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Avoidance behaviour response and esterase inhibition in the earthworm, *Lumbricus terrestris*, after exposure to chlorpyrifos

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Abstract The avoidance response of earthworms to polluted soils has been standardised using a simple and low-cost test, which facilitates soil toxicity screening. In this study, the avoidance response of *Lumbricus terrestris* was quantified in chlorpyrifos-spiked soils, depending on the pesticide concentration and exposure duration. The inhibition of acetylcholinesterase (AChE) and carboxylesterase (CbE) activities was also determined as indirect measures of pesticide bioavailability. The effects of different chlorpyrifos concentrations were examined in a standardised test (two-chamber system) with 0.6, 3 and 15 mg/kg chlorpyrifos. A modification of the test involved a pre-exposure step (24, 48 or 72 h) in soils spiked with 15 mg/kg. In both protocols, earthworms were unable to avoid the contaminated soils. However, the esterase activities showed that all earthworms were exposed to chlorpyrifos. Acetylcholinesterase activity did not change in earthworms in the standardised behavioural test (0.58 ± 0.20 U/mg protein, mean ± SD; $n = 72$), whereas the CbE activity was significantly inhibited (62–87 % inhibition) in earthworms exposed to 3 and 15 mg/kg. In the modified test, earthworms had greatly inhibited AChE activity (0.088 ± 0.034 U/mg protein, $n = 72$), which was supported by reactivation of the inhibited enzyme activity in the presence of pralidoxime (2-PAM). Similarly, the CbE activity was significantly inhibited in earthworms with all treatments. This study suggests that the avoidance behaviour test for organophosphorus-contaminated soils could be supported by specific biomarkers to facilitate a better understanding of pesticide exposure and toxicity during this test.

Keywords Behaviour toxicity · *Lumbricus terrestris* · Pesticide detoxification · Environmental risk assessment · Native polyacrylamide electrophoresis

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Introduction

The chemical control of agricultural pests can lead to a loss of soil quality due to the accumulation of pesticides or their metabolites in soil (Hussain et al. 2009). This environmental risk justifies the high number of toxicological bioassays currently required by the regulatory decision-making process for agrochemical authorisation (Walker 2006; European Commission 2002). Earthworms are suitable organisms for toxicity testing during environmental risk assessments of pesticides, and many acute and chronic toxicity tests are available that use these soil organisms (Spurgeon et al. 2003). In the last decade, the avoidance behaviour response (ABR) test has been used for screening as a low-cost and rapid sublethal toxicity assay (ISO 2008). This test evaluates unfavourable conditions in soil (e.g.,

pollution) by comparing the earthworm proportions in two compartments, i.e., a reference soil and a contaminated soil.

The ABR test has been used as a complementary tool for assessing soils spiked with, or historically contaminated by, agrochemicals (Marques et al. 2009; Olvera-Velona et al. 2008; Garcia et al. 2008; Amorim et al. 2005). However, some studies have shown that this sublethal assay is not as sensitive to organophosphorus (OP) pesticides as expected. For example, juvenile *Aporrectodea caliginosa* do not avoid soils sprayed with chlorpyrifos or diazinon at the recommended application rates (Hodge et al. 2000). Similarly, *Eisenia fetida* cannot discriminate soils spiked with 1–80 mg/kg dry weight (dw) chlorpyrifos or 7.5–56 mg/kg dw methamidophos (García-Santos and Keller-Forrer 2011). Jordaan et al. (2012) also found that juvenile *Eisenia andrei* could not avoid soils spiked with azinphos-methyl at concentrations (12 mg/kg dw) where maturation, growth and reproduction were significantly affected. Indeed, some researchers have suggested that the test indicates repellence rather than being a true toxicological bioassay (Capowiez and Bérard 2006). The lack of OP avoidance response in earthworms may reflect the mechanisms of pesticide detoxification or the disruption of physiological function in cholinergic synapses, thereby leading to hyperactivity (Pereira et al. 2010).

The mode of toxic action for OP pesticides and their main detoxification pathways share a group of esterases enzymes. The acute toxicity of these compounds involves the inhibition of acetylcholinesterase (AChE, Enzyme Commission [EC] 3.1.1.7) activity in nervous tissues (Fukuto 1990), but their detoxification is mediated primarily by two groups of esterases, i.e., carboxylesterases (CbEs, EC 3.1.1.1) and phosphotriesterases (EC 3.1.8.1) (Sogorb and Vilanova 2002). Many studies have shown that CbE activity exhibits a higher affinity for OPs than AChE activity, suggesting thereby a protective role (Wheelock et al. 2008). This stoichiometric mechanism of OP detoxification (irreversible inhibition) could modulate the pesticide toxicity during the ABR test.

The primary aim of this study was to examine whether the avoidance response of earthworms to OP-contaminated soils was associated with the inhibition of AChE activity. AChE is a key enzyme in the normal functioning of cholinergic synapses in the nervous system and neuromuscular junctions. A second aim was to determine the role of CbE activity as a pesticide sink that reduces the impact of OPs on AChE activity and the avoidance response. Thus, the inhibitions of both esterase activities were determined in the body wall muscle of earthworms after two behavioural tests: a standardised protocol (ISO 2008) and a modification of this assay, which included a pesticide pre-exposure step (24, 48 and 72 h). Earthworm esterases have multiple isoforms with marked variations in their hydrolytic activity (Sanchez-Hernandez and Wheelock 2009) and sensitivity to OPs (Aamodt et al. 2007), so the analysis of the enzyme activity using non-denaturing electrophoretic gels (zymograms) and the reactivation of the OP-inhibited cholinesterase activity using pyridine-2-aldoxime methochloride (2-PAM, pralidoxime) were included as complementary and specific methods of determining OP exposure.

Materials and methods

Earthworms and test soil samples

Adult and clitellated earthworms were purchased from a local supplier (Poisson Fenag, Madrid, Spain), kept in plastic containers (345 × 325 × 150 mm) for 1 month (15 °C and continuous dark) and fed weekly with wet cow manure, which was applied onto the soil surface. During this acclimatisation period, the earthworms were kept in soil samples (pH_{H_2O} = 7.2 ± 0.08, conductivity = 116.7 ± 19 µS/cm and total organic carbon = 5.9 ± 2.0 %; mean ± SD of 10 samples previously dried at 105 °C for 48 h) collected from an abandoned agricultural land located near the National Park of Cabañeros (Montes de Toledo, Spain), which is an area with no known history of pesticide application. This soil type (10.7 % clay, 10.8 % silt, 54.5 % coarse sand and 23.7 % fine sand) was also used in the subsequent bioassays.

The organophosphorus chlorpyrifos (Dursban 5G, 5 % w/w chlorpyrifos, granulated formulation obtained from Compo Agricultura S.L., Barcelona, Spain) was used as the test pesticide because it is one of the most commonly used anticholinesterase insecticides in the European Union (Eurostat 2007). The chlorpyrifos concentrations were selected based on their sublethal effects on earthworms (Eijsackers 2004; Reinecke and Reinecke 2007) and assuming a predicted environmental concentration (PEC) of 3.3 mg active ingredient (a.i.) kg^{-1} dw, which was calculated based on the lowest recommended application rate, a soil layer depth of 5 cm with pesticide penetration, no crop interception and a bulk soil density of 1.5 g/cm^3 (Rault et al. 2008). The concentrations of chlorpyrifos in the test soils were equal to the PEC (3 mg a.i./kg dw), PEC/5 (0.6 mg a.i./kg dw) and 5× PEC (15 mg a.i./kg dw). Soil spiking was performed by mixing 1.5 kg of dry soil with the insecticide for 2 min using a plastic bag to ensure the uniform distribution of the pesticide in the bulk soil. The soil water content was adjusted to 25 % w/v, which corresponded to approximately 80 % of the maximum water-holding capacity (0.29 ± 0.03 g H_2O/g dry soil, mean ± SD of five replicates).

Avoidance behaviour response tests

The avoidance response of earthworms was examined using two experimental protocols. The first trial was adopted from the standardised ABR test prescribed by the International Organization for Standardization (ISO 2008), which involved separating a rectangular box (265 × 162 × 100 mm) into two equal sections using a removable plastic divider. Half of the box was filled with 0.5 kg wet weight of uncontaminated (control) soil and the other half was filled with 0.5 kg of chlorpyrifos-spiked soil. Each pesticide concentration was run in three replicates. The test containers were kept at 15 °C in the dark for 48 h to allow equilibration. Next, the plastic divider was removed and eight earthworms per replicate were placed on the line of direct contact between both soils. The containers were kept in the acclimatisation chamber and earthworms moved freely in both soil sections for a period of 48 h. Finally, the divider was replaced before counting the earthworms in each compartment.

In the second experimental design, earthworms were previously exposed to soil spiked with 15 mg/kg chlorpyrifos. Worms (eight individuals per replicate) were confined to the container section holding the chlorpyrifos-spiked soil and kept there for a fixed period of time (24, 48 or 72 h). Next, control soil (0.5 kg wet weight) was placed in the adjacent section and the separator was removed. From this point, the test procedure was the same as that described above. In both experimental approaches, individuals found in the middle of the test container were allocates based on the head position (De Silva and van Gestel 2009; Garcia et al. 2008). Earthworms from both bioassays were frozen immediately at −80 °C before subsequent biochemical analysis. A group of 17 adults and clitellated earthworms were taken directly from the acclimatisation containers and used as a reference group, which were not used in the ABR tests.

Esterase activities

The body wall muscle was dissected from the clitellum towards the anus and a sample of tissue (~0.5 g wet weight) was homogenised (1:10, w/v) in ice-cold 25 mM Tris–HCl buffer (pH = 8.0) containing 0.1 % Triton X-100 using a glass–PTFE Potter–Elvehjem tissue grinder connected to a Heidolph type ST1 homogeniser (10,000 rpm). The homogenates were centrifuged at 9,000 g for 20 min at 4 °C and the supernatant or post-mitochondrial fraction (range of total proteins = 2.4–6.7 mg/ml) was used in the esterase assays.

The acetylcholinesterase activity was determined according to the method of Ellman et al. (1961), which was adapted to the microplate format (Wheelock et al. 2005). The enzymatic reaction was performed in 96-well flat bottom plates by mixing 235 µl of 0.1 M Na phosphate buffer (pH = 8.0) containing 320 µM 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) and 5 µl of sample. After 2 min of equilibration, 10 µl of 60 mM acetylthiocholine iodide (AcSCh) was added to the reaction mixture and the absorbance was read at 412 nm for 10 min (1 min intervals) at 22 °C using an Asys HiTech UVM340 plate reader (Asys HiTech GmbH, Eugendorf, Austria). The esterase activity was calculated using a calibration curve produced with DTNB and serial concentrations of reduced glutathione (6.25–100 µM), as described by Eyer et al. (2003). The hydrolysis of AcSCh was attributed to AChE activity whereas the contributions of other cholinesterases such as butyrylcholinesterase (BChE, EC 3.1.1.8) were neglected (Rault et al. 2007). The carboxylesterase activity was assayed using two substrates, i.e., α-naphthyl acetate (αNA) and 4-nitrophenyl valerate (4NPV). The hydrolysis of αNA (αNA-CbE) was determined as described by Thompson (1999). The hydrolytic reaction was initiated by mixing 170 µl of 25 mM Tris–HCl (pH 7.6), 10 µl of 2 mM (final concentration) αNA and 20 µl of the sample, and it was stopped after 10 min (22 °C and continuous agitation) by the addition of 50 µl of a solution that contained 2.5 % SDS in 0.1 % Fast Red ITR/2.5 % Triton X-100. The absorbance of the naphthol–Fast Red ITR complex was read at 530 nm and the specific activity was calculated using an external curve produced with α-naphthol (1.5–100 nmol/ml). The hydrolysis of 4NPV (4NPV-CbE) was determined according to the method of Carr and Chambers (1991). This enzymatic reaction was initiated by mixing 170 µl of 50 mM Tris–HCl (pH 7.5), 10 µl of 1 mM (final concentration) 4NPV and 20 µl of the sample. After 15 min of incubation (22 °C and continuous agitation), the reaction was stopped by adding 50 µl of a solution that contained 2 % (w/v) SDS and 2 % (w/v) Tris base. The formation of 4-nitrophenolate was read at 405 nm and quantified using an external calibration curve (5–100 nmol 4-nitrophenolate/ml).

All hydrolytic reactions were run in triplicate and blanks (reaction medium without a sample) were periodically assayed to discount non-enzymatic formation of the reaction products. The esterase activities were expressed as units per milligram of total proteins (U/mg protein). One unit (U) of enzyme activity was defined as that catalysing the formation of 1 µmol of product in 1 min in the experimental conditions described above. The concentrations of total proteins were determined using the Bradford method (Bradford 1976) with bovine serum albumin as the standard.

Chemical reactivation of cholinesterase activity

The phosphorylation of AChE activity by chlorpyrifos-oxon was tested by incubating the sample with 2-PAM, according to the method of Rodríguez-Castellanos and Sanchez-Hernandez (2007). Two aliquots (40 µl) of the muscle homogenate for each sample were incubated separately with 5×10^{-4} M (F.C.) of 2-PAM or distilled water (control) for 60 min at 25 °C. The AChE activity was then measured in both samples and an increase in the esterase activity was judged as significant by comparing the mean esterase activities of OP-exposed earthworms and their corresponding controls.

Electrophoretic zymography

Non-denaturing polyacrylamide gel electrophoresis (native-PAGE) was used to analyse multiple esterase isoforms with overlapping substrate specificity and to determine their sensitivity to chlorpyrifos exposure. An assay of the in-gel AChE activity was performed using samples of earthworms that had been exposed to 15 mg/kg-spiked soils for 24, 48 and 72 h. Samples were divided into two aliquots: one aliquot (40 µl) was spiked with 10 µl of 2-PAM (5×10^{-4} M, final concentration), whereas the second received 10 µl of distilled water (control). Samples were incubated at 25 °C for 60 min and diluted with 25 µl of 60 mM Tris–HCl (pH = 6.8) containing 25 % glycerol and 0.1 % bromophenol blue. Finally, the samples were loaded onto 4 % stacking and 9 % resolving 1.0 mm polyacrylamide gels (25 mM Tris, 192 mM glycine as running buffer), and the gels were electrophoresed using a Bio-Rad Tetra Cell Electrophoresis Unit (Bio-Rad, USA) at a constant voltage of 30 V for 30 min, followed by 150 V until the tracking dye reached the bottom of the gel. Protein bands with AChE activity were visualised after incubation (overnight at 22–23 °C using an orbital shaker) of the gels with a staining solution prepared according to the method of Karnovsky and Roots (1964). The reagents were mixed in the following order: 32.5 ml of 0.1 M Na phosphate buffer (pH = 6.0), 25 mg AcSCh, 2.5 ml of 0.1 M sodium citrate, 5 ml of 30 mM $CuSO_4$, 5 ml of 5 mM potassium ferricyanide and 5 ml of distilled water. Electric eel AChE type V–S (Sigma–Aldrich, catalogue no. C2888-500UN, lot no. 087K7002) was used as a standard to confirm the in-gel hydrolysis of AcSCh. Gels were scanned using a Gel Doc™ EZ Imager system (Bio-Rad) and the protein bands were separated using the Image Lab software system (version 3.0.1, Bio-Rad Laboratories).

The in-gel staining CbE activity was determined separately using two staining solutions (Manchenko 2003). Samples of earthworms exposed to 15 mg/kg chlorpyrifos for 24, 48 and 72 h were used to determine the effects of OP on CbE isozymes. Initially, the electrophoresed gel was incubated for 8 min at 22 °C with a solution of 0.1 M Na phosphate buffer (pH = 7.2) containing 100 µM 4-methylumbelliferyl acetate. The gel was then scanned, washed with Na phosphate buffer and incubated (10 min, 22 °C) again with a second staining solution (filtered immediately before use), which contained 0.1 mg/ml αNA, 0.1 mg/ml βNA and 0.5 mg/ml Fast Blue RR in 0.1 M Na phosphate buffer (pH = 7.2).

Determination of the chlorpyrifos concentration in soil samples

The nominal concentrations of chlorpyrifos were confirmed by liquid chromatography with spectrophotometric detection (HPLC–UV/Vis) after both ABR tests had been completed. The half-life of chlorpyrifos in soil ranges from 30 to 60 days when incorporated into the soil profile (Racke et al. 1996), so the degradation of chlorpyrifos was assumed to be non-significant during the tests under the experimental conditions (15 °C and darkness). The QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) method, which was adapted for soil samples by Asensio-Ramos et al. (2010), was used to analyse chlorpyrifos residues and its main metabolites, chlorpyrifos-oxon and 3,5,6-trichloro-2-pyridinol. Air-dried soil samples (2.5 g) were extracted by the addition of 5 ml acetonitrile (HPLC-grade), using paraoxon-ethyl (50 µg/ml in acetonitrile) as an internal standard. Samples were shaken vigorously for 1 min, followed by the addition of $MgSO_4 \cdot 7H_2O$, NaCl and citrate salts, sonication for 5 min (ultrasonic bath, 50 W) and centrifugation (5,000×*g* for 5 min). The supernatants were cleaned up by dispersive solid phase extraction with $MgSO_4$ and primary-secondary amine (particle size = 40 µm). The mixture was agitated for 1 min and centrifuged (5,000×*g* for 5 min). Finally, the supernatant was filtered (Cameo 3 N nylon: 0.22 µm, 3 mm) and 20 µl of the supernatant was injected into an Agilent 1200 Series HPLC system, which comprised a manual injector (7725i injection valve, 20 µl loop), a vacuum degasser, a quaternary pump and a multiple wavelength detector. The mobile phase consisted of H_2O with 0.05 % acetic acid (solvent A) and acetonitrile with 0.05 % acetic acid (solvent B). Chlorpyrifos and its metabolites were separated using an LC-8 column (0.46 cm × 25 cm × 5 µm particle size) at a flow rate of 0.5 ml/min with the following solvent program: 65 % B at $t = 0$ min, which was increased to 95 % B up to 7 min and maintained for 3 min, followed by 65 % B for 5 min. The multiple wavelength detector was set to 290 nm (chlorpyrifos, chlorpyrifos-oxon and internal standard) and 310 nm (3,5,6-trichloro-2-pyridinol).

The recovery of chlorpyrifos was assessed by spiking control soils with two levels of chlorpyrifos (3.0 and

13.5 μg/g dw). The spiking procedure was as follows: 1 ml of each chlorpyrifos standard in acetonitrile was added to 5 g of dried soil. The solvent was allowed to evaporate and the spiked soils were mixed vigorously in a 15 ml centrifuge tube for 1 min to ensure sample homogenisation. A 2.5 g subsample of the spiked soil was used to determine the chlorpyrifos concentration. The recovery rates ranged between 108 ± 3.8 % ($n = 3$ samples spiked with 3.0 μg/g dw) and 103 ± 4.7 % ($n = 3$ samples spiked with 13.5 μg/g dw).

Data analysis

The minimum sample size was 24 individuals per treatment, which was calculated using the power analysis module in Statistica (version 6, StatSoft, Inc., Tulsa, OK, USA) with a significance level of $\alpha = 0.05$ at 0.5 power. The non-parametric Mann–Whitney test was used to test for significant differences in the earthworm distribution between both sections (control and spiked soils). The effects of the chlorpyrifos concentration and the duration of pre-exposure on the avoidance response were also tested using the Kruskal–Wallis ANOVA test. First, the avoidance response in each replicate was calculated using the following expression: $NR = [(C-T)/N] \times 100$, where NR is the net response (%), C is the number of earthworms in control soil, T is the number of individuals in the chlorpyrifos-spiked soils and N is the total number of earthworms per replicate.

General linear models were used to test for differences in the esterase activities (logarithmic-transformed data) due to the chlorpyrifos concentration (standardised ABR test) and the duration of OP exposure (modified ABR test). Multivariate analysis of variance (MANOVA) tests were followed by post hoc LSD Fisher's or Dunnett's tests to make pairwise comparisons among treatments or with the reference group, respectively. The increase in the AChE activity following 2-PAM treatment was compared with the corresponding inhibition of the esterase activity using the Mann–Whitney test. If this increase was statistically significant, the percentage of reactivation (angular transformed data) in the experimental groups (0.6, 3 and 15 mg/kg, or 24, 48 and 72 h) and those in the control and OP-spiked soils were compared using the LSD post hoc test. The percentage AChE reactivation was calculated using the following equation: Reactivation (%) $= ([R–I]/[C-I]) \times 100$, where R is the AChE activity of the chlorpyrifos-exposed earthworm after 60 min of 2-PAM (5×10^{-4} M) incubation, I is the inhibited enzyme activity of the chlorpyrifos-exposed earthworm and C is the mean AChE activity of the reference group (i.e., non-exposed earthworms). In all statistical tests, $P < 0.05$ was assumed to be statistically significant.

Results

Chlorpyrifos concentrations in soils

The observed concentrations of chlorpyrifos were 17.8 ± 4.1 mg/kg dw (5 × PEC group, $n = 6$ samples), 2.4 ± 1.6 mg/kg dw (PEC group, $n = 6$) and 0.44 ± 0.5 mg/kg dw (PEC/5 group, $n = 7$), which were within 16–30 % of the nominal concentrations. This wide inter-assay variation was probably due to the pesticide formulation (granules), which may have led to a heterogeneous distribution of the active ingredient in the soil matrix. The observed OP concentrations in the soil samples after the pre-exposure trial were 13.1 ± 3.9 mg/kg dw ($t =$ 24 h group, $n = 6$ soil samples), 9.1 ± 1.7 mg/kg dw ($t = 48$ h group, $n = 6$) and 14.8 ± 3.2 mg/kg dw ($t = 72$ h group, $n = 6$). Chlorpyrifos metabolites were not detected in the spiked soils using the current analytical procedure.

Avoidance behaviour response

No mortality was observed after the avoidance tests were completed and no earthworms were observed on the soil surface. Figure 1 shows the mean percentage of earthworms recovered in the control soils after both avoidance behaviour tests. There were no significant differences (Mann–Whitney test, $P < 0.05$) in the earthworm distributions in both soil sections, irrespective of the chlorpyrifos concentration and the duration of pesticide pre-exposure. Nevertheless, the earthworm group exposed to soils spiked with 15 mg/kg chlorpyrifos for 72 h before the standardised ABR test had a preference for the control soil (71 % of earthworms), although this response was not statistically significant (Mann–Whitney test, $P = 0.08$). The average net response was not significantly affected by the chlorpyrifos concentration ($H_{2,9} = 1.08$, $P = 0.582$) or by the duration of pesticide pre-exposure ($H_{2,9} = 3.25$, $P = 0.196$).

Acetylcholinesterase activity

The muscle AChE activity of earthworms in the standardised ABR test was not inhibited, although individuals were present in the compartment holding the pesticide-spiked soil. The mean (±SD) enzyme activity ranged from 0.47 ± 0.19 to 0.71 ± 0.16 U/mg protein (Fig. 2a). These levels of esterase activity were not statistically different ($P > 0.17$, Dunnett's post hoc test) from the mean AChE activity of the reference group (0.55 ± 0.15 U/mg protein). In addition, treatment of the muscle homogenates with 5×10^{-4} M 2-PAM supported the lack of observed enzyme inhibition because there was no significant

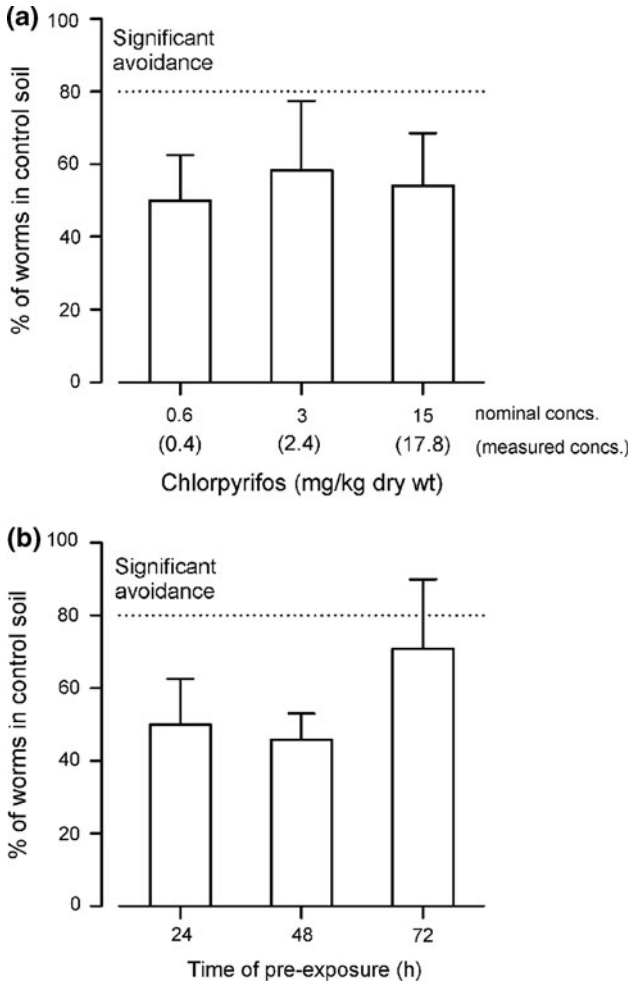

Fig. 1 Percentage of earthworms, *Lumbricus terrestris*, found in the control soil compartments after the 48 h standardised avoidance test (**a**), and the modified 48 h avoidance test (**b**), which included previous continuous (24–72 h) exposure to chlorpyrifos (15 mg/kg)-spiked soils. The effect of the chlorpyrifos concentration or the duration of pre-exposure on the avoidance response was not statistically significant (Kruskal–Wallis test, $P < 0.05$). The mean percentage of earthworms that avoided the pesticide-spiked soils did not exceed the threshold value (80 %) proposed by Hund-Rinke et al. (2003) as the soil habitat limit

increase ($P > 0.130$, Mann–Whitney test) in the esterase activity (Fig. 2a). However, the esterase activity was strongly inhibited ($F_{6,76} = 75.86$, $P < 0.0001$) in earthworms exposed to chlorpyrifos for 24, 48 or 72 h before the ABR test (Fig. 2b). Treatment of the homogenates with 2-PAM caused a significant (Mann–Whitney test, $P \leq 0.03$) increase in the enzyme activity relative to their corresponding original values (phosphorylated AChE). However, 2-PAM was unable to fully restore the phosphorylated esterase activity to its normal levels. The 2-PAM-induced AChE activities were compared with the mean enzyme activity of the reference group, which showed that the oxime caused only a 10–20 % reactivation (Table 1). This 2-PAM-induced increase in the AChE activity was reproduced in the native-PAGE gels. The electrophoretic zymograms contained two broad bands and the mean staining intensity measured using the Image Lab software (Bio-Rad) detected increases of 87 and 193 % in band-I and band-II, respectively, compared with the corresponding bands in the control gel (Fig. 2c).

Carboxylesterase activity

The effect of chlorpyrifos on CbE activity depended on the type of substrate used in the kinetic assays and the avoidance test protocol (Fig. 3). Compared with the reference group, the hydrolysis of αNA was significantly lower ($P < 0.001$, Dunnett's post hoc test) in the experimental groups exposed for 72 h (47–58 % inhibition) and in the earthworms exposed for 24 h and present in the OP-spiked soil (48 % inhibition). However, there was no significant decrease in αNA hydrolysis in the earthworms in the standardised ABR test ($P > 0.141$, Dunnett's post hoc test), irrespective of the insecticide concentration (Fig. 3a). The hydrolysis of 4NPV was strongly inhibited (62–87 % inhibition compared with the reference group) in earthworms exposed to 3 and 15 mg/kg chlorpyrifos, and in all earthworms exposed for 24, 48 and 72 h to soils spiked with 15 mg/kg chlorpyrifos (57–79 % inhibition relative to controls) before the ABR test (Fig. 3b).

The electrophoretic zymograms contained multiple bands that corresponded to CbE activity and there were comparable abundances and relative mobilities in the gels of the substrates (4-MUBA and αNA/βNA) used to determine the in-gel activity staining (Fig. 3c). Chlorpyrifos caused a slight reduction in the staining intensity of some protein bands compared with control earthworms. However, the high variation in the staining intensity and isozyme abundance of the controls (C1 and C2 in the gel, Fig. 3c) made it difficult to separate the effect of chlorpyrifos on specific CbE isozymes.

Discussion

Earthworms did not avoid soils treated with chlorpyrifos, which is a behavioural response to OP-contaminated soils that has already been documented by others (Hodge et al. 2000; Jordaan et al. 2012). The concentrations of chlorpyrifos in the current soils, although environmentally realistic, may have been insufficient to produce a significant avoidance response within 48 h. Indeed, some studies have shown that the outcome of the ABR test with pesticides is concentration-dependent (Slimak 1997; Pereira et al. 2010; García-Santos and Keller-Forrer 2011). Chlorpyrifos residues were not measured in earthworms,

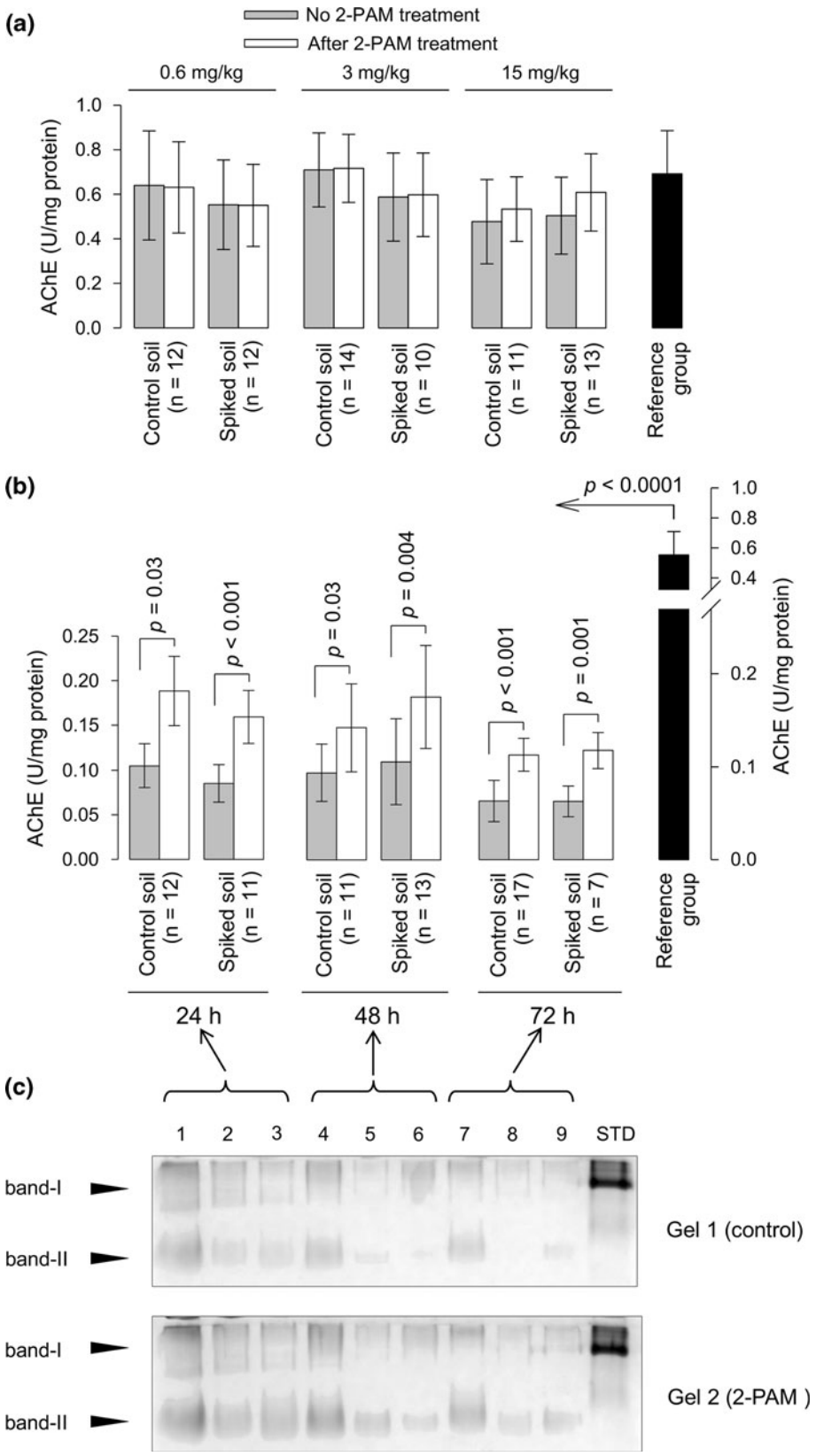

Fig. 2 Mean (±standard deviation) acetylcholinesterase (AChE) activity of earthworms, *Lumbricus terrestris*, after the standardised avoidance behaviour response test (**a**) and after the modified version of this avoidance test (**b**). *Gray bars* represent the enzyme activity of earthworms found in the control and chlorpyrifos-spiked soils, whereas *white bars* represent the AChE activity of these earthworms after 2-PAM treatment (see Sect. "Materials and methods" for further details). The *black bar* shows the mean AChE activity of the reference group (0.55 ± 0.15 U/mg protein, $n = 17$ earthworms). **c** Native-PAGE of samples ($n = 3$) corresponding to the 24, 48 and 72 h exposed groups with (gel 1) and without (gel 2) previous 2-PAM (pralidoxime) treatment. *Lanes* 1, 2 and 3: earthworms exposed for 24 h to soils spiked with 15 mg/kg (41, 38 and 47 µg protein charged on gel). *Lanes* 4, 5 and 6: earthworms exposed for 48 h (33, 26 and 23 µg protein). *Lanes* 7, 8 and 9: earthworms exposed for 72 h (42, 50 and 39 µg protein). *Lane* 10: electric eel AChE type V–S. A MANOVA test followed by Dunnett's post hoc test was used to detect significant differences between the reference group and treated groups, whereas the Mann–Whitney test was used for pairwise comparisons of the AChE activity in each treatment

but the measurements of AChE inhibition and 2-PAM-induced reactivation were used as sensitive and specific indexes, respectively, of OP exposure (Fukuto, 1990; Rodríguez-Castellanos and Sanchez-Hernandez 2007). In both ABR protocols, the response of the AChE activity differed markedly. The earthworms in the standardised test

Table 1 Pralidoxime (2-PAM)-induced increase in the acetylcholinesterase (AChE) activity of earthworms, *Lumbricus terrestris*, after exposure to soils spiked with 15 mg/kg chlorpyrifos

	% Increase relative to the corresponding inhibited AChE activity[a]				% Increase relative to the reference mean AChE activity[b]			
	Control soil		Chlorpyrifos-spiked soil		Control soil		Chlorpyrifos-spiked soil	
Group	*N*	%[c]	*N*	%	*N*	%	*N*	%
24 h	12	$81 \pm 23^{(NS)}$	11	$90 \pm 19^{(NS)}$	12	$19 \pm 5.5^{(A)}$	11	$16 \pm 3.1^{(A,B)}$
48 h	11	$61 \pm 20^{(NS)}$	13	$74 \pm 32^{(NS)}$	11	$13 \pm 5.8^{(B)}$	13	$17 \pm 6.4^{(B)}$
72 h	17	$92 \pm 48^{(NS)}$	7	$96 \pm 45^{(NS)}$	17	$10 \pm 3.8^{(B)}$	7	$12 \pm 2.7^{(A)}$

[a] 'NS' denotes non-significant differences between the mean percentages (angular transformed data) of the experimental groups (24, 48 and 72 h) and between both soil compartments (control vs. chlorpyrifos-spiked soils) using the post hoc LSD test ($P < 0.05$). The percentage of increased AChE activity was calculated using the following expression: Reactivation (%) = [(R−I)/I] × 100, where I is the AChE activity of the chlorpyrifos-exposed earthworm and R is the enzyme activity after pralidoxime treatment

[b] The mean (±standard deviation) AChE activity of the reference group was 0.55 ± 0.15 U/mg protein ($n = 17$ individuals). The percentage increased AChE activity was calculated using the following equation: Reactivation (%) = ([R−I]/[C−I]) × 100 (R and I are as above, and C is the mean AChE activity of the reference group). Different letters ('A' and 'B') in the experimental groups indicate significant differences ($P < 0.05$, LSD post hoc test). No significant differences were detected between the control and pesticide-spiked soils in each experimental group

[c] Percentage values as the mean and standard deviation

experienced no inhibition of their AChE activity, which was confirmed by the absence of any activity increase following 2-PAM treatment. The mean AChE activity of these earthworms was similar to that of the reference group (550 ± 150 mU/mg protein, mean ± SD, $n = 17$), which was also comparable to the AChE activity reported by others in this earthworm species (363 ± 47 to 512 ± 144 mU/mg protein [Collange et al. 2010], 391 ± 10 mU/mg protein [Sanchez-Hernandez and Wheelock 2009] and 209 ± 57 mU/mg protein [Rault et al. 2007]). However, the inclusion of a pre-exposure step in this standardised protocol produced strong inhibition (>80 % of control activity) of AChE activity in all earthworms, irrespective of the duration of exposure (24–72 h). Thus, it may be assumed that the systemic concentration of chlorpyrifos-oxon (i.e., the main metabolite of chlorpyrifos with a high affinity for the active site of esterases) in the standardised ABR design was not sufficiently high to depress the AChE activity. This finding may reflect the low residence of earthworms in the box compartment holding the chlorpyrifos-spiked soil or sporadic episodes of OP exposure during the 48 h test duration.

To the best of our knowledge, only two studies have tried to link the avoidance response of OP-exposed earthworms to the inhibition of AChE activity. For example, Pereira et al. (2010) reported a significant AChE inhibition in *E. andrei* when exposed to methomyl concentrations >0.86 mg/kg, whereas a significant avoidance response occurred at 5.62 mg/kg. Similarly, Jordaan et al. (2012) found that *E. andrei* was not able to avoid soils contaminated with 6 or 12 mg/kg azinphos-methyl, although these concentrations strongly inhibited (>90 % of controls) the cholinesterase activity. However, these studies cannot be compared directly with the current study because of differences in the experimental procedures and earthworm species. In these two studies, cholinesterase inhibition was measured in earthworms that were used in parallel experiments rather than the ABR test (48 h of continual exposure to methomyl [Pereira et al. 2010] or intermittent 12 week exposures to azinphos-methyl [Jordaan et al. 2012]).

Despite the high level of AChE inhibition in the earthworms in the modified ABR design, there was no clear pattern of soil selection. Assuming that cholinergic transmission is the main neural network involved in the ability of earthworms to detect and avoid contaminated soils (Rosenbluth 1972), a high degree of AChE activity inhibition should imply an overstimulation of postsynaptic cells (e.g., muscle fibres), thereby leading to hyperactivity. This neurotoxic effect has been observed in organisms such as the amphipod *Gammarus fossarum* exposed to methomyl (Xuereb et al. 2009), zebrafish larvae (*Danio rerio*) exposed to chlorpyrifos (Kienle et al. 2009) and eastern rainbow fish (*Melanotaenia duboulayi*) exposed to profenofos (Kumar and Chapman 1998). A similar effect may have occurred in the earthworms in the modified ABR test, where the chlorpyrifos-induced hyperactivity may have led the earthworms to adopt an irregular locomotion pattern, thereby leading to no clear avoidance response. This hypothesis was also postulated by Pereira et al. (2010) with the earthworm *E. andrei* exposed to the carbamate insecticide methomyl.

In this study, reactivation of AChE activity with 2-PAM was used as a complementary and specific index of chlorpyrifos exposure. Previous in vitro experiments have shown that this oxime is a suitable reactivating agent for earthworm cholinesterases (Rodríguez-Castellanos and Sanchez-Hernandez 2007). However, 2-PAM was unable

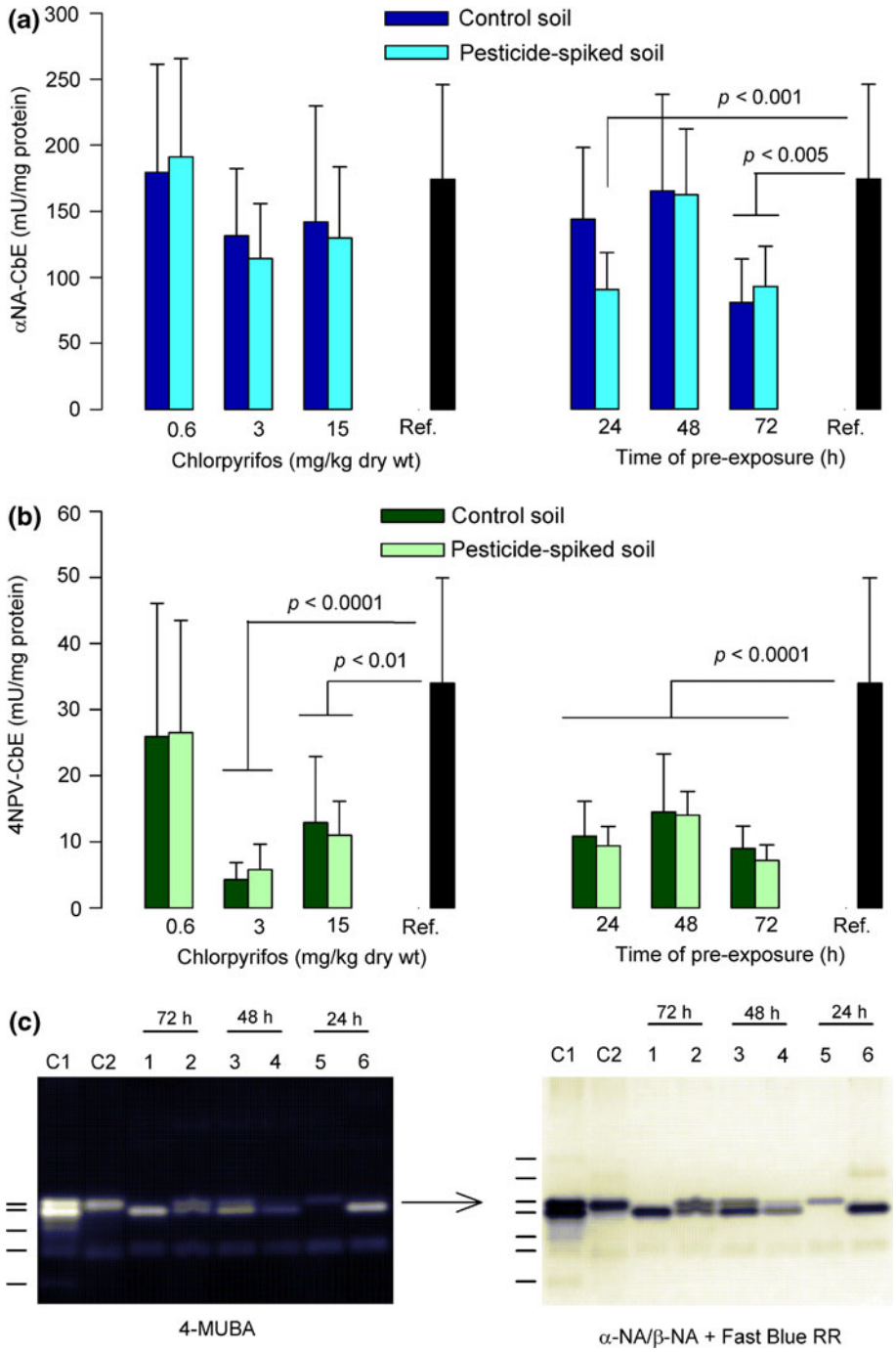

Fig. 3 Mean (±standard deviation) carboxylesterase (CbE) activity with α-naphthyl acetate (αNA, graph **a**) and 4-nitrophenyl valerate (4NPV, graph **b**) in the body wall muscle of earthworms, *Lumbricus terrestris*, after the standardised avoidance behaviour response test and the modified version of the avoidance test. *Black bars* show the mean CbE activity of the reference group (αNA–CbE = 174 ± 71.6 mU/mg protein, 4NPV–CbE = 34 ± 16 mU/mg protein, $n = 17$ earthworms). (**c**) Native-PAGE of the muscle homogenates from earthworms exposed for 24, 48 and 72 to chlorpyrifos (15 mg/kg)-spiked soils. *Lanes* C1 and C2: control individuals (71 and 72 µg protein charged on gel). *Lanes* 1 and 2: earthworms exposed for 72 h (34 and 35 µg protein). *Lanes* 3 and 4: earthworms exposed for 48 h (34 and 42 µg protein). *Lanes* 5 and 6: earthworms exposed for 24 h (23 and 25 µg protein). Significant differences were determined using a MANOVA test followed by Dunnett's post hoc test

to fully recover the phosphorylated AChE activity in the earthworms from the modified ABR test. The aging phenomenon in the cholinesterase activity following inhibition by OPs is believed to be the main reason for this low potency of 2-PAM in recovering the AChE activity (Collange et al. 2010). Despite this limitation of 2-PAM, the high inter-individual variation of AChE activity in non-exposed earthworms (coefficient of variation = 28 %, $n = 17$ individuals) and the absence of data on chlorpyrifos residues in earthworm tissue suggest that the use of chemical reactivation methods with oximes may allow a more reliable data interpretation.

Another goal of this study was to determine whether CbEs contributed to reducing the effect of chlorpyrifos on the AChE activity. It is well known that CbEs promote the non-catalytic detoxification of OP compounds by the formation of a stable enzyme-inhibitor complex (Sogorb and Vilanova 2002). Current results support this protective

action. In the standardised ABR protocol, the AChE activity did not change compared with the reference group, but the earthworms were definitely exposed to this OP because significant inhibition of the CbE activity was detected. This higher sensitivity of CbE activity to OP exposure compared with the AChE activity has been demonstrated in earthworms (Sanchez-Hernandez and Wheelock 2009; Collange et al. 2010). The detoxification of chlorpyrifos by CbEs was more prominent in earthworms exposed to chlorpyrifos for 24 to 72 h before the ABR test. Indeed, all individuals exhibited a strong inhibition of 4NPV–CbE activity, as well as αNA–CbE activity, although to a lesser extent. In addition to the higher affinity of CbEs for OP pesticides compared with cholinesterases (Wheelock et al. 2008), the number of enzyme molecules is also critical for the efficacy of this stoichiometric mechanism of OP detoxification (Chanda et al. 1997). The strong inhibition of CbE activity suggests that there were insufficient enzyme molecules available to bind free insecticide, so the inhibition of AChE activity occurred. This may have been the situation for the earthworms in the modified ABR test, so their AChE activity was also strongly inhibited.

The analysis of the in-gel activity staining showed that 2-PAM induced an increase in the AChE activity, which was markedly distinct in both of the protein bands detected. This difference in 2-PAM reactivation suggests the presence of two different types of AChE activity in the muscle tissues of earthworms. Indeed, some studies have shown that the total cholinesterase activity of earthworm species such as *E. fetida* is attributable to two different cholinesterases (Stenersen 1980, Aamodt et al. 2007). Similarly, the in-gel hydrolysis of naphthyl esters is another common post-electrophoresis method for examining the isozyme profiles of esterases (Manchenko 2003). This technique allows the analysis of the isozyme-specific sensitivities of CbE activity in earthworms after exposure to OP insecticides (Sanchez-Hernandez and Wheelock 2009). In the present study, however, it was not possible to detect a pronounced effect of chlorpyrifos exposure in the multiple CbE staining bands observed in the native-PAGE gels, even when different substrates (e.g., 4-MUBA and αNA/βNA) were used for in-gel activity staining. The two individuals used as controls also exhibited a marked difference in the staining intensity and isozyme abundance, which led to a lack of definitive conclusions about the effects of the OP on these esterases. However, these results reproduced the high inter-individual variation in the CbE activity observed in the kinetic assays. Thus, although the use of electrophoretic zymograms aided the data interpretation, care must be taken when considering the CbE activity.

Conclusions

Measurements of the CbE and AChE activities in the standardised avoidance behaviour assay and the inclusion of a pre-exposure step allowed the following conclusions. (1) The standardised ABR test is not simply a sensory response to toxicants. Instead, it is a sublethal toxicity bioassay where the absence of an avoidance response does not indicate that the pesticide concentrations in soil are harmless to earthworms. (2) The inhibition of AChE activity, i.e., a key enzyme in the chemical synapses involved in locomotion, did not necessarily imply an avoidance response. (3) The use of multiple substrates identified a fraction of the CbE activity that had a high sensitivity to chlorpyrifos inhibition, which accounted for the lack of AChE inhibition in the standardised ABR test.

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