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

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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⁻¹ 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

1. Introduction

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

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

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

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

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

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

2. Materials and methods

2.1. Chemicals

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

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

2.2. Soil and earthworms

A silt loamy soil (23.4% clay, 57% silt, 19.6% sand, 28.3 g kg⁻¹ organic matter, pH 8.3), and healthy adults 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. Earthworms were collected manually, weighed and kept in the laboratory 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 solutions (40 ml) to yield the final concentrations of 0.1, 1 and 10 mg active ingredient (a.i.) kg⁻¹ wet soil. These ethyl-parathion concentrations were chosen according to the recommended application rates by the supplier, and the calculations of the Predicted Environmental Concentration (PEC) using the equation (FOCUS, 2006): $PEC_{S,O} = A \times (1 - fint) / (100 \times depth \times bd)$, where $PEC_{S,O}$ is the initial soil concentration (mg kg⁻¹) of the insecticide immediately following a single application, A is the application rate (g ha⁻¹), $fint$ is the fraction of pesticide intercepted by the crop canopy, $depth$ is the mixing depth (cm), and bd is the dry bulk soil density (g cm⁻³). The $PEC_{S,O}$ was calculated according to a bulk soil density of 1.5 g cm⁻³, a depth soil layer of 5 cm of pesticide penetration, and no crop interception. Therefore, recommended application rates of ethyl-parathion corresponded to an initial PEC of 0.7 mg a.i. kg⁻¹ soil, which is close to the nominal concentration of 1 mg kg⁻¹ we used. A control soil was prepared under the

same conditions but free of pesticide. All treated soils were split into 100-g subsamples, which were placed into plastic pots (10-cm d × 3-cm h), to yield 20 replicates per treatment.

The earthworms were washed in tap water, blotted dried on filter paper, weighed (without gut voiding) and individually placed into the test containers (control and ethyl-parathion-treated). For each treatment (control, 0.1, 1 and 10 mg ethyl-parathion) 20 earthworms were placed individually into the containers to prevent cascade death (Sheppard et al., 1992), and they were kept in a dark cold chamber ($12\pm1^{\circ}\text{C}$) for 7 days. After this incubation time, earthworms were weighed and either frozen at -20°C until biochemical measurements ($n=10$ per treatment) or placed in a two-dimensional terrarium for recording behavioral responses ($n=10$ per treatment). Earthworm-free containers ($n=10$) containing 100 g of soil were used as controls to assess cast production. Body weight variations were expressed as a percentage of the initial average weight.

2.3. Earthworm behavior

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

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

2.4. Homogenate preparation

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

2.5. Esterase activity assays

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

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

Because the sensitivity of carboxylesterase activity to OP is highly dependent on the substrate, this activity was assayed using three different substrates, which differ in their carbon-ester chains: 1-naphtyl-acetate (1-NA), 4-nitrophenyl acetate (4-NPA) and 4-nitrophenyl valerate (4-NPV) to characterize multiple CbE isoenzymes following the microplate-scale assay by Thompson (1999). The hydrolysis of 1-NA was determined in a reaction medium (200 μL final volume) contained 25 mM Tris-HCl (pH 7.6), 1 mM CaCl_2 and 2 μL of diluted (1/20) homogenate. The reaction was initiated by adding 2 mM 1-NA (final conc.), and stopped after 10 min of incubation at 25°C by the addition of 50 μL 2.5% SDS containing 0.1% Fast Garnet in 2.5% Triton X-100. The solutions were left to stand for 10 min at room temperature and dark, and the absorbance of the 1-naphthol-Fast Garnet complex was read at 530 nm. Enzyme activity was calculated from a standard curve ($A_{530} = f([1\text{-naphthol}])$) containing α -naphthol in the same reaction mixture free of substrate. Hydrolysis of 4-NPV and 4-NPA was determined in a reaction mixture (250 μL , final volume including 2 μL of enzyme extract) contained 1 mM 4-NPV and 50 mM Tris-HCl (pH 7.5), or 5 mM 4-NPA in 20 mM Tris-HCl (pH 8.0) and 1mM EDTA (to inhibit Ca^{2+} dependent phosphotriesterases). In both assays, the formation of 4-nitrophenol was monitored for 5 min at 405 nm, 25°C and quantified using a 4-nitrophenol standard curve.

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

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

2.6. Acetylcholinesterase inhibition kinetics

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

2.7. Native polyacrylamide electrophoresis for esterase isoenzymes

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

2.8. Statistical analysis

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

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

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

3. Results

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

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

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

3.2. Effect of ethyl-parathion on esterase activities

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

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

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

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

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

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

4. Discussion

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

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

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

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

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

5. Conclusions

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

caliginosa was more impacted by the OP insecticide than *A. chlorotica* was. The dose-dependent inhibition of both AChE and CbE activities of *A. caliginosa* was reflected in a significant decrease of body weight (as an indicator of feeding activity) and cast production. Despite a lower number of CbEs isoenzymes and a lower total specific activity observed in *A. chlorotica* species, we confirmed that CbE activity could act as a detoxifying enzyme able to reduce the impact of ethyl-parathion on the target enzyme (AChE). This suggested that *A. chlorotica* CbE should be highly efficient in scavenging OP. Moreover, the *in vitro* outcomes revealed that the sensitivity of AChE to ethyl-paraoxon could be an additional mechanism in the species-specific differences observed in the selected toxicity endpoints. Our results suggest that these two species are highly recommended in the field monitoring of OP contamination, not only because they are among the most abundant species in the agricultural lands, but also because the high sensitivity of behavioral responses (e.g., cast production) that 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⁻¹ 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 ⁻¹) 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

Figure captions

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

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

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

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

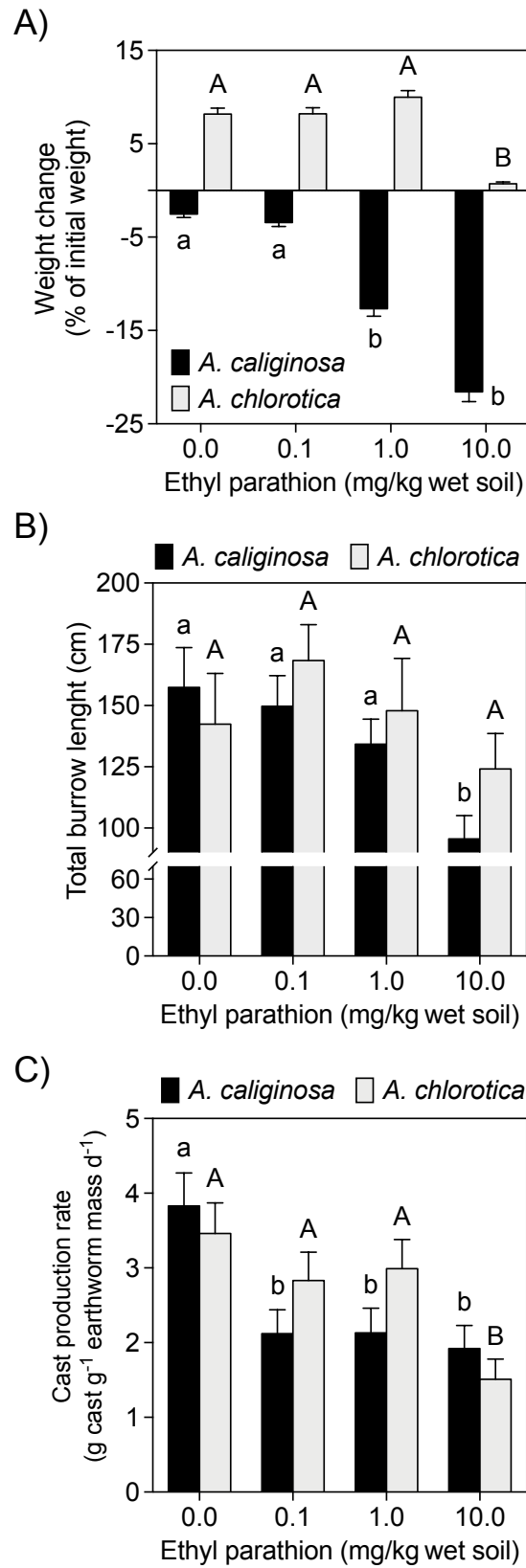
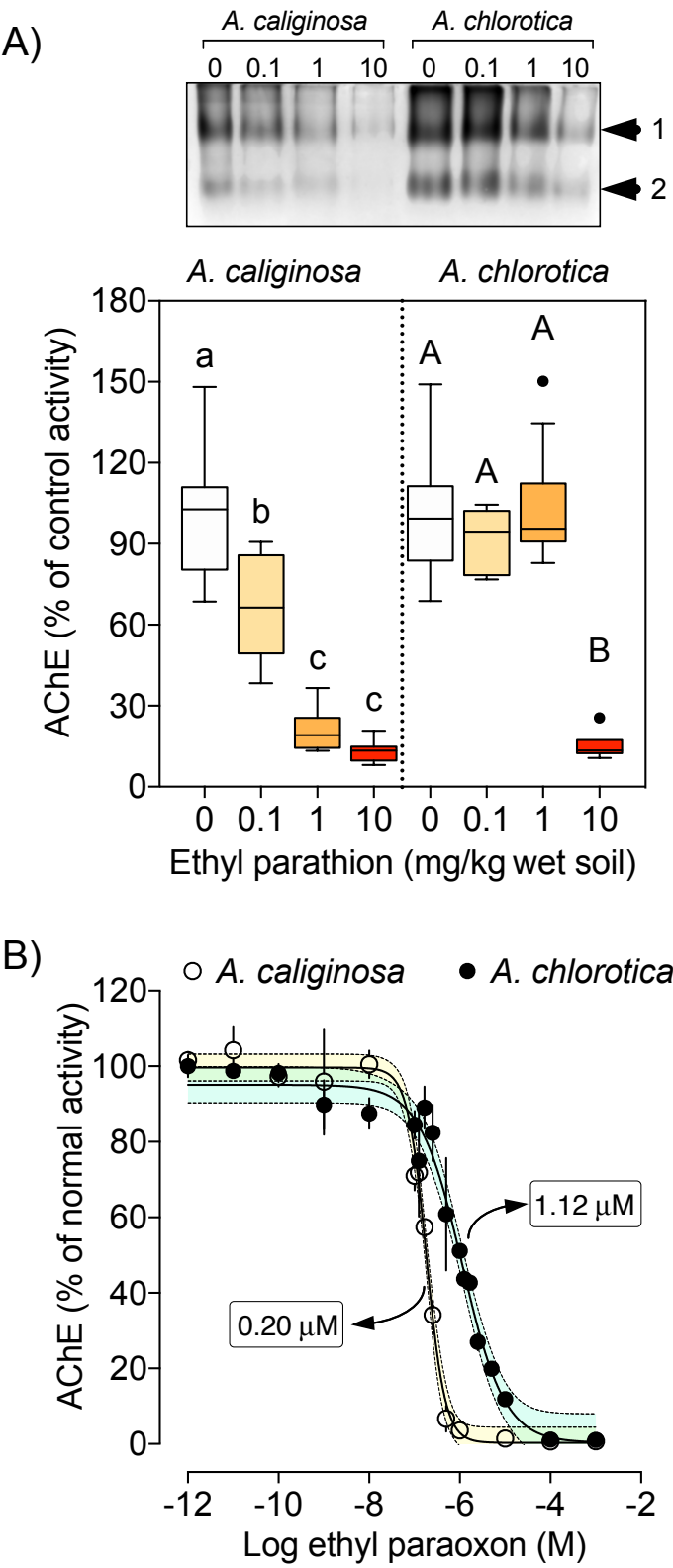


Figure 1

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Figure 2

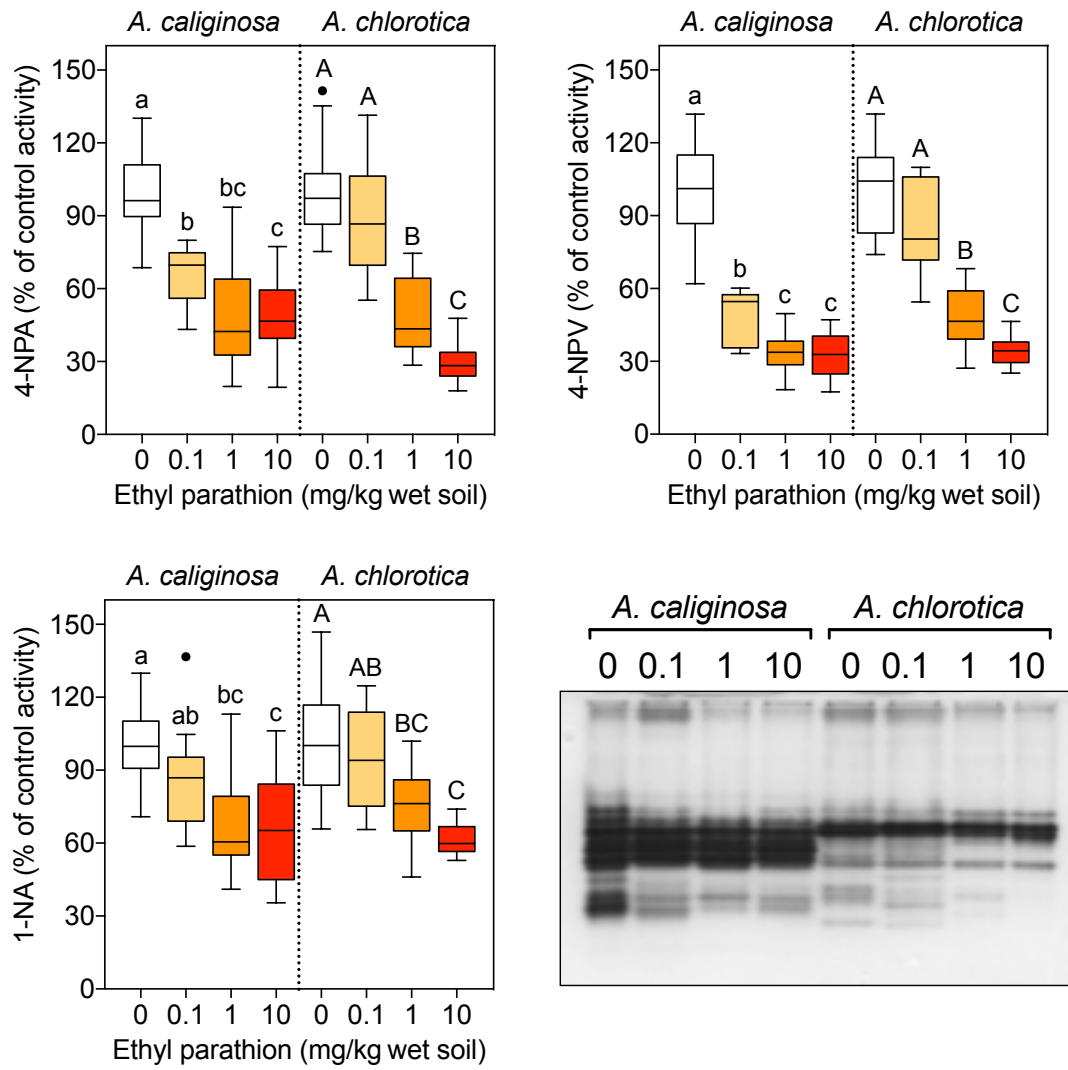


Figure 3

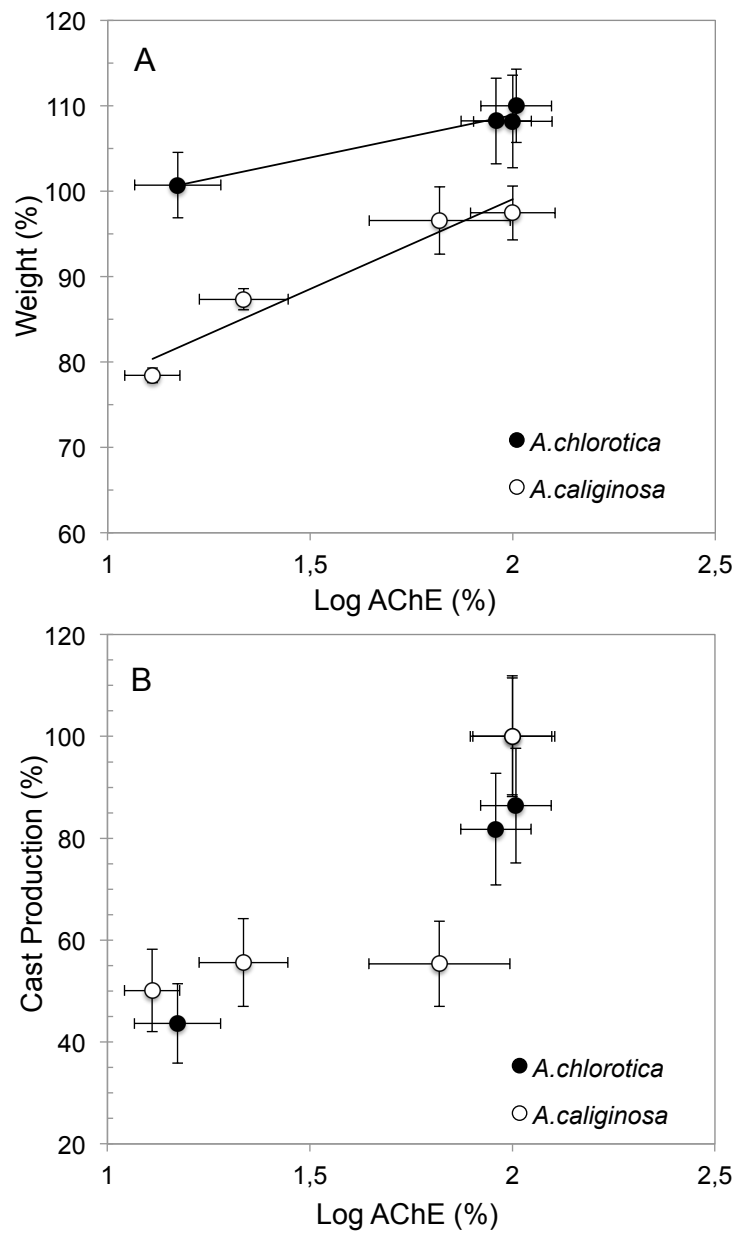


Figure 4