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Integrated biomarker analysis of chlorpyrifos metabolism and toxicity in the earthworm *Aporrectodea caliginosa*

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HIGHLIGHTS

- Carboxylesterases were an efficient bioscavenger for chlorpyrifos-oxon.
- Chlorpyrifos increased CYP450 and GST activities at short-term exposure.
- Chlorpyrifos bioactivation was measured by a simple and low-cost enzymatic assay.

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ABSTRACT

To increase our understanding about the mode of toxic action of organophosphorus pesticides in earthworms, a microcosm experiment was performed with *Aporrectodea caliginosa* exposed to chlorpyrifos-spiked soils (0.51 and 10 mg kg^{-1} dry soil) for 3 and 21 d. Acetylcholinesterase (AChE), carboxylesterase (CbE), cytochrome P450-dependent monooxygenase (CYP450), and glutathione *S*-transferase (GST) activities were measured in the body wall of earthworms. With short-term exposure, chlorpyrifos inhibited CbE activity (51–89%) compared with controls in both treated groups, whereas AChE activity was depressed in the 10-mg kg^{-1} group (87% inhibition). With long-term exposure, chlorpyrifos strongly inhibited all esterase activities (84–97%). Native electrophoresis revealed three AChE isozymes, two of which showed a decreased staining corresponding to the level of pesticide exposure. The impact of chlorpyrifos on CbE activity was also corroborated by zymography. CYP450 activity was low in unexposed earthworms, but it increased (1.5- to 2.4-fold compared to controls) in the earthworms exposed to both chlorpyrifos concentrations for 3 d. Bioactivation of chlorpyrifos was determined by incubating the muscle homogenate in the presence of chlorpyrifos and $NAD(H)_2$. The mean ($\pm$SD, $n = 40$) bioactivation rate in the unexposed earthworms was 0.74 ± 0.27 nmol $NAD(H)_2$ oxidized min^{-1} mg^{-1} protein, and a significant induction was detected in the low/short-term exposure group. GST activity significantly increased (33–35% of controls) in earthworms short-term exposed to both chlorpyrifos concentrations. Current data showed that CYP450 and GST activities had a prominent role in the initial exposure to the organophosphorus. With short-term exposure, CbE activity was also a key enzyme in the non-catalytic detoxification of chlorpyrifos–oxon, thereby reducing its impact on AChE activity, before it became saturated at $t = 21$ d. Results indicate that *A. caliginosa* detoxify efficiently chlorpyrifos, which would explain its tolerance to relatively high exposure levels to chlorpyrifos.

1. Introduction

Earthworms are suitable organisms in many standardized ecotoxicity tests to assess soil pollution, and *Eisenia fetida* and *Eisenia andrei* are the recommended species. However, some studies have shown that these earthworm species are not the most sensitive to environmental contaminants such as agrochemicals. For example, Pelosi et al. (2013) revealed through a meta-analysis that *Eisenia* species are less sensitive to pesticides than *Lumbricus terrestris* and *Aporrectodea caliginosa*. Previously, Ma and Bodt (1993) examined the toxicity of several pesticides to six earthworm species and concluded that species-specific variations in pesticide sensitivity were not attributed to body size as a determinant factor of the exposure level. They suggested, however, that physiological factors could contribute to variations of pesticide toxicity between species, which was also supported by others (Pereira et al., 2010; Henson-Ramsey et al., 2007). Furthermore, some

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earthworm species seem to tolerate relatively high concentrations of organophosphorus (OP) pesticides, judging by their lack of response in the standardized avoidance behavior test, i.e., a sublethal screening assay that measures the earthworm ability to detect and avoid contaminated soils (García-Santos and Keller-Forrer, 2011; Jordaan et al., 2012; Martínez Morcillo et al., 2013). This apparent tolerance to OP pesticides might be accounted for by the bioactivation of OPs to yield highly toxic metabolite "oxon", as well as their metabolism by the phase-I and -II biotransformation enzymes.

It is well established that the mechanism for acute toxicity of OP insecticides as chlorpyrifos is the inhibition of the enzyme acetylcholinesterase (AChE, EC 3.1.1.7) located in the nervous tissue and in the neuromuscular junction (Thompson and Richardson, 2004). However, this interaction is highly efficient with the 'oxon' metabolite of OP, which is formed through an oxidative desulfuration reaction catalyzed mainly by the cytochrome P450-dependent monooxygenases (CYP450s, EC 1.14.14.1) or the flavin-dependent monooxygenases (FMOs, EC 1.14.13.8) (Hodgson, 2010). The oxon metabolites have a high affinity for a group of esterases called carboxylesterases (CbEs, EC 3.1.1.1). The phosphorylation of CbE activity is considered a non-catalytic and stoichiometric mechanism of OP detoxification, in which the number of enzyme molecules and the sensitivity of the enzyme for OP govern the efficacy of this detoxification system (Chanda et al., 1997; Wheelock et al., 2008). Besides these detoxifying enzymes, OP pesticides and their metabolites are also metabolized by phase-II biotransformation enzymes as glutathione *S*-transferases (GSTs, EC 2.5.1.18) (Testai et al., 2010).

Former studies with earthworms have demonstrated the occurrence of the major enzymes, i.e., CYP450s, GSTs and CbEs, involved in the bioactivation and metabolism of OPs (Haites et al., 1972; Stenersen et al., 1979; Stenersen and Øien, 1981; Stenersen, 1984; Berghout et al., 1991). Most of these enzymes have been used as biomarkers of contaminant exposure and effect in laboratory toxicity tests (Cao et al., 2012; Booth et al., 2002; Ribera et al., 2001; Velki and Hackenberger, 2013a, 2013b) or, to a lesser extent, in field monitoring of soil pollution (Łaszczyca et al., 2004). As far as we know, little is known about the earthworm capacity to bioactivate and detoxify OPs through the integrated analysis of biomarkers that are involved in these processes. This information would increase our understanding about the mode of action of these agrochemicals upon earthworms, and would support the selection of suitable species for toxicity testing and environmental monitoring purposes. In addition, some soil-dwelling earthworms, such as *L. terrestris* or *A. caliginosa*, are frequently used in the bioremediation of pesticide-contaminated soils (Hickman and Reid, 2008; Rodriguez-Campos et al., 2014). A deeper knowledge on the metabolizing capacity of earthworms for OPs would help to predict sublethal effects during the bioremediation or selection of the most suitable species for this purpose.

The present study is part of a broader project to evaluate the role of earthworms in the enzymatic bioremediation of OP pesticide residues in soil. It has been long recognized that earthworms facilitate contaminant degradation by their stimulatory effect upon soil microbial activity (Hickman and Reid, 2008). Nevertheless, most of studies have focused on the bioremediation of polycyclic aromatic hydrocarbons, polychlorinated biphenyls, herbicides (i.e., atrazine) and organochlorine pesticides, and their role on OP degradation has been little investigated (Rodriguez-Campos et al., 2014). Some laboratory studies have shown that anecic earthworms such as *L. terrestris* are able to secrete CbEs in their gastrointestinal tract, suggesting thereby a potential enzymatic system for degrading OPs in the ingested soil (Sanchez-Hernandez et al., 2009). With this project scope, a challenge that requires attention is to know to what extent earthworms will be able to tolerate relatively high sublethal OP concentrations during bioremediation. Therefore, the aim of this study was to examine the response of selected metabolizing enzymes belonging to phase-I (CYP450, CbEs) and phase-II (GST) of the pesticide biotransformation, and their role in the ability of *A. caliginosa* to tolerate an exposure to chlorpyrifos-spiked soils. Inhibition of AChE activity was included in this study as a reference of OP toxicity depending on the bioactivation rate and detoxification of chlorpyrifos.

2. Materials and methods

2.1. Chemicals

Reagents were purchased from Sigma-Aldrich (Madrid, Spain). The organophosphorus pesticide Dursban 5G (5% w/w, chlorpyrifos) was obtained from Compo Agricultura S.L. (Barcelona, Spain). The substrates for CbE assays, i.e., α-naphthyl butyrate (α-NB) and 4-nitrophenyl butyrate (4-NPB) were prepared in ethanol at a stock concentration of 20 mM and kept at 4 °C. The substrates for CYP450 activity ethoxyresorufin, pentoxyresorufin, and benzylozyresorufin, were prepared in dimethyl sulfoxide at stock concentrations of 300, 350 and 330 μM, respectively, and stored at −20 °C. Solvents for chromatographic analysis were HPLC-grade and purchased from Scharlab S.L. (Barcelona, Spain).

2.2. Earthworms and soil

Adult and clitellated *A. caliginosa* were collected from lawn gardens in the Faculty of Environmental Sciences and Biochemistry (Univ. Castilla-La Mancha, Toledo). Earthworms were acclimatized in the laboratory (15 °C and darkness) using the same soil as that used in the toxicity tests. Soil was collected from an abandoned agricultural area close to the rural town of Polán (Toledo), with no known pesticide contamination history. Soil was air-dried for three weeks and subsequently sieved to a <2 mm particle size. The physicochemical properties of this soil (n = 4) were pH = 7.96 ± 0.08, electrical conductivity = 220.4 ± 5.1 μS cm^{-1}, total organic carbon = 2.9 ± 0.1% and maximum water holding capacity (WHC) = 0.34 ± 0.01 g H_2O g^{-1} dry soil, and texture = 7.95% clay, 11.65% silt, 62.43% coarse sand and 17.97% fine sand.

2.3. Exposure setup

Effects of chlorpyrifos on selected biomarkers were examined using two nominal concentrations 1 and 10 mg kg^{-1} dry soil, which were selected based upon the predictive environmental concentration of 3.3 mg kg^{-1} dry soil. This concentration was estimated taking into account a single application rate of Dursban 5G (2.5 kg active ingredient ha^{-1}, according to the manufacturer), a homogeneous distribution of the pesticide in the first 5 cm of soil, no crop interception and a soil density of 1.5 g cm^{-3} (Collange et al., 2010). Moreover, some studies (reviewed in Racke, 1993) on chlorpyrifos persistence in agriculture systems showed that initial chlorpyrifos concentrations in sandy loam soils ranged between 1.1 and 32 mg kg^{-1}, when the OP was soil-incorporated as a granular formulation (application rates varying between 0.75 and 5 kg ha^{-1}).

In a first experimental trial, earthworms were exposed to the test soils for 3 d (short-term exposure), while in a second toxicity trial the exposure duration was extended to 21 d (long-term exposure). Soil samples (250 g dry soil) were placed in plastic bottles (300 ml volume), to obtain 5 replicates per treatment. Soil was spiked with the appropriate amount of Dursban 5G (taking into account the concentration of the active ingredient) to yield the nominal concentrations of 1 mg kg^{-1} (5 mg Dursban 5G/250 g soil) and 10 mg kg^{-1} (50 mg Dursban 5G/250 g soil). Vessels were capped and shaken vigorously for 1 min to facilitate distribution of pesticide granules throughout the bulk soil. Water was added to each container to adjust soil water content to 50% of the maximum WHC, and subsequently kept for 48 h in the acclimatized chamber (15 °C) for equilibration. A total of 120 earthworms were taken from the culture, weighed

(0.60 ± 0.19 g, mean ± SD), and released in four-individual groups in each test container, to yield 20 earthworms per treatment and exposure duration. Before releasing the earthworms, a soil subsample (2.5 g) was taken from each replicate to verify the nominal chlorpyrifos concentrations by high-performance liquid chromatography with a diode-array detector (HPLC-DAD). In the case of long-term exposure, soils were weekly renewed with freshly spiked soils that were previously kept at 15 °C and dark for 48 h for equilibration. Chemical analysis of chlorpyrifos was performed in the freshly spiked soils before transferring the earthworms. Earthworms from both toxicity assays were fed with ≈2 g of plant debris (fallen leaves from *Morus alba*) per container, which was renewed with each soil change in the case of the long-term assay. Earthworms were frozen immediately at −80 °C before subsequent biochemical analysis.

2.4. Tissue homogenization

Biomarker analysis was focused on the wall muscle because it represents one of the main uptake routes for environmental contaminants and, also because it provides a sufficient sample amount for multiple biochemical determinations. Wall muscle tissue (0.27 ± 0.07 g, mean ± SD, n = 120 samples) was carefully removed under a stereomicroscope, avoiding the nervous ganglia, gastrointestinal tract, and reproductive tissues. After removal, the samples were washed in the homogenization buffer to remove rests of coelomic fluid, blood and other undesirable materials. Muscle tissue was homogenized (1:10, w/v) in ice-cold 20 mM Tris–HCl buffer (pH = 7.6) containing 1 mM EDTA using a glass-PTFE Potter-Elvehjem tissue grinder connected to a Heidolph type ST1 homogenizer. The homogenates were centrifuged at 9000 *g* at 4 °C for 20 min, and the post-mitochondrial fraction (total proteins = 3.27 ± 0.57 mg ml^{-1}, n = 120) was used for biomarker analysis.

2.5. Esterase activities

AChE activity was determined according to the method of Ellman et al. (1961) adapted to the 96-well microplate format by Wheelock et al. (2005). Reaction medium (200 μl) was composed of 0.1 M Na phosphate buffer (pH = 8.0), 320 μM (final concentration, f.c.) 5,5′-dithiobis-2-nitrobenzoic acid, 3 mM (f.c.) acetylthiocholine iodide and 5 μl of sample, and kinetics were read for 10 min (1-min intervals) at 412 nm and 22 °C. Carboxylesterase activity was determined using the esters α-NB and 4-NPB, following the method by Thompson (1999). Reaction medium (200 μl) contained 0.1 M Tris–HCl (pH = 7.4), 2 mM (f.c.) α-NB or 4-NPB and the sample, and was incubated for 10 min at 22 °C and continuous agitation. The hydrolysis of α-NB was stopped by adding 25 μl of a solution made mixing 0.1% Fast Red ITR in 2.5% (w/v) SDS and 2.5% Triton X-100. Microplates were left in the dark for 30 min for color development. The hydrolysis of 4-NPB was concluded by adding 25 μl of a solution that contained 2% SDS and 2% Tris base. The product of the reaction, i.e., 4-nitrophenolate, was immediately read at 405 nm.

Esterase kinetic assays were run in triplicate and read using an Asys HiTech UVM340 plate reader (Asys HiTech GmbH, Eugendorf, Austria). Blanks (sample-free) were periodically assayed to discount non-enzymatic formation of the reaction products. The esterase activities were expressed as nmol min^{-1} mg^{-1} of total protein. AChE activity was calculated using a calibration curve produced with DTNB and serial concentrations of GSH (6.25–100 μM). The specific activity of CbE (α-NB) was calculated using an external curve produced with α-naphthol (1.5–100 μM), whereas the hydrolysis of 4-NPB was quantified using a calibration curve made with 5–100 μM 4-nitrophenolate. The concentration of total proteins was determined using the Bradford method (Bradford, 1976) with bovine serum albumin as the standard.

2.6. Biotransformation enzyme activities

Glutathione *S*-transferase activity was determined according to Habig et al. (1974) in a reaction mixture (1 ml) containing 0.1 M Na-phosphate buffer (pH 6.5), 2 mM (f.c.) CDNB, 5 mM (f.c.) reduced glutathione, and the sample. The product of the reaction was read at 340 nm and a molar extinction coefficient of 9.6 mM^{-1} cm^{-1} was used to express the specific enzyme activity.

The method described by Hanioka et al. (2000), with slight modifications, was used for measuring CYP450 activity in the post-mitochondrial fraction. Aliquots (50 μl) of the samples were pre-incubated for 5 min at 22 °C with 50 mM Na-phosphate buffer (pH = 7.4) and the substrate (8.2 μM ethoxyresorufin, 6.5 μM benzyloxyresorufin or 7.0 μM pentoxyresorufin, f.c.). Afterward, 10 μl of 25 mM NADPH was added to the reaction mixture (500 μl, final volume), and samples were incubated for 30 min. The reaction was stopped by adding 500 μl of cold methanol, followed by hand-shaking, centrifugation (6000 ×g, 20 min, 4 °C), and filtration (0.45 μm) of the supernatants before injection into the HPLC system. Blanks were routinely made with all components of the reaction mixture and the peak areas of resorufin in the samples were substrated by the peak areas in the blanks. A calibration curve of resorufin (1.25–10 nM) was made daily by diluting stock solutions with a mixture of 50 mM Na-phosphate buffer (pH = 7.4):methanol (50:50, v/v). Preliminary analyses were carried out using calibration curves that included the sample (a pooled post-mitochondrial fraction) to correct the matrix effect. However, such an effect did not differ from those calibration curves without the sample. Detection and quantitation of resorufin were performed on an Agilent Eclipse Plus LC-18 column (0.46 × 150 mm × 5 μm particle size) set up at 30 °C, a mobile phase composed of 20 mM Na-phosphate buffer (pH = 6.8), methanol, and acetonitrile in the proportions 52:45:3 (v/v), and a flow rate of 0.5 ml min^{-1}. A fluorescence detector was set up at E_X = 560 nm and E_M = 585 nm.

The oxidation of $NAD(H)_2$ in the presence of chlorpyrifos was measured through a continuous kinetic assay to determine if the muscle post-mitochondrial fraction were able to bioactivate chlorpyrifos (Dzul-Caamal et al., 2012). Monooxygenation reactions of xenobiotic by CYP450 or FMO isozymes involve the reduction of one atom of O_2 to water and the incorporation of the other oxygen atom into the xenobiotic. NADPH or NADH is an electron donor in this reaction (Hodgson, 2010; Chambers et al., 2010). Oxidation of $NAD(H)_2$ was measured according to the method by Dzul-Caamal et al. (2012), adapted to the microplate format. The reaction mixture (200 μl), containing 50 μl of the post-mitochondrial fraction, 0.1 M Tris–HCl buffer (pH = 7.4), and 500 μM chlorpyrifos (dissolved in ethanol), was pre-incubated for 5 min at 22 °C; the subsequent reaction was initiated with the addition of 10 μl of 50 mM $NAD(H)_2$ (2.5 mM, f.c.). Oxidation of $NAD(H)_2$ was observed at 1.5-min intervals over 20 min. Blanks (chlorpyrifos-free reaction mixture) were used for checking the spontaneous oxidation of $NAD(H)_2$ and no decomposition was detected. NADH-dependent monooxygenase activity was expressed as nmol $NAD(H)_2$ consumed min^{-1} mg^{-1} protein, using 6.22 mM^{-1} cm^{-1} as the molar extinction coefficient (Dzul-Caamal et al., 2012).

2.7. Native polyacrylamide gel electrophoresis

Non-denaturing polyacrylamide gel electrophoresis (native-PAGE) was used to test isozyme-specific sensitivity to OP. Two samples representing each treatment were loaded onto 4% stacking and 9% resolving 1.0 mm non-denaturing polyacrylamide gels. Detailed descriptions of electrophoresis conditions and in-gel activity staining for both AChE and CbE activities are provided in Martínez Morcillo et al. (2013). Gels were scanned using a Gel Doc™ EZ Imager system (Bio-Rad Laboratories, Hercules, CA, USA) and the protein bands were separated using the Image Lab software system (version 3.0.1, Bio-Rad

Table 1
Measured concentrations of chlorpyrifos by high-performance liquid chromatography in the test soils compared with the nominal concentrations (mean ± SD, n = 5).

Nominal concentration	Measured concentration (mg kg^{-1})[a]			
	3 d	21 d (1st week)	21 d (2nd week)	21 d (3rd week)
1 mg kg^{-1}	0.51 ± 0.22	0.68 ± 0.64	0.60 ± 0.58	0.53 ± 0.43
10 mg kg^{-1}	10.5 ± 4.1	10.1 ± 3.8	9.29 ± 2.3	10.8 ± 3.5

[a] Statistical differences between sampling times were tested using the non-parametric test Mann–Whitney test ($P < 0.05$), and no significant differences were found.

Laboratories). A standard of native proteins (NativeMark™ Protein Standard, Invitrogen) covering molecular weights from 20 to 480 kDa was used to compare the relative migration of multiple esterase isozymes between samples and estimate their apparent native molecular mass.

2.8. Chemical analysis of chlorpyrifos in soil

Chlorpyrifos was extracted following the method by Asensio-Ramos et al. (2010), with modifications. Soil samples (2.5 g wet soil) were vigorously shaken with 5 ml acetonitrile (HPLC-grade) for 1 min followed by sonication (5 min, 50 W) and centrifugation (5000 ×*g* for 5 min). The supernatants (1 ml) were cleaned up by dispersive solid phase extraction with 25 mg primary–secondary amine (particle size = 40 μm) and afterward centrifuged at 10,000 rpm for 5 min. A 20-μl aliquot was injected into an Agilent 1200 Series HPLC system (Agilent Technologies, Santa Clara, CA, USA). Chlorpyrifos was separated at a flow rate of 0.8 ml min^{-1} on a C-18 column (0.46 × 150 mm × 5 μm particle size) heated at 40 °C under the following solvent gradient: 65% acetonitrile (solvent A): 35% water (solvent B) at t = 0 min, which was changed to 100% solvent A up to 8 min and maintained for 2 min, followed by 65% solvent A in 1 min and maintained for 5 min. Chlorpyrifos was detected at 290 nm and quantified using an external curve made with 0.5–10 μg ml^{-1} chlorpyrifos. Paraoxon ethyl (2 μg ml^{-1}) was used as the internal standard.

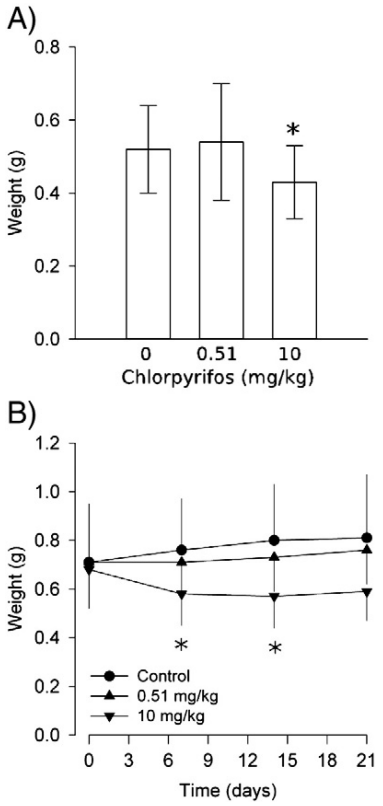

Fig. 1. Changes of body weight of *Aporrectodea caliginosa* exposed to chlorpyrifos-spiked soils for 3 d (A) and 21 d (B). Data are the mean and standard deviation (n = 20 individuals per experimental group). Asterisks denote significant differences with the control groups *$P < 0.05$, Dunnett's test.

2.9. Data analysis

Data were log transformed to meet the assumptions of normality (Shapiro–Wilk *W* test, $P < 0.05$) and homoscedasticity (Levene test, $P < 0.05$). The effect of chlorpyrifos on biomarker responses was examined using a two-way between-groups analysis of variance (ANOVA), taking the chlorpyrifos concentration and time of exposure as the factors. ANOVA tests were followed by the Dunnett post hoc test for comparisons with the corresponding controls.

3. Results

3.1. Chlorpyrifos concentrations and earthworm body weight

Table 1 summarizes the measured concentrations (mean ± SD) of chlorpyrifos in the test soils sampled from the short- and long-term experiments. The exposure concentrations in the soils spiked with 1 mg kg^{-1} dry soil were half the estimated values. The reason for this lack of agreement between the nominal and the measured concentrations is not clear. The pesticide formulation used in this study (i.e., granulated) may account for this inconsistency because of the small amount of pesticide (5 mg) added to 250 g of soil. Indeed, the relationship between estimated and measured concentrations of OP improved at the highest concentration. Because the mean measured concentrations in the 1-mg kg^{-1} group were similar for both exposure times (Table 1), an average OP concentration of 0.51 mg kg^{-1} dry soil was assumed to be the real exposure level.

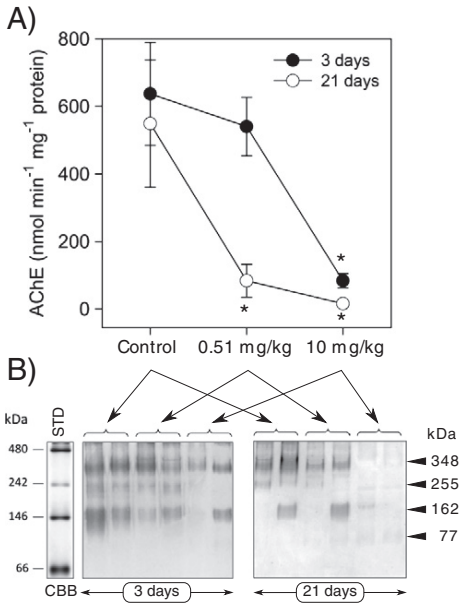

Fig. 2. A) Acetylcholinesterase (AChE) activity of *Aporrectodea caliginosa* exposed to chlorpyrifos-spiked soils. Data are the mean and standard deviation (n = 20). *$P < 0.0001$, Dunnett's test. B) Native PAGE gels of muscle post-mitochondrial supernatants (2 individuals per treatment) stained for in-gel AChE activity. Protein charge was 16–18 μg (control lanes), 13–14 μg (0.51-mg kg^{-1} lane) and 12–18 μg (10-mg kg^{-1} lanes). Triangles indicate the protein bands showing in-gel activity staining. Protein standard (5-μl load volume) was bovine serum albumin (66 kDa), lactate dehydrogenase (146 kDa), B-phycoerythrin (480 kDa) and apoferritin (480 kDa), and visualized after Coomassie brilliant blue (CBB) staining.

No mortality was observed in either experiment. The highest chlorpyrifos concentration significantly reduced ($F_{2,59} = 4.05$, $P = 0.023$) the body mass of the earthworms exposed for 3 d (Fig. 1A). Compared to body weights recorded at the beginning of the experiment, the earthworms treated with 10 mg kg^{-1} for 21 d experienced a slight, but significant ($F_{3,79} = 3.13$, $P = 0.03$) loss of body weight during the first two weeks, although it was not statistically significant by the end of the experiment (Dunnett's test, $P = 0.084$) (Fig. 1B). The controls and 0.51-mg kg^{-1} groups in the long-term (21 d) experiment did not suffer any significant change of their body weight ($F_{3,79} = 1.18$, $P = 0.32$ for controls and $F_{3,79} = 0.74$, $P = 0.53$ for the 0.51-mg kg^{-1} group).

3.2. Esterase activities

Chlorpyrifos significantly inhibited the AChE activity ($F_{2,114} = 479$, $P < 0.0001$). Moreover, there was a significant interaction effect between the OP concentration and the time of exposure ($F_{2,114} = 58.16$, $P < 0.0001$); the esterase inhibition was more pronounced after 21 d of exposure (Fig. 2A). However, the group treated with 0.51 mg kg^{-1} for 3 d did not show inhibition of the AChE activity compared with controls (Dunnett's test, $P = 0.12$). The maximum AChE inhibition (87–97% of control activity) was reached in earthworms exposed at 10 mg kg^{-1} at both sampling times. Zymograms revealed the presence of three main stained bands corresponding to AChE activity, which had apparent native molecular masses of 162, 255 and 348 kDa (Fig. 2B). The insecticide caused a decrease of the staining intensity of these three protein bands, corroborating the enzyme kinetic outcomes. Thus, the 162- and 255-kDa bands decreased their staining intensity (<64.5% of controls) in the 0.51-mg kg^{-1} samples, whereas the percentages of staining decreased were almost 100% of controls for the 10-mg kg^{-1} groups (Fig. 2B). However, the 162-kDa band showed a variable intensity that did not correspond to the level of chlorpyrifos exposure. This band was even absent in some control earthworms. Interestingly, a fourth protein band (≈77 kDa) was evident in the pesticide-exposed earthworms after 21 d of exposure.

Chlorpyrifos had a strong inhibitory effect on CbE activities toward α-NB ($F_{2,114} = 390$, $P < 0.0001$) and 4-NPB ($F_{2,114} = 955$, $P < 0.0001$) compared with the corresponding controls (Fig. 3A and B). Moreover, hydrolysis of 4-NPB was more sensitive to chlorpyrifos than that with α-NB, judging by their mean percentages of remaining CbE activity (19.2 ± 3.2% for 4-NPB, and 47.3 ± 16.7% for α-NB, mean ± SD) in the low/short-term exposure group (0.51 mg kg^{-1} and 3 d). Zymograms evidenced multiple CbE isozymes that can be grouped in three ranges of native molecular mass: 23–26 kDa, 52–98 kDa and 297–476 kDa. The staining intensity of all these isozymes decreased with OP exposure (Fig. 3C). However, there was a high variation in the profile of CbE isozymes between controls that made it difficult to assess changes in isozyme abundance relative to chlorpyrifos treatment.

One main aim of this study was to evaluate the role of CbE activity as an endogenous scavenger for chlorpyrifos–oxon, thereby protecting AChE activity. Thus, percentages of residual esterase activity were plotted, and a clear relationship was obtained between AChE and CbE responses to chlorpyrifos exposure (Fig. 4). This relationship was still evident after 21 d of chlorpyrifos exposure (Fig. 4B), despite all esterases were strongly inhibited (>70% of controls).

3.3. CYP450 and glutathione S-transferase activities

CYP450 activity was measured by the *O*-dealkylation of ethoxyresorufin (EROD), benzyloxyresorufin (BROD), and pentoxyresorufin (PROD) to yield a single product, resorufin, which was eluted in a sharp and well-defined peak at 6.25 min (Fig. 5). There was a significant main effect of chlorpyrifos concentration on CYP450 activities ($F_{2,114} =$

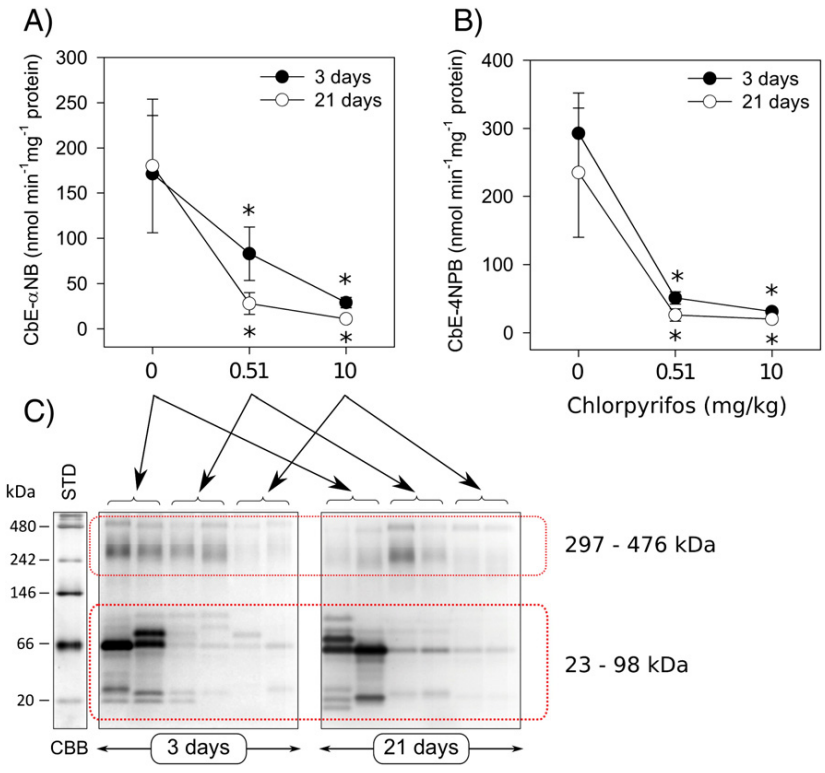

Fig. 3. Carboxylesterase (CbE) activity toward α-naphthyl butyrate (α-NB, graph A) and 4-nitrophenyl butyrate (4-NPB, graph B) of *Aporrectodea caliginosa* exposed to chlorpyrifos-spiked soils. Data are the mean and standard deviation (n = 20). *$P < 0.0001$, Dunnett's test. C) In-gel CbE activity after native PAGE electrophoresis of muscle post-mitochondrial supernatants (2 earthworms per treatment). Protein charge and native protein standard as in Fig. 2; soybean trypsin inhibitor (20 kDA) was also included in the standard.

16.84, $P < 0.0001$ for EROD activity; $F_{2,113} = 23.93$, $P < 0.0001$ for BROD activity; $F_{2,113} = 5.93$, $P = 0.004$ for PROD activity). Moreover, there were significant interaction effects between the time of exposure and the OP concentration for the three CYP450 activities ($F_{2,114} = 15.21$, $P < 0.0001$ for EROD; $F_{2,113} = 11.31$, $P < 0.0001$ for BROD; $F_{2,113} = 17.20$, $P < 0.0001$ for PROD). This indicated that short- and long-term responses of CYP450 activities were affected differently by the chlorpyrifos concentration (Fig. 5). Unexposed earthworms contributed to this interaction effect because their CYP450 activities significantly increased at t = 21 d compared with the short-term exposure ($P < 0.001$, Mann–Whitney test).

The capacity of muscle post-mitochondrial fraction to bioactivate OP pesticides was assessed through $NAD(H)_2$ oxidation in the presence of chlorpyrifos (Fig. 6A). Mean (±SD, n = 20) bioactivation rate of unexposed earthworms varied from 0.76 ± 0.26 nmol $NAD(H)_2$ oxidized min^{-1} mg^{-1} protein at t = 3 d to 0.80 ± 0.29 nmol $NAD(H)_2$ oxidized min^{-1} mg^{-1} protein at t = 21 d. There was a significant interaction effect between the time of exposure and the chlorpyrifos concentration ($F_{2,107} = 5.52$, $P = 0.005$) on the bioactivation rate, despite the absence of a main effect of the OP concentration ($F_{2,107} = 2.20$, $P = 0.115$). The Dunnett's test revealed that the low/short-term exposure group had a higher chlorpyrifos bioactivation rate ($P = 0.034$) than the corresponding control. Although this bioactivation rate was 34% higher in the high/long-term exposed group than the controls, it was not statistically significant ($P = 0.06$, Dunnett's test). Glutathione *S*-transferase activity significantly varied with the chlorpyrifos concentration ($F_{2,114} = 3.20$, $P = 0.044$) and the time of exposure ($F_{1,114} = 33.0$, $P < 0.0001$). With the short-term exposure, GST activity was significantly higher in both treated groups compared with controls ($P < 0.001$, Dunnett's test) (Fig. 6B), however there were not meaningful differences with the long-term exposure ($P > 0.91$).

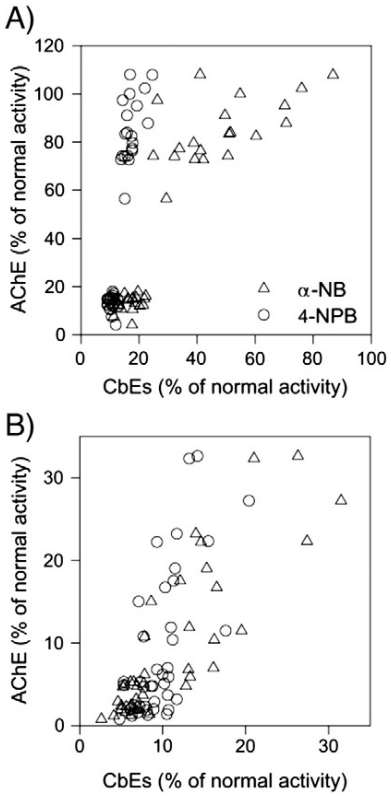

Fig. 4. Correlation between acetylcholinesterase (AChE) and carboxylesterase (CbE) activities in *Aporrectodea caliginosa* exposed to chlorpyrifos for 3 d (graph A) and 21 d (graph B). Data are the percentage of residual enzyme activity respect to the mean values of the corresponding control groups.

4. Discussion

4.1. Esterase responses

In the present study, the mean AChE activity of the control *A. caliginosa* groups fall within the same range of variation as that reported by Olvera-Velona et al. (2008), although it was higher than AChE activities reported by others in the same species (Table 2). Modifications of the original method by Ellman et al. (1961) could account for this wide variation between studies. Nevertheless, the available data in the literature show that *A. caliginosa* and *L. terrestris* display AChE activities one order of magnitude higher than those measured in *Eisenia* species (Table 2). In this study, the AChE activity of *A. caliginosa* was severely inhibited by 10 mg kg^{-1} chlorpyrifos. Inhibition of this esterase activity has been well documented in earthworms through in vitro (Stenersen, 1980a), microcosm (Reinecke and Reinecke, 2007; Aamodt et al., 2007; Collange et al., 2010) or mesocosm (Booth et al., 2000) approaches, in which its use as a biomarker of OP exposure has been widely established. Some authors have suggested that cholinesterase (ChE) inhibition may provide an early warning of adverse effects at individual level as measured by changes in body weight, reproduction rate or survival (Booth and O'Halloran, 2001; Reinecke and Reinecke, 2007). Current data support this likely linkage. The highest chlorpyrifos concentration caused a significant decrease of weight in both the short-term (17.3% of controls) and long-term (23.7–28.7% of controls) exposed earthworms, although inhibition of AChE and CbE activities was already detected in the low/short-term exposure group. It has been postulated that contaminants may indirectly decrease body mass, or growth rate, of earthworms because of the energy costs of detoxifying the contaminants (Spurgeon and Hopkin, 1996; Booth and O'Halloran, 2001). The significant weight loss in the 10-mg kg^{-1}-groups, particularly that short-term exposed, could be reflecting this energy costs because of the enhanced detoxifying capacity.

Whether inhibition of AChE activity is the primary mechanism of OP acute toxicity in earthworms, why do these organisms survive with a minimal AChE activity? This question is still more intriguing whether one takes into account the slow recovery rate of phosphorylated AChE activity that generally follows acute exposure (Booth et al., 2000; Aamodt et al., 2007; Rault et al., 2008; Collange et al., 2010). The presence of multiple AChE forms with different sensitivity to OP could be a plausible answer. For example, Stenersen (1980a) described two different ChE activities in *E. fetida*; the one named E1 by the author was resistant to in vitro inhibition by carbaryl. Indeed, this carbaryl-resistant E1 enabled both *E. fetida* and *E. andrei* to survive carbaryl exposure compared with *Eisenia veneta* who does not have this esterase activity (Stenersen et al., 1992). In the present study, the muscle of *A. caliginosa* showed three AChE forms that were evidenced by zymography. This finding is in agreement with previous results by Principato et al. (1978) who, using the same earthworm species, isolated and identified three AChEs that were believed to be a monomeric (≈180 kDa), dimeric (≈350 kDa), and tetrameric (≈700 kDa) form. The esterase E1 of *E. fetida* was also expressed as four isoforms with molecular weights of about 65, 105, 235 and 312 kDa (Stenersen, 1980b) an isozyme pattern that was later confirmed by Scaps et al. (1996). In our study, a fourth AChE isoform of about 77 kDa was slightly stained in the earthworms exposed to OP during 21 d. Although native PAGE electrophoresis does not accurately estimate the molecular mass of proteins because they are separated according to their size and charge, the AChE isoform of 77 kDa in our earthworms could be a monomeric form that, by assembling, would give the dimeric, trimeric and tetrameric forms (Fig. 2B). This hypothesis has been already postulated by others (Stenersen, 1980b; Scaps et al., 1996). The AChE band corresponding to about 162 kDa was unstable among individuals, being present in

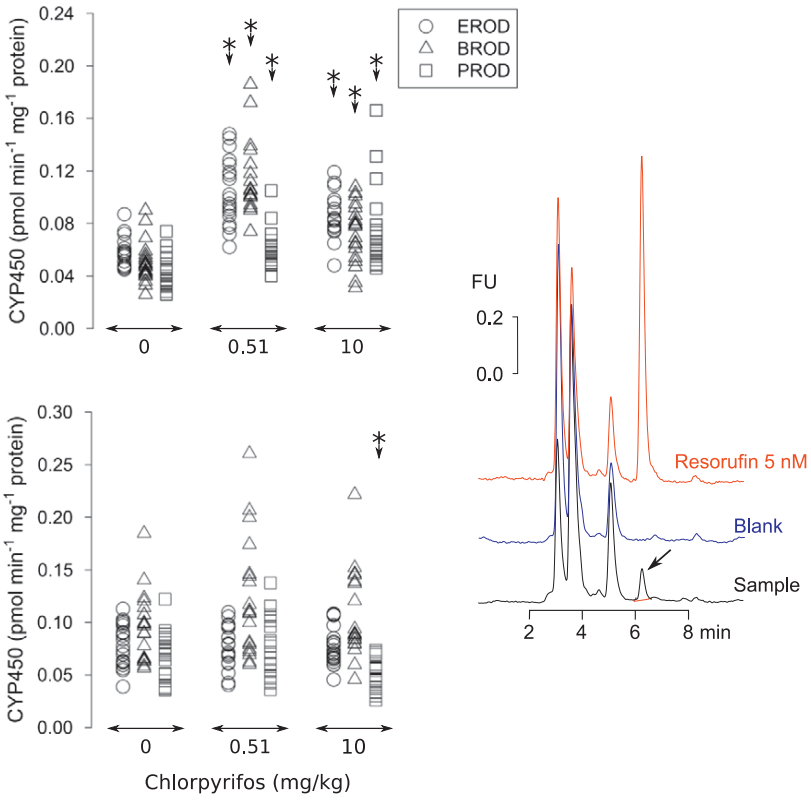

Fig. 5. A) Examples of chromatographic profiles of the resorufin standard (5 nM), blank (NADPH-free), and a sample after EROD assay (see the Materials and methods section for details). The resorufin standard was added the reaction mixture containing all reagents except the substrate. B) Ethoryresorufin-*O*-deethylase (EROD), C) benzyloxyresorufin-*O*-deethylase (BROD) and D) pentoxyresorufin-*O*-deethylase (PROD) activities in *Aporrectodea caliginosa* exposed to chlorpyrifos-spiked soils. Data are the mean and standard deviation (n = 20). *$P < 0.005$, Dunnett's test.

some chlorpyrifos-exposed earthworms while absent in some controls. Kaloustian (1981) also observed the occurrence of a new AChE isozyme in estivating *A. caliginosa* compared to non-estivating individuals that showed only three isozymes. It is unclear whether changes in the AChE isozyme pattern are an acclimation response of *A. caliginosa* to pesticide-contaminated environments.

The inclusion of AChE activity in our study was to assess, however, the capability of CbE activity as a bioscavenger for chlorpyrifos–oxon. It has been long demonstrated that mammalian CbEs are an efficient non-catalytic detoxification system, by which the 'oxon' metabolite of OPs is bound to the active site of the enzyme, thus reducing the available OP molecules able to inhibit the AChE activity (Tang and Chambers, 1999; Chanda et al., 1997). Little is known, however, about a similar role for earthworm CbEs. So far, only a few studies have included CbE inhibition as a complementary biomarker of OP exposure (Table 2). Results in the present study show that muscle CbE activity is more sensitive to chlorpyrifos than AChE activity, which confirms previous findings in the literature (Wheelock et al., 2008). Although the number of experimental groups was low in our study, the relationships between AChE and CbE activities evidenced a "protective capacity" of the latter esterases, reducing the impact of chlorpyrifos–oxon on AChE activity (Fig. 4). In the low/short-term exposure group, the CbE activity could be binding the chlorpyrifos–oxon, which is reflected in the high inhibition percentages of this esterase activity (51 ± 17.2% inhibition for αNB-CbE and 83 ± 3.0% inhibition for 4NPB-CbE, mean ± SD) compared with controls, and in the absence of AChE inhibition (Fig. 4A). However, at 10 mg kg^{-1} chlorpyrifos or when the time of exposure extended up to 21 d, the AChE activity was severely inhibited (85 ± 9.0 to 97 ± 1.6% inhibition compared with controls, mean ± SD). In that state, it can be assumed that the detoxification capacity of CbE activity was almost saturated (inhibition percentages higher than 70% of controls), although the relationship between both esterase activities was kept (Fig. 4B).

Levels of basal CbE activity have been suggested as a determinant of the differences both within and between species. A comparison with data in the literature show that the basal levels of CbE activity is similar between earthworm species (Table 2), except for those reported by Łaszczyca et al. (2004). However, the relatively low substrate concentration (5 μM 4-NPA) used in that study likely accounts for the low CbE activity compared with other earthworm species (Table 2). The affinity of earthworm CbE to bind OPs should be, therefore, a more determinant factor for species-specific differences in pesticide toxicity; however, available data is still too scarce for solid conclusions. In our study, inhibition of CbE activity using 4-NPB was equal to that reported for *L. terrestris* exposed for 2 d to 3 mg kg^{-1} (83% inhibition) and 12 mg kg^{-1} (87%) chlorpyrifos, although 4-nitrophenyl valerate (4-NPV) was used as a substrate (Collange et al., 2010). Comparisons should be done with cautions because of differences in the substrates used for CbE determinations. For example, the substrates 4-NPA and α-naphthyl acetate (α-NA) are commonly used for CbE measurements (Table 2); however, their hydrolysis rates are not as severely affected by OPs as those of longer-chain carbon esters (e.g., 4-NPB, 4-NPV or α-NB) (Sanchez-Hernandez and Wheelock, 2009; Collange et al., 2010). This difference in substrate-dependent CbE sensitivity could explain why the CbE activity of *E. andrei* is less sensitive to inhibition by dimethoate than AChE activity (Velki and Hackenberger, 2013a).

Another factor affecting the modulation of OP toxicity is CbE isozyme abundance (Wheelock et al., 2008). In the present study, multiple CbE isozymes were evidenced by in-gel staining using α-NB as a substrate. Using α-NA in the in-gel staining, however, Haites et al. (1972) found several CbE isozymes in the wall muscle of *L. terrestris* with a molecular weight varying between 60 and 72 kDa in 7.5% acrylamide gels. These

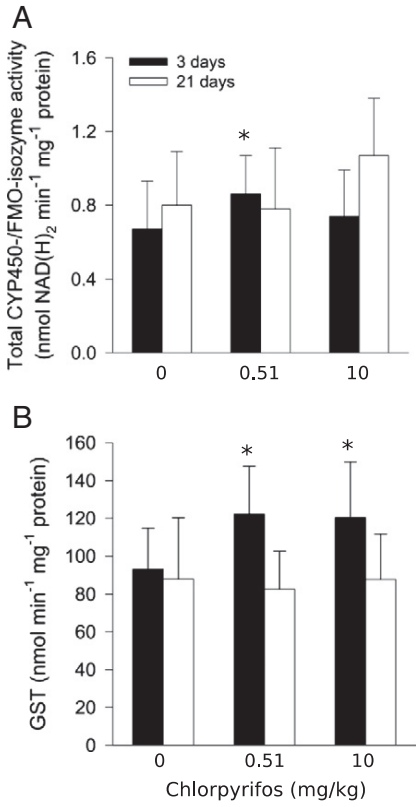

Fig. 6. A) Chlorpyrifos bioactivation rate (graph A) and glutathione *S*-transferase activity (graph B) of *Aporrectodea caliginosa* exposed to chlorpyrifos-spiked soils. Data are the mean and standard deviation (n = 20). *$P < 0.001$, Dunnett's test.

bands were very close to the strongest staining bands in our gels that had a native molecular mass ranging from 52 to 98 kDa (Fig. 3C). Likewise, most protein bands were sensitive to chlorpyrifos exposure; this contrasts with previous observations found in *L. terrestris* exposed to chlorpyrifos-spiked soils (Martínez Morcillo et al., 2013). In that study, a slight or absent decrease of staining was found in the zymograms of pesticide-exposed earthworms. The use of α-NB in the present study was, therefore, a better reporter to evidence pesticide-sensitive isozymes in the gel.

4.2. Biotransformation enzymes

CYP450-dependent monooxygenase and GST activities were measured in this study as indicators of chlorpyrifos activation and metabolism, respectively. It is well known that phosphorothioate-type OPs such as chlorpyrifos need to be activated to their oxygen analogs (e.g., chlorpyrifos–oxon) to gain affinity for ChEs and CbEs; an oxidative desulfuration reaction mainly catalyzed by CYP450 and FMO isozymes (Hodgson, 2010). In this study, control earthworms displayed a lower CYP450 activity toward the substrates ethoxyresorufin (0.057 ± 0.011 and 0.077 ± 0.020 pmol min^{-1} mg^{-1} protein, mean ± SD of both control groups) pentoxyresorufin (0.043 ± 0.012 and 0.068 ± 0.023), and bezyloxyresorufin (0.050 ± 0.016 and 0.096 ± 0.032), compared with other earthworm species. For example, microsomal CYP450 activity of *E. fetida* varied between 0.26 and 1.05 pmol min^{-1} mg^{-1} protein for PROD activity, and between 0.14 and 0.30 pmol min^{-1} mg^{-1} protein for BROD activity at 25 °C and pH = 7.8 (Achazi et al., 1998). Similarly, the microsomal fraction of *L. terrestris* midgut displayed a mean (±SE) BROD activity of 1.45 ± 0.25 pmol min^{-1} mg^{-1} protein at 25 °C and pH = 8.0 (Berghout et al., 1991). Microsomal ethoxycoumarin-*O*-dealkylase activity of *A. caliginosa* ranged from 0.025 to 0.1 pmol 7-hydroxycoumarin produced min^{-1} mg^{-1} protein measured at optimal assay conditions (10 °C and pH = 7.6). Nevertheless, the detection of CYP450 activity in our *A. caliginosa* contrasts with previous studies. Eason et al. (1998) did not detect EROD, BROD and PROD in the post-mitochondrial fraction of *A. caliginosa* midgut at 15 °C and pH = 7.4. Brown et al. (2004) also failed to detect EROD activity in the earthworm *Lumbricus rubellus*. Assuming that there are differences in CYP450 expression between earthworm species, a possible reason for this discrepancy could be also related to the analytical technique. Detection of earthworm CYP450 activity by fluorimetric enzymatic assays is often a difficult task, not only because of the low enzyme activity, but also because of the existence of interfering compounds such as respiratory pigments that may not be fully removed during microsome isolation (Zhang et al., 2006; Cao et al., 2012).

In mammals and fish, both CYP1A and CYP2B isozymes are actively involved in the metabolism of environmental contaminants, and ethoxyresorufin and pentoxyresorufin are their selective reporters, respectively (Burke et al., 1985). Benzyloxyresorufin is another common substrate dealkylated by both isozymes. Results in the present study showed that both CYP1A and CYP2B isozymes might occur in the muscle of *A. caliginosa*. The EROD and BROD activities had a similar response to chlorpyrifos exposure suggesting the intervention of a single isozyme, likely the CYP1A, in the dealkylation reaction. However, the PROD activity showed a different dose–response relationship that would support the occurrence of a second CYP450 isozyme, probably a CYP2B-like isoform. Given the likelihood that multiple CYP450 isozymes are involved in the chlorpyrifos bioactivation, current results suggest the use of a battery of fluorimetric reporters for a better assessment of CYP450 induction. Moreover, the bioactivation of phosphorothionate-type OP pesticides may be also catalyzed by the FMO system (Hodgson, 2010). Thus, the method by Dzul-Caamal et al. (2012) was used for corroborating the bioactivation of chlorpyrifos in the *A. caliginosa* muscle. Results showed that chlorpyrifos bioactivation occurred in unexposed earthworms at a similar rate that reported in fish (Dzul-Caamal et al., 2012), for instance. In addition, this bioactivation rate was increased in the low/short-term exposure group and, although not statistically significant, in the high/long-term exposure group. Therefore, there was not any relationship between $NAD(H)_2$ oxidation and dealkylation of alkoxyphenoxazones, suggesting that chlorpyrifos bioactivation in *A. caliginosa* involves detoxifying enzymes other than CYP1A or CYP2A such as the FMO system.

Chlorpyrifos is an inducer of mammalian monooxygenase activity (Testai et al., 2010). Current results also suggest an induction of earthworm CYP450 activity by this OP pesticide with short-term exposure. These enhanced biotransformation enzyme activities would explain the strong inhibition of CbE activity already detected after 3 d of OP exposure. Induction of CYP450 activity is a common response in earthworms exposed to environmental contaminants. For example, EROD activity increased in *E. fetida* exposed to metals (Cao et al., 2012), benzo(a)pyrene (Booth et al., 2002; Zhang et al., 2006), and carbaryl (Ribera et al., 2001), or in *Aporrectodea tuberculata* exposed to metals (Lukkari et al., 2004). Nevertheless, statistical analysis did not reveal any significant induction of CYP450 activities in the long-term exposed earthworms compared with controls (Fig. 5). The higher *O*-dealkylation activities found in the control group sampled at 21 d compared to that at 3 d probably did not enabled to achieve statistical differences in the treated groups. The time of exposure affected the CYP450 activities of the control groups, but the reason for this difference was not clear.

Interestingly, both EROD and BROD activities showed a biphasic response in the short-term exposure group, defined by an increase of activity at 0.51 mg kg^{-1} chlorpyrifos followed by a decrease of the catalytic activity at the highest concentration. This inverted U-shaped, dose-dependent relationship seems to be a common response in earthworms. Zhang et al. (2006) found that the concentration of CYP450 increased in *E. fetida* exposed to low concentrations of benzo(a)pyrene,

Table 2
Baseline levels of esterase activity in non-exposed adult earthworms.

Species	Tissue or organ	AChE activity[a]		CbE activity		Ref.
		Assay conditions[b]	Specific activity[c] (nmol min^{-1} mg^{-1} prot.)	Assay conditions[b]	Specific activity[c] (nmol min^{-1} mg^{-1} prot.)	
A. caliginosa	Whole organism	3000 ×*g* — supernatant, 0.1 M phosphate (pH = 7.0), 1 mM AcSCh, 20 °C, λ = 412 nm	380–500 (n = 20)	–	–	(1)
A. caliginosa	Whole organism	3000 ×*g* — supernatant, 0.1 M phosphate (pH = 7.0), 3 mM AcSCh, 20 °C, λ = 412 nm	92 ± 7 (n = 20)	–	–	(2)
A. caliginosa[d]	Whole organism	10,000 ×*g* — supernatant, 33 mM phosphate (pH = 8.0), 2 mM AcSCh, 20 °C, λ = 410 nm	98 ± 141 (n = 17)	50 mM phosphate (pH = 7.5), 5 μM 4-NPA, 25 °C, λ = 400 nm	9.29 ± 8.03 (n = 8)	(3)
A. caliginosa	Muscle	9000 ×*g* — supernatant, 0.1 M phosphate (pH = 8.0), 2.4 mM AcSCh, 22 °C, λ = 412 nm	593 ± 174 (n = 40)	0.1 M Tris–HCl (pH = 7.4), 2 mM α-NB or 4-NPB, 22 °C, λ = 405 nm (4-NPB) or 530 nm (α-NB)	α-NB = 176 ± 69 (n = 40) 4-NPB = 264 ± 83 (n = 40)	This study
L. terrestris	Whole organism	3000 ×*g* — supernatant, 0.1 M phosphate (pH = 7.0), 3 mM AcSCh, 20 °C, λ = 412 nm	209 ± 57 (n = 20)	–	–	(2)
L. terrestris	Muscle	9000 ×*g* — supernatant, 25 mM Tris–HCl (pH = 7.6), 2 mM AcSCh, 25 °C, λ = 412 nm	363 ± 47 to 512 ± 144 (n = 36)	25 mM Tris–HCl (pH = 7.6), 46 μM α-NA, λ = 530 nm 20–50 mM Tris–HCl (pH = 7.4–8.0), 0.5 mM 4-NPA or 4-NPV, λ = 405 nm	α-NA = 63.8 ± 7.6 to 97.2 ± 41.7 (n = 36) 4-NPA = 81.8 ± 37 to 113 ± 64 (n = 36) 4-NPV = 95.7 ± 16 to 142 ± 72 (n = 34)	(4)
L. terrestris	Muscle	9000 ×*g* — supernatant, 0.1 M phosphate (pH = 8.0), 2.4 mM AcSCh, 22 °C, λ = 412 nm	550 ± 150 (n = 17)	25 mM Tris–HCl (pH = 7.6), 2 mM α-NA, λ = 530 nm 50 mM Tris–HCl (pH = 7.5), 1 mM 4-NPV, λ = 405 nm	α-NA = 174 ± 71 (n = 17) 4-NPV = 34 ± 16 (n = 17)	(5)
E. andrei	Whole organism	9000 ×*g* — supernatant, 0.1 M phosphate (pH = 7.4), 0.5 mM AcSCh, 25 °C, λ = 405 nm	40–45 (n = 60)	–	–	(6)
E. andrei	Whole organism	9000 ×*g* — supernatant, 0.1 M phosphate (pH = 7.2), 156 mM AcSCh, 25 °C, λ = 412 nm	51.5 ± 4.6 to 54.2 ± 4.5 (n = 21)	1 M Tris–HCl (pH = 8.0), 1 mM 4-NPA, 25 °C, λ = 405 nm	176 ± 17 to 187 ± 17 (n = 21)	(7)
E. andrei	Whole organism	9000 ×*g* — supernatant, 0.1 M phosphate (pH = 7.2), 156 mM AcSCh, 25 °C, λ = 412 nm	28.6 ± 3.2 to 32.2 ± 4.4 (n = 21)	1 M Tris–HCl (pH = 8.0), 1 mM 4-NPA, 25 °C, λ = 405 nm	271 ± 21 to 299 ± 33 (n = 21)	(8)
E. fetida	Whole organism	15,000 ×*g* — supernatant, 0.1 M phosphate (pH = 8.0), 75 mM AcSCh, 20 °C, λ = 410 nm	13.61 ± 2.6 (n = 6)	–	–	(9)

References: (1) Olvera-Velona et al. (2008), (2) Rault et al. (2007), (3) Łaszczyca et al. (2004), (4) Collange et al. (2010), (5) Martínez Morcillo et al. (2013), (6) Gambi et al. (2007), (7) Velki and Hackenberger (2013a), (8) Velki and Hackenberger (2013b), (9) Scaps et al. (1996).
[a] According to Ellman et al. (1961).
[b] AcSCh = acetylthiocholine, α-NA = alpha-naphthyl acetate, α-NB = alpha-naphthyl butyrate, 4-NPA = 4-nitrophenyl acetate, 4-NPB = 4-nitrophenyl butyrate, 4-NPV = 4-nitrophenyl valerate.
[c] Data are mean and standard deviation or range of mean values. Number of earthworms between brackets.
[d] Temperature values are not reported in the study, and they were taken from the references cited in Łaszczyca et al. (2004).

whereas the response of CYP450 was inverted at higher hydrocarbon concentrations. Similarly, Cao et al. (2012) observed the same Gaussian-type response in the EROD activity measured in the gastrointestinal tract of *E. fetida* exposed to metals such as cadmium. Carbaryl also decreased the methoxyresorufin-*O*-deethylase activity (a selective probe for mammalian CYP1A2-like isozyme) in *E. fetida* exposed to high concentrations of the carbamate (Ribera et al., 2001). In line with these studies, there is not a plausible explanation why exposure to high contaminant concentrations leads to a decrease of CYP450 activity.

Glutathione *S*-transferases actively detoxify OP pesticides as well as their oxon metabolites (Testai et al., 2010; Hodgson, 2010). This phase-II metabolizing enzyme is present in earthworms at activity levels, and with an isozyme profile, comparable to those found in vertebrates (Stenersen and Øien, 1981; LaCourse et al., 2009). In the present study, the mean GST activity of the unexposed earthworms was 90.31 ± 27.4 nmol min^{-1} mg^{-1} protein (n = 40), which was similar to that previously reported for *A. caliginosa* (49–101 nmol min^{-1} mg^{-1} protein) (Booth and O'Halloran, 2001), although lower than that in *E. andrei* [296 ± 5.0–424 ± 106 nmol min^{-1} mg^{-1} protein (Ribera et al., 2001); 204 ± 26–220 ± 25 min^{-1} mg^{-1} protein (Velki and Hackenberger, 2013a)]. Both chlorpyrifos concentrations caused a significant increase of GST activity (33–35% of controls) in *A. caliginosa* short-term exposed to the OP, which is in line with previous data. For example, Velki et al. (2014) found a significant increase of GST activity in the earthworm species *E. andrei*, *L. terrestris*, *L. rubellus* and *Octolasium lacteum* exposed for 28 d to pirimiphos methyl using the paper contact test (2.5–250 mg m^{-2}). However, other researchers have found that chlorpyrifos (4–28 mg kg^{-1}, 28-d exposure) did not change the GST activity of *A. caliginosa* although, conversely, the OP diazinon (12–60 mg kg^{-1}, 28-d exposure) inhibited it (Booth and O'Halloran, 2001). Likewise, some studies have shown that the GST response to pesticide is dependent on the duration of exposure. In a microcosm study, the OP dimethoate caused a significant increase of this enzyme activity in *E. andrei* after two weeks of exposure at concentrations ranging between 0.03 and 3.0 mg kg^{-1}, although no significant variations were recorded for the other sampling times (1, 3, 6, 10, 21 and 28 d) compared with the corresponding controls (Velki and Hackenberger, 2013a). In our study, no significant variation of GST activity was found in *A. caliginosa* after 21 d of chlorpyrifos exposure compared with controls. This lack of GST response at longer exposure duration has been also observed by Hans et al. (1993), who reported an increase of GST activity in *Pheretima posthuma* exposed for one week to organochlorine pesticides, but found that enhanced GST activity returned to control levels after 4 weeks of exposure. Similar findings were reported by Schreck et al. (2008) who found an increase of GST activity in *A. caliginosa nocturna* exposed for 3 d to soil contaminated with both chlorpyrifos-ethyl and λ-cyhalothrin, but it significantly decreased after 14 d of exposure compared with unexposed earthworms. Results in the present study support this time-dependent response of GST activity against chlorpyrifos exposure, thereby suggesting a higher detoxification role of GST activity in the initial exposure of the OP.

Inoculation of anecic and endogeic earthworms in contaminated soils is becoming an attractive environmental approach for recovering soil quality (Rodriguez-Campos et al., 2014). But it is needed to determine the sublethal effects (e.g., changes in body weight and reproduction rate, avoidance response or changes in biomarkers linked to

adverse effects at individual level) on earthworms to assess the long-term efficacy of the earthworm-assisted bioremediation. In the present study, chlorpyrifos caused a low impact on the *A. caliginosa*'s health at environmentally realistic concentrations. Particularly, the high sensitivity of CbEs for the highly toxic chlorpyrifos–oxon was one of the molecular mechanisms that accounted for the tolerance of *A. caliginosa* to chlorpyrifos. Therefore, this endogeic species might be a suitable candidate in the bioremediation actions of OP-contaminated soils.

5. Conclusions

Three main conclusions can be drawn when *A. caliginosa* is exposed to sublethal concentrations of chlorpyrifos. First, CbE is an efficient endogenous bioscavenger for chlorpyrifos–oxon, reducing its impact on AChE activity. However, the effectiveness of CbE activity is limited to short-term exposure periods or under low pesticide concentrations, which may represent the most realistic environmental scenarios. Second, with short-term exposure, the main detoxifying enzymes CYP450s and GSTs had a prominent role in the metabolism of chlorpyrifos. As a consequence, the rapid formation of chlorpyrifos–oxon would explain the strong inhibition of CbE activities during this initial period of exposure. Third, chlorpyrifos bioactivation was detected in the muscle of unexposed earthworms through a simple and low-cost spectrophotometric method (Dzul-Caamal et al., 2012). However, the increase of this bioactivation rate did not correspond with the enhanced CYP450 activity measured by synthetic fluorimetric reporters (e.g., ethoxyresorufin). This finding suggests the intervention of detoxifying enzymes other than CYP450s in the chlorpyrifos bioactivation, such as the FMO system. Current results suggest that *A. caliginosa* is able to metabolize efficiently the OP chlorpyrifos, which would explain its tolerance to relatively high levels of pesticide exposure.

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