensitivity of behavioral responses (e.g., cast production) that  
400 may have ecologically consequences for soil fertility and degradation.

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524

525

526 Table 1. Specific esterase activities, expressed as U.mg<sup>-1</sup> proteins (mean±SEM) are measured  
 527 in homogenates from the whole body of the endogeic earthworms *Aporrectodea caliginosa*  
 528 and *Allolobophora chlorotica*.

529

Species	Acetylcholinesterase	Carboxylesterases		
	(U.mg <sup>-1</sup> ) AcSCh	4-NPA	4-NPV	1-NA
<i>A. caliginosa</i>	0.147±0.026	0.300±0.038	0.182±0.034	0.882±0.114
<i>A. chlorotica</i>	0.073±0.020	0.157±0.026	0.095±0.015	0.386±0.077

530 AcSCh=acetylthiocholine, 4-NPA=4-nitrophenyl acetate, 4-NPV=4-nitrophenyl valerate and  
 531 1-NA=1-naphthyl acetate.

532

533 **Figure captions**

534 Fig 1. Effect of ethyl-parathion exposure (7 days) on earthworm weight (A), burrow length  
535 (B) and cast production (C). Bars represent the mean and the standard error of the mean  
536 (SEM). Different letters (lowercases for *Aporrectodea caliginosa* and uppercases for  
537 *Allolobophora chlorotica*) denote significant differences between treatments ( $p < 0.05$ , Mann-  
538 Whitney).

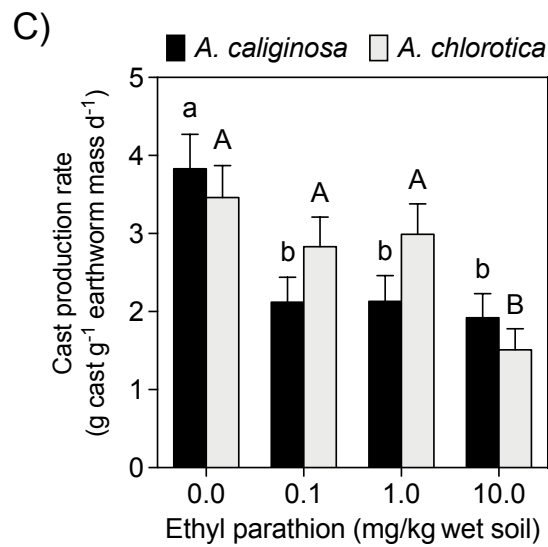
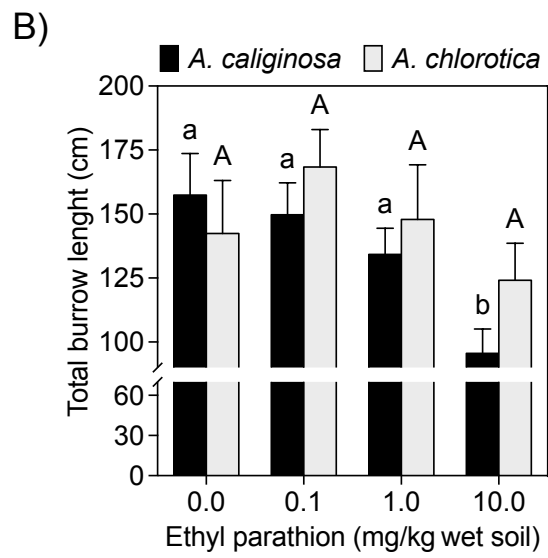
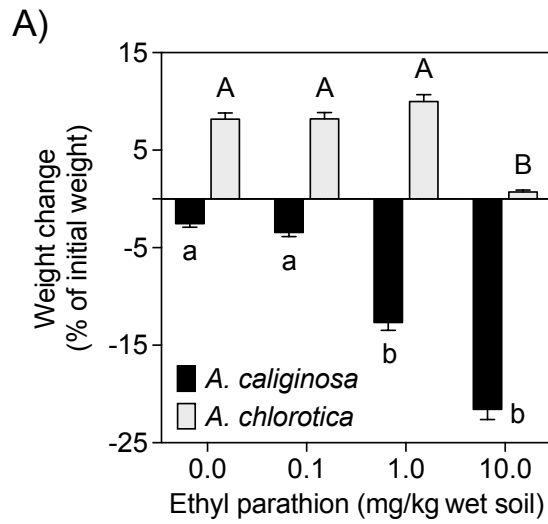
539 Fig 2. A) Response of acetylcholinesterase (AChE) activity in earthworms exposed to ethyl-  
540 parathion-contaminated soils for 7 days, and in-gel staining activity after native PAGE  
541 electrophoresis (protein charge=100–119  $\mu\text{g}/\text{lane}$  for *A. chlorotica*, and 87–110  $\mu\text{g}/\text{lane}$  for *A.*  
542 *caliginosa*). Tukey box plots indicate the median, the 25<sup>th</sup> and 75<sup>th</sup> percentiles (box edges),  
543 the range (whiskers) and outliers (black dots). Significant difference between treatments is  
544 indicated by different letters as in Fig. 1 ( $p < 0.05$ , Mann-Whitney test). B) Dose-dependent  
545 inhibition curves for acetylcholinesterase (AChE) of *Aporrectodea caliginosa* and  
546 *Allolobophora chlorotica* in the presence of serial molar concentration of ethyl-paraoxon.  
547 Symbols are the mean  $\pm$  SEM, the bands show the 95% confidence intervals of the non-linear  
548 regression curves, and the arrows mark the  $IC_{50}$  values.

549 Fig 3. Response of carboxylesterase (CbE) activity in earthworms exposed to ethyl-parathion  
550 -contaminated soils for 7 days. The esterase activity was measured using the substrates 4-  
551 nitrophenyl acetate (4-NPA), 4-nitrophenyl valerate (4-NPV), and 1-naphthyl acetate (1-NA).  
552 In-gel staining activity after native PAGE electrophoresis was performed with homogenates  
553 (protein load=100–119  $\mu\text{g}/\text{lane}$  for *A. chlorotica*, and 87–110  $\mu\text{g}/\text{lane}$  for *A. caliginosa*) of all  
554 treatments. Tukey box plots indicate the median, the 25<sup>th</sup> and 75<sup>th</sup> percentiles (box edges), the  
555 range (whiskers) and outliers (black dots). Significant difference between treatments is  
556 indicated by different letters as in Fig. 2 ( $p < 0.05$ , Mann-Whitney test).

557

558 Fig 4. Relationship between acetylcholinesterase (AChE) activity and body weight change  
559 (A) or cast production (B) after exposure to ethyl-parathion (mean  $\pm$  SEM). AChE activity is  
560 expressed as % of control values.





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Figure 1

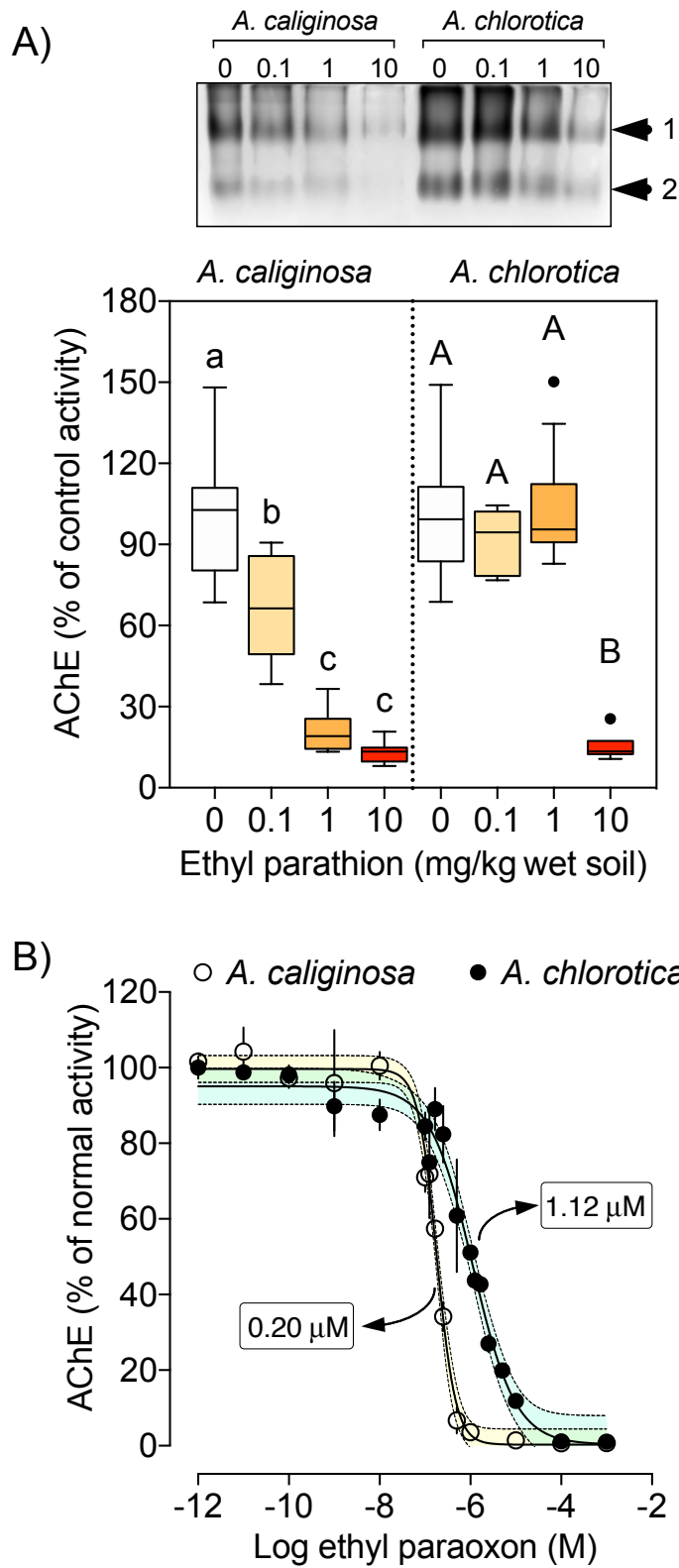
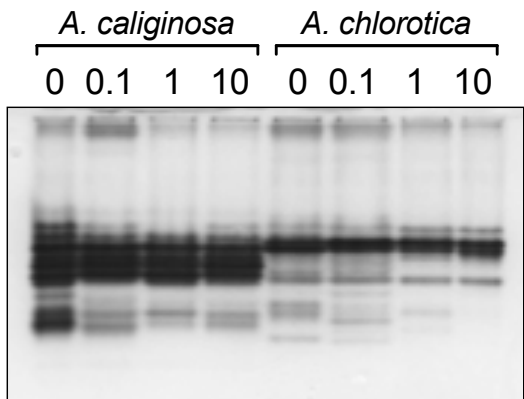
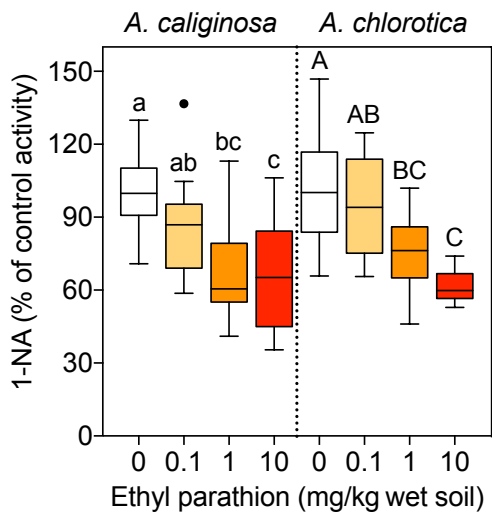
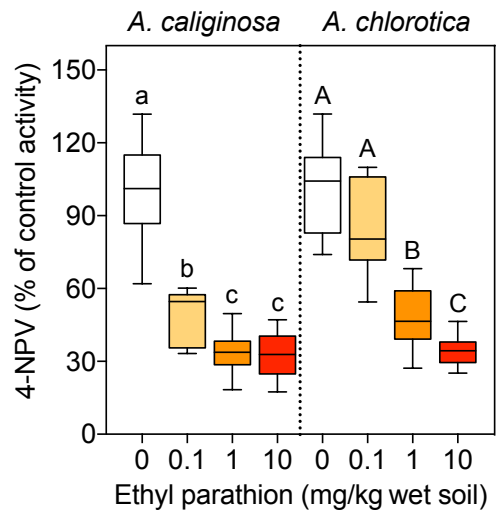
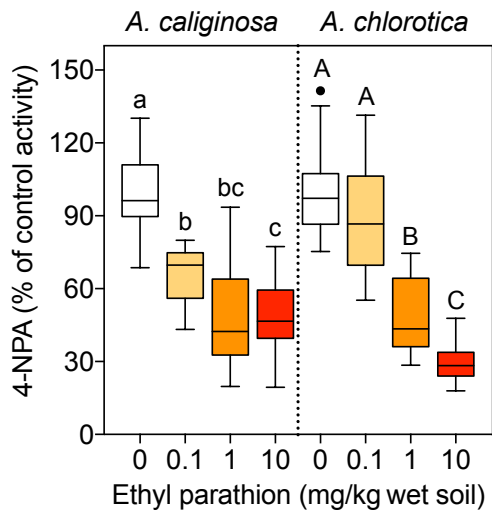
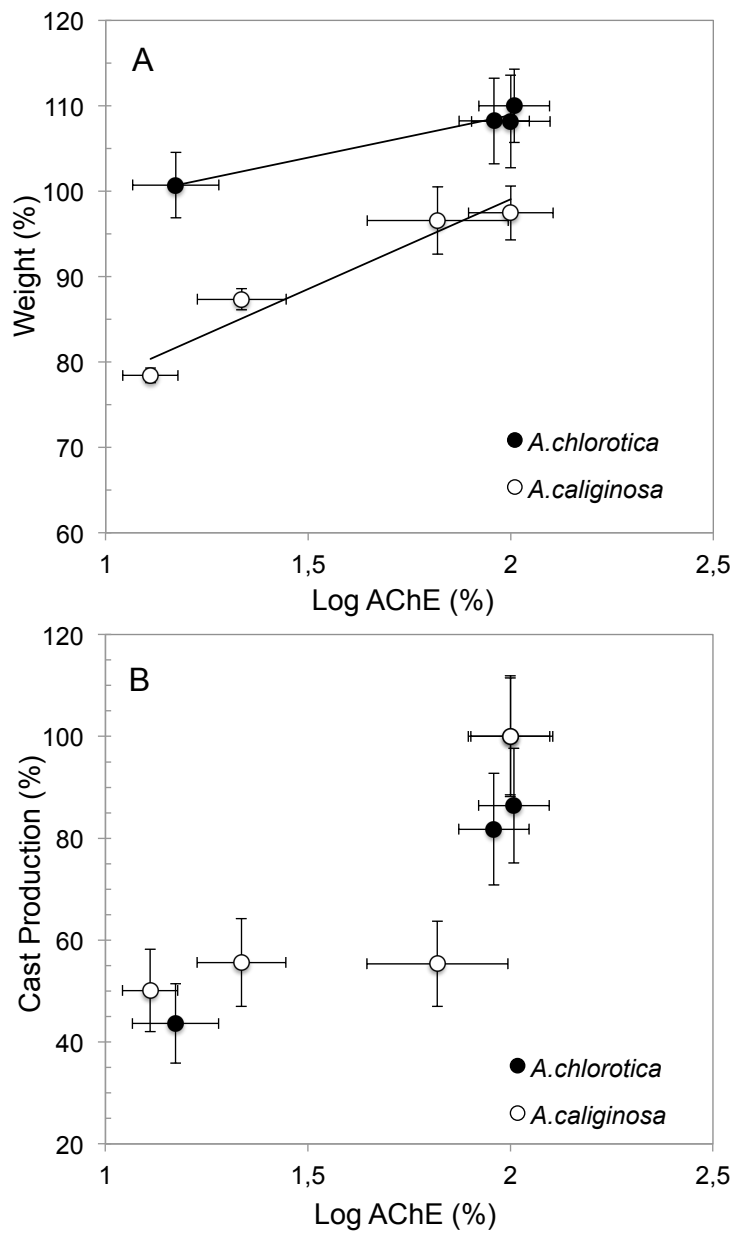


Figure 2



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Figure 3



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Figure 4