



## Inhibition, recovery and oxime-induced reactivation of muscle esterases following chlorpyrifos exposure in the earthworm *Lumbricus terrestris*

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*Esterase inhibition combined with oxime reactivation methods is a suitable approach for monitoring organophosphate contamination*

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### ABSTRACT

Assessment of wildlife exposure to organophosphorus (OP) pesticides generally involves the measurement of cholinesterase (ChE) inhibition, and complementary biomarkers (or related endpoints) are rarely included. Herein, we investigated the time course inhibition and recovery of ChE and carboxylesterase (CE) activities in the earthworm *Lumbricus terrestris* exposed to chlorpyrifos, and the ability of oximes to reactivate the phosphorylated ChE activity. Results indicated that these esterase activities are a suitable multibiomarker scheme for monitoring OP exposure due to their high sensitivity to OP inhibition and slow recovery to full activity levels following pesticide exposure. Moreover, oximes reactivated the inhibited ChE activity of the earthworms exposed to 12 and 48 mg kg<sup>-1</sup> chlorpyrifos during the first week following pesticide exposure. This methodology is useful for providing evidence for OP-mediated ChE inhibition in individuals with a short history of OP exposure ( $\leq 1$  week); resulting a valuable approach for assessing multiple OP exposure episodes in the field.

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### 1. Introduction

Determination of cholinesterase (ChE) inhibition is an important sublethal endpoint in the exposure and effect assessment of organophosphorus (OP) and carbamate (CB) pesticide contamination. Many studies have demonstrated the utility of this biomarker in identifying OP-exposed organisms in the field (Fulton and Key, 2001; Galloway et al., 2002). However, the knowledge of ChE-OP interactions is still scarce and fragmentary in earthworms. In the past, some laboratory investigations characterized enzymatically the ChE activity in *Eisenia fetida* (Stenersen, 1980) and *Allolobophora caliginosa* (Principato et al., 1978), and they described multiple forms or different ChEs in those earthworm species. More recently, the substrate specificity and inhibition selectivity of ChEs were investigated in several earthworm species (Caselli et al., 2006; Rault et al., 2007), and some studies have reported a slow recovery rate of phosphorylated ChE activity (Aamodt et al., 2007; Rault et al., 2008), which is in agreement with previous investigations (Mikalsen et al., 1982).

Carboxylesterases (CEs) are another group of esterases that are used as biomarkers of pesticide exposure (Wheelock et al., 2008).

Interaction of CE with OPs yields a stable enzyme-inhibitor complex which is considered a stoichiometric mechanism of OP inactivation (Chambers et al., 1990). Carboxylesterases are also key enzymes in the hydrolytic breakdown of pyrethroid and CB insecticides (Crow et al., 2007; Wheelock et al., 2008). As with earthworm ChEs, only a few studies have identified and described the CE activity in these invertebrates (Haïtes et al., 1972; Øien and Stenersen, 1984; Sanchez-Hernandez and Wheelock, 2009), and to the best of our knowledge, no studies have examined the in vivo response of earthworm CE activity to OP exposure.

Comparison of ChE activity levels between OP- and non-exposed individuals in the field often requires a significant sampling effort in order to identify individuals with ChE activity inhibited by OPs. These observations are at least partially explained by the high interindividual variation in basal ChE activity (Walker, 1995). This situation is further complicated by the fact that it is not always possible to distinguish between OP- and CB-exposed individuals, and chemical analysis of pesticide residues in the body or carcass can be challenging due to rapid dissipation of pesticide, i.e., metabolism and irreversible inhibition of esterases. The use of oxime-induced reactivation of phosphorylated ChE activity has been used to resolve some of these limitations (Martin et al., 1981). Some field studies with birds and reptiles have shown

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the advantages of these ChE-reactivator agents in the assessment of OP pesticide impact on wildlife (Parsons et al., 2000; Sanchez-Hernandez et al., 2004; Maul and Farris, 2005; Fildes et al., 2006). Likewise, an in vitro study showed that reactivation of chlorpyrifos-oxon-inhibited ChE using the oxime pyridine-2-aldoxime methochloride (2-PAM) was a workable methodology in earthworms during the first week following acute exposure to chlorpyrifos (Rodriguez and Sanchez-Hernandez, 2007).

This study was designed to examine the inhibition and recovery of muscle ChE and CE activities in *Lumbricus terrestris* after exposure to Dursban 5G (chlorpyrifos) towards the aim of developing an appropriate biomarker system of OP exposure to be used in field monitoring of OP contamination. We focused on a single tissue because 1) ChE and CE activities in earthworms vary widely between tissues and 2) esterase sensitivity to OP inhibition is tissue-dependent (Rault et al., 2007; Sanchez-Hernandez and Wheelock, 2009). Moreover, wall muscle was selected as target tissue for esterase measurements because earthworm skin represents a potential route for pesticide uptake (Jager et al., 2003). A second aim was to validate the use of oxime-induced reactivation of phosphorylated earthworm ChE as a complementary methodology in the assessment of OP exposure.

## 2. Materials and methods

### 2.1. Reagents and stock solutions

Acetylthiocholine iodide (AcSch), 5,5'-dithiobis-2-nitrobenzoic acid (DTNB),  $\alpha$ -naphthyl acetate ( $\alpha$ -NA), 4-nitrophenyl acetate (4-NPA), 4-nitrophenyl valerate (4-NPV) and *p*-nitrophenol were purchased from Sigma–Aldrich (Madrid, Spain). Pyridine-2-aldoxime methochloride (2-PAM), obidoxime chloride (OBx) were supplied by Scharlab (Barcelona, Spain). Dursban 5G (5% w/w of chlorpyrifos, granulated formulation) was obtained from Compo Agricultura S.L. (Barcelona, Spain). The substrates 4-NPA and 4-NPV were prepared initially at 50 mM in dimethyl sulfoxide (DMSO), whereas  $\alpha$ -NA was dissolved in acetone to a concentration of 1.85 mM. Stock aqueous solutions of oximes were prepared at a concentration of 100 mM immediately prior to use.

### 2.2. Exposure to chlorpyrifos-spiked soils

In vivo experiments were performed using a field non-contaminated soil (<4 mm particle size). This soil was slightly alkaline (pH = 7.64  $\pm$  0.08), non-saline (EC = 249  $\pm$  22.7  $\mu$ S cm<sup>-1</sup>), contained 2.26  $\pm$  0.24% organic carbon, and 18% w/v moisture in the moment of collection (physicochemical properties were determined in five subsamples previously dried at 105 °C for 48 h). Concentrations of chlorpyrifos were chosen based upon the predicted environmental concentration (PEC) of 3.3 mg a.i. kg<sup>-1</sup> dry soil, which was calculated according to a recommended application rate of 5 g of Dursban 5G/m<sup>2</sup> (2500 g a.i. ha<sup>-1</sup>), a depth soil layer of 5 cm of pesticide penetration, no crop interception and a bulk soil density of 1.5 g/cm<sup>3</sup> (Rault et al., 2008). Chlorpyrifos concentrations in the test soils were equal to PEC (3 mg a.i. kg<sup>-1</sup> dw), 4  $\times$  PEC (12 mg a.i. kg<sup>-1</sup> dw) and 16  $\times$  PEC (48 mg a.i. kg<sup>-1</sup> dw). We selected chlorpyrifos concentrations higher than the PEC based upon the LC50 for *L. terrestris* (458 mg a.i. kg<sup>-1</sup> of soil dw; Ma and Bodt, 1993) and previous studies on sublethal effects of chlorpyrifos on earthworm ChE inhibition and recovery (Supplementary Table 1). Soil samples containing chlorpyrifos were mixed thoroughly using plastic containers to ensure uniform distribution of pesticide granules in the bulk soil. After pesticide spiking, soil moisture was adjusted to 25% w/v, which was maintained throughout the experiment. Three replicates (1.2 kg soil, wet weight) of each test concentration, including controls, were prepared and placed in plastic containers (14.5  $\times$  14  $\times$  12 cm) and left for 48 h at 12 °C and dark to equilibrate prior to addition of the earthworms.

Earthworms (*L. terrestris*) were purchased from a local supplier (Armeria20, Toledo, Spain), who imported them from a commercial vermiculture supplier (Vivastic, Elsenheim, France). In the laboratory, they were acclimatized in uncontaminated soil identical to that used in the experiments, in the dark for two weeks at 12 °C.

Mature worms were selected for the test (3.73  $\pm$  0.87 g fresh weight,  $n$  = 144 earthworms), and they were placed in petri dishes (14.5 mm diameter) containing moist filter paper, and kept at 12 °C for 24 h for voiding of their gut. Groups of 12 individuals were released in the testing containers and maintained for two days at 12 °C and dark. Subsequently, control and exposed earthworms were transferred to clean soils (1.2 kg fresh soil each replicate) and kept for 35 d under the same experimental conditions as for OP exposure period. Two earthworms were randomly collected from each test container at 0 (immediately prior to release in

clean soil), and 2, 4, 8, 18 and 35 d after transferring earthworms to clean soil. The frequency of sampling was selected to examine the potency of oximes to reverse OP-inhibited ChE activity, which was believed to primarily occur during the initial week following OP exposure (Rodriguez and Sanchez-Hernandez, 2007).

### 2.3. Tissue homogenization

Earthworms were cooled (4 °C) for 10–15 min to allow easy dissection because of muscle relaxing. Animals were sacrificed by the dissection process, which was performed on the dorsal side from clitellum towards the mouth. Wall muscle samples (0.45 to 0.66 g wet weight) were dissected and washed to remove soil particles with an isotonic buffer balanced for *L. terrestris* (Stein and Cooper, 1981) containing (mM): 71.5 NaCl, 4.8 KCl, 3.8 CaCl<sub>2</sub>, 1.1 MgSO<sub>4</sub>, 0.4 NaH<sub>2</sub>PO<sub>4</sub>, 0.4 Na<sub>2</sub>HPO<sub>4</sub>, 4.2 NaHCO<sub>3</sub>. Muscle samples were then immersed in liquid nitrogen and stored frozen at –80 °C until use in esterase assays. Weighted samples were added (1:10 w/v ratio) to ice-cold 25 mM Tris–HCl buffer (pH = 8.0) containing 0.1% Triton X-100. Samples were homogenized at 4 °C using a glass-Teflon Potter–Elvehjem homogenizer. The homogenate was then centrifuged at 9000  $\times$  g at 4 °C for 10 min for the postmitochondrial fraction (range of total proteins = 4.4–8.7 mg ml<sup>-1</sup>). We also dissected gut and reproductive tissues for time-course dynamic of CE activities. Results with these tissues have been already reported in a related paper (González Vejares et al., 2010).

### 2.4. Esterase activities

Cholinesterase activity was determined according to the Ellman method (Ellman et al., 1961). The reaction medium included 25 mM Tris–HCl (pH 7.6), 0.3 mM DTNB, 2 mM AcSch and 10  $\mu$ l of diluted supernatant (35–70  $\mu$ g protein). Kinetics were recorded at 412 nm for 1 min at 25 °C, and specific ChE activity was calculated using a molar absorption coefficient of 14.15  $\times$  10<sup>3</sup> M<sup>-1</sup> cm<sup>-1</sup> (Eyer et al., 2003).

Carboxylesterase activity was assayed using three substrates,  $\alpha$ -NA, 4-NPA and 4-NPV. Hydrolysis of  $\alpha$ -NA ( $\alpha$ -NA-CE activity) was determined according to Gomori (1953), as adapted by Bunyan and Jennings (1968). The reaction medium contained 25 mM Tris–HCl (pH 7.6), the sample (17–35  $\mu$ g protein) and 46  $\mu$ M  $\alpha$ -NA. The formation of naphthol was stopped after 10 min of incubation at 25 °C by the addition of 500  $\mu$ l 2.5% SDS and subsequently 500  $\mu$ l 0.1% Fast Red ITR in 2.5% Triton X-100 in water. The absorbance of the naphthol–Fast Red ITR complex (molar extinction coefficient of 33.225  $\times$  10<sup>3</sup> M<sup>-1</sup> cm<sup>-1</sup>) was read at 530 nm after 30 min at 22 °C in the dark. Hydrolysis of 4-NPV by CE (4-NPV-CE activity) was determined as described by Carr and Chambers (1991). Samples (17–35  $\mu$ g protein) were pre-incubated in 50 mM Tris–HCl (pH 7.4) for 5 min at 25 °C, and the reaction was initiated by the addition of 4-NPV (5  $\times$  10<sup>-4</sup> M). The reaction was stopped after 15 min by the addition of a solution containing 2% (w/v) SDS and 2% (w/v) Tris base. The 4-nitrophenolate ion liberated was read at 405 nm and quantified by a calibration curve (5–100  $\mu$ M). Carboxylesterase activity using 4-NPA (4-NPA-CE) was determined by the continuous spectrophotometric assay described by Chanda et al. (1997). The incubation mixture contained 20 mM Tris–HCl (pH 8.0), 1 mM EDTA to avoid hydrolysis by Ca<sup>2+</sup>-dependent phosphotriesterases (Vilanova and Sogorb, 1999), and the sample (5–10  $\mu$ g protein). The reaction was initiated by the addition of 4-NPA (5  $\times$  10<sup>-4</sup> M), and the formation of 4-nitrophenolate ion was monitored for 1 min at 405 nm and quantified using the 4-nitrophenol standard curve.

Enzyme activities were determined using a Jenway 6400 spectrophotometer (Barloworld Scientific, Essex, UK). Specific activity was expressed as U mg<sup>-1</sup> of total protein (1 Unit = 1  $\mu$ mol of substrate hydrolyzed per minute under the experimental conditions for each esterase). Continuous kinetic assays were supported with blanks (reaction mixture free of sample) to check for non-enzymatic hydrolysis of the substrates and no significant hydrolysis was observed. Total protein concentration was quantitated by the Bradford method (Bradford, 1976) with bovine serum albumin as the standard protein.

### 2.5. Chemical reactivation of phosphorylated ChE

Reactivation of phosphorylated ChE activity was performed as described by Rodriguez and Sanchez-Hernandez (2007). Three aliquots of the muscle homogenate corresponding to each sample were used for testing reactivation using 2-PAM and OBx. Two of them were incubated separately with 5  $\times$  10<sup>-4</sup> M of 2-PAM or OBx for 60 min at 25 °C, whereas the third aliquot was diluted with dH<sub>2</sub>O (dilution factor = 1/5) as those spiked with oximes to serve as a control.

### 2.6. Data analysis

A general linear model was applied to examine the impact of both the chlorpyrifos concentration and the time of recovery on esterase activities (logarithmically transformed data). The individual contribution of each independent biomarker to the global response was tested by univariate analyses of variance (ANOVA) followed by the LSD post-hoc test. Discriminant analysis was performed to differentiate between the chlorpyrifos treatments according to the esterase responses, and further to examine the significant contribution of each esterase activity to discriminate between chlorpyrifos treatments during the recovery period. The

impact of both oximes on OP-inhibited ChE activity was evaluated by comparison of the activity means (log transformed data) before and after oxime treatment ( $t$ -test for independent variables). However, we judged inhibition of the ChE activity in single samples when the percentage of ChE increase after oxime treatment was higher than 10% respective to the corresponding controls (the interassay coefficient of variation of nontreated samples was < 7.6%). The latter criterion enabled to investigate the *in vivo* aging kinetics of the chlorpyrifos-inhibited ChE activity by plotting the percentage of oxime-induced ChE reactivation against time (days). This relationship was fitted to the non-linear regression model  $y = ae^{-bx}$ , where the coefficient  $a$  corresponds to the maximal ChE activity, at  $t = 0$  d expressed as the percentage of increase of the enzyme activity after oxime treatment and the coefficient  $b$  is the observed aging rate constant ( $k_a$ ), expressed as  $\text{day}^{-1}$ . The capability of both oximes to reactivate the phosphorylated ChE activity was also evaluated by the median aging time (AT50). This parameter was defined as the time elapsed since OP exposure (assuming maximal ChE inhibition at the end of the OP exposure period) at which the oxime caused a 50% of activity increase over the maximal percentage of oxime-induced reactivation, i.e., immediately after chlorpyrifos exposure. Statistic tests were performed using Statistica software (version 6.0, StatSoft, Inc., Tulsa, OK, USA), whereas graphs and non-linear fitting were carried out using SigmaPlot software (v. 9.0, Systat Software, Point Richmond, CA, USA).

### 3. Results

#### 3.1. Cholinesterase inhibition and recovery

Specific ChE activity in the control groups varied from  $363 \pm 47.5$  to  $512 \pm 144$   $\text{mU mg}^{-1}$  protein (mean  $\pm$  SD), with coefficients of variation within the experimental groups ranging from 13 to 39% (Fig. 1, supplementary Table 2). No significant difference was detected between the control groups at different sampling times ( $F_{1,5} = 0.75$ ,  $P = 0.59$ ). Chlorpyrifos exposure exhibited a dose-response effect on ChE activity ( $F_{1,3} = 70.37$ ,  $P < 0.00001$ ), with maximum ChE inhibition observed in the group exposed to  $48 \text{ mg kg}^{-1}$  (Fig. 1). This relationship was consistent throughout the entire 35 d recovery period ( $F_{1,5} = 4.71$ ,  $P < 0.001$ ). No full recovery of ChE activity was observed after 35 d following exposure to 12 and  $48 \text{ mg kg}^{-1}$  dw chlorpyrifos (Fig. 1). Percentages of ChE

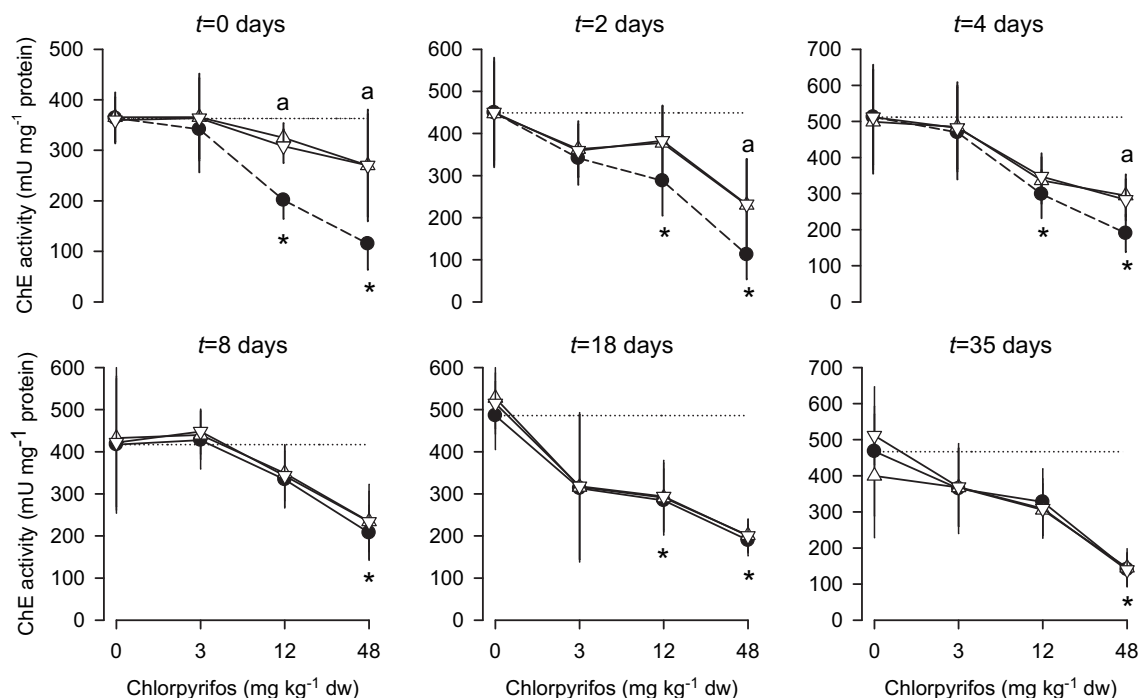
inhibition in these earthworm groups varied from 45% to 68% at  $t = 0$  d, whereas they ranged from 30% to 70% at the end of the recovery period.

#### 3.2. Chemical reactivation of phosphorylated ChE

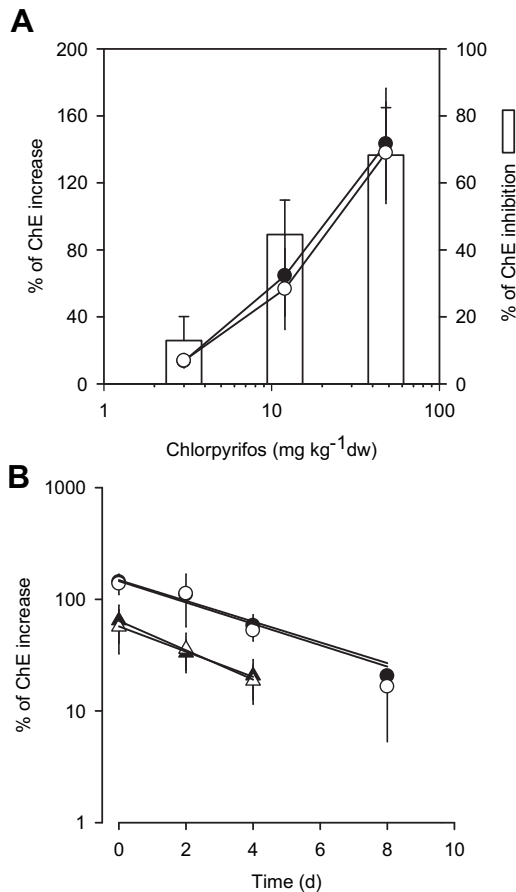
Muscle extracts of earthworms exposed to the OP-spiked soils were incubated in the presence of  $5 \times 10^{-4}$  M 2-PAM or OBx for reactivation of the phosphorylated ChE activity. All samples corresponding to the 12 and  $48 \text{ mg kg}^{-1}$  groups exhibited a ChE increase higher than 20% during the first 4 d after transferring earthworms to clean soil (Fig. 1). No apparent differences were found in the reactivation potency between both oximes. There was a linear relationship between the capability of oximes to reactivate the phosphorylated ChE and the percentage of inhibited ChE at  $t = 0$  d (Fig. 2A), however such a relationship was lacked when time after OP exposure increased. This decrease in the oxime efficiency followed the exponential decay model  $y = ae^{-bx}$  (Fig. 2B). No significant chemical reactivation of ChE activity was observed in any OP-exposed groups following 10 d of the recovery period (Fig. 1). The time-course of chemical reactivation of OP-inhibited ChE activity enabled the assessment of the *in vivo* ChE aging. Chlorpyrifos-inhibited ChE activity aged with an observed  $k_a$  ranging between 0.21 and  $0.30 \text{ day}^{-1}$  (Table 1). The AT50s for both 2-PAM and OBx were the same in the earthworm group exposed to the highest chlorpyrifos concentration ( $\sim 7$  d), whereas they were lower in the group exposed to  $12 \text{ mg kg}^{-1}$  (AT50 < 3.0 d).

#### 3.3. Carboxylesterase inhibition and recovery

The dynamics of muscle CE inhibition and recovery in *L. terrestris* following chlorpyrifos exposure is shown in Fig. 3. Inter-individual variation of CE activity in non-exposed earthworms ranged from 12 to 43% for  $\alpha$ -NA-CE, from 23 to 62% for 4-NPA-CE and from



**Fig. 1.** Time-course inhibition (black-circle plots) and oxime-induced reactivation (white-symbol plots) of muscle cholinesterase (ChE) activity in *Lumbricus terrestris* after two days of exposure to chlorpyrifos-spiked soils (0, 3, 12 and  $48 \text{ mg kg}^{-1}$  dw). Horizontal dotted lines show the average ChE activity of the control groups. Symbols are the mean and standard deviation of six earthworms. Asterisks denote significant difference of the chlorpyrifos-exposed earthworms compared to the controls (post-hoc LDS test,  $P < 0.05$ ), whereas the letter "a" indicates significant increase of the mean ChE activity in the oxime-treated groups compared to that of the corresponding controls ( $t$ -test,  $P < 0.05$ ).



**Fig. 2.** Reactivation of phosphorylated cholinesterase (ChE) activity by pralidoxime (2-PAM) and obidoxime (OBx). A) Relationship between the potency of 2-PAM or OBx to reverse the ChE activity (black and white circles) and the percentage of ChE inhibition in the earthworm group exposed to 48 mg kg<sup>-1</sup> dw of chlorpyrifos (bars). B) Effect of the time elapsed since chlorpyrifos exposure on the efficiency of oximes to reverse phosphorylated ChE activity. Kinetics were fitted to the nonlinear model  $y = ae^{-bx}$  (statistic parameters are summarized in Table 1).

16 to 50% for 4-NPV-CE. No significant differences in esterase activities were detected between the control groups at the multiple sampling times for  $\alpha$ -NA-CE ( $F_{1,5} = 1.64$ ,  $P = 0.17$ ), 4-NPA-CE ( $F_{1,5} = 0.29$ ,  $P = 0.91$ ) or 4-NPV-CE ( $F_{1,5} = 1.29$ ,  $P = 0.29$ ). However, the inhibitory effect of chlorpyrifos on CE activity was strongly dependent on the substrate used. Although the pesticide treatment had a significant effect on CE activity using  $\alpha$ -NA ( $F_{1,3} = 15.73$ ,  $P < 0.00001$ ) or 4-NPA ( $F_{1,3} = 7.63$ ,  $P < 0.001$ ), no concentration-

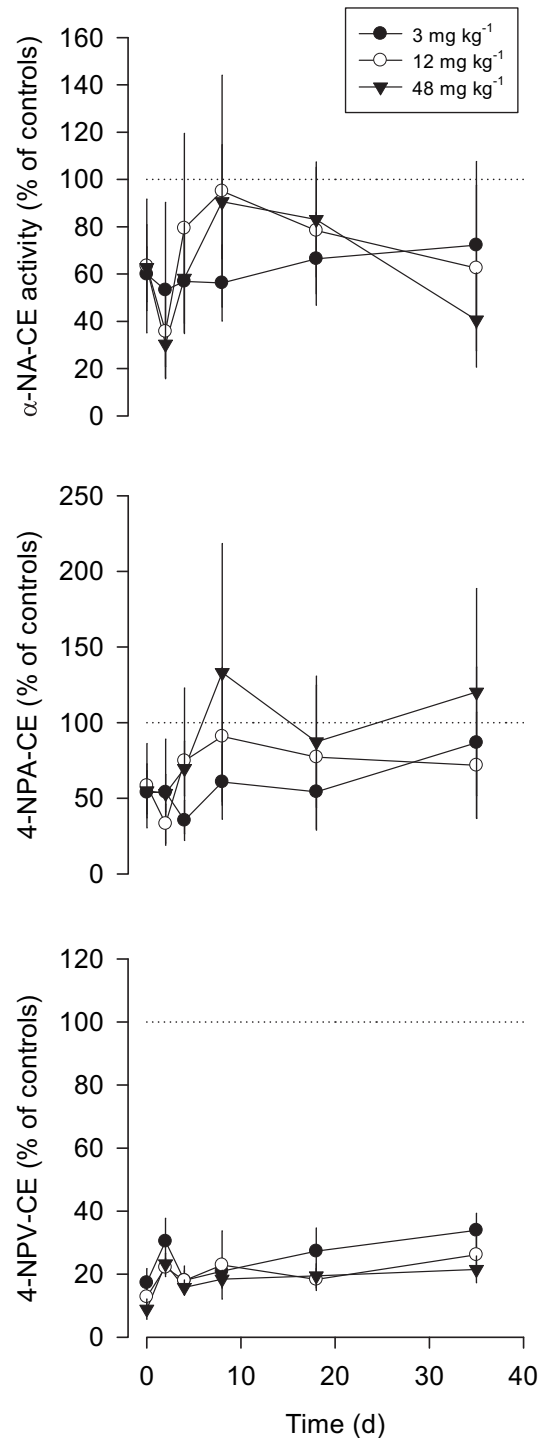
**Table 1**  
Statistics for non-linear regression of cholinesterase (ChE) aging kinetics as a function of the time elapsed since exposure to chlorpyrifos-spiked soils.

Oxime	Treatment (mg kg <sup>-1</sup> dw)	Regression statistics <sup>a</sup>				AT50 (d) <sup>b</sup>
		a	b	r	P	
Pralidoxime	12	117 ± 27.3	0.30 ± 0.08	0.78	0.0004	2.8
	48	224 ± 36.5	0.21 ± 0.04	0.79	<0.0001	7.2
Obidoxime	12	95.8 ± 22.5	0.25 ± 0.07	0.7	0.0018	2.5
	48	223 ± 37.6	0.21 ± 0.05	0.79	<0.0001	6.9

<sup>a</sup> Statistics obtained from data in Fig. 2B, which were fitted to the non-linear regression model  $y = ae^{-bx}$ . Coefficients are the mean ± standard error. The coefficient “b” is the observed aging rate constant ( $k_a$ ).

<sup>b</sup> AT50 = median aging time expressed in days (i.e., the time elapsed since cholinesterase inhibition at which oxime [ $5 \times 10^{-4}$  M] caused a 50% of increase in the ChE activity).

response relationship was observed (supplementary Table 2). Indeed, no significant inhibition of 4-NPA-CE activity was observed in the OP-exposed groups after 4 d of the recovery period. Similarly, inhibition of  $\alpha$ -NA-CE activity was sporadically observed in the exposed groups over the recovery period (Fig. 3). Nevertheless, chlorpyrifos had a marked inhibitory effect on 4-NPV-CE activity of



**Fig. 3.** Recovery of muscle carboxylesterase (CE) activities in earthworms following two days of chlorpyrifos exposure. The symbols are the mean ( $\pm$  SD) percentages of residual esterase activity respect to the mean activity of the corresponding controls, which was set to 100% (horizontal dotted line). Esterase activity was measured using the substrates  $\alpha$ -naphthyl acetate ( $\alpha$ -NA), 4-nitrophenyl acetate (4-NPA) and 4-nitrophenyl valerate (4-NPV).

all treated groups ( $F_{1,3} = 262.3$ ,  $P < 0.00001$ ), which remained strongly inhibited over the recovery period (Fig. 3).

We also examined whether ChE and CE activities were able to differentiate exposure to chlorpyrifos in *L. terrestris*. Considering all esterase responses and analyzing all sampling periods (0–35 d), it was difficult to differentiate between the three levels of chlorpyrifos exposure, although the global esterase response discriminated between controls and treated groups (Wilk's  $\lambda = 0.087$ ,  $F_{12,344} = 43.2$ ,  $P < 0.00001$ ) (Fig. 4). However, when the contribution of esterase responses to differentiating chlorpyrifos contamination scenarios was examined at each sampling period, the response of ChE and 4-NPV-CE activities enabled us to distinguish between chlorpyrifos treatments (supplementary Table 3). After 8 d of recovery period however, no distinction could be established between levels of chlorpyrifos exposure based on ChE inhibitory response, whereas 4-NPV-CE activity was a more potent discriminating factor throughout the full recovery period.

## 4. Discussion

### 4.1. Inhibition and recovery of B-esterases

Earthworm ChE activity often exhibits a slow recovery rate to normal levels following inhibition by OP pesticides (Aamodt et al., 2007; Rault et al., 2008), which was supported by the observations in the current study. Muscle ChE activity of *L. terrestris* remained depressed during the 35 d recovery period in the group exposed to the highest concentration of chlorpyrifos ( $48 \text{ mg kg}^{-1} \text{ dw}$ ). Similarly no sign of recovery was observed for the 4-NPV-CE activity in the three treated groups. This slow or absent recovery makes earthworm esterases suitable biomarkers for field monitoring because OP-exposed individuals could be detected several weeks following pesticide application. The most plausible explanation for this slow recovery of OP-inhibited esterase activities is the low rate of synthesis of new enzyme because spontaneous reactivation of the phosphorylated enzyme is considered negligible (Rodriguez and Sanchez-Hernandez, 2007). Alternatively, the continued presence of toxicologically active pesticide residues in the earthworm fluids and tissues could account for a slow recovery of ChE activity. We found inhibition of V-S type AChE activity from electric eel when was incubated in the presence of filtered muscle extracts of

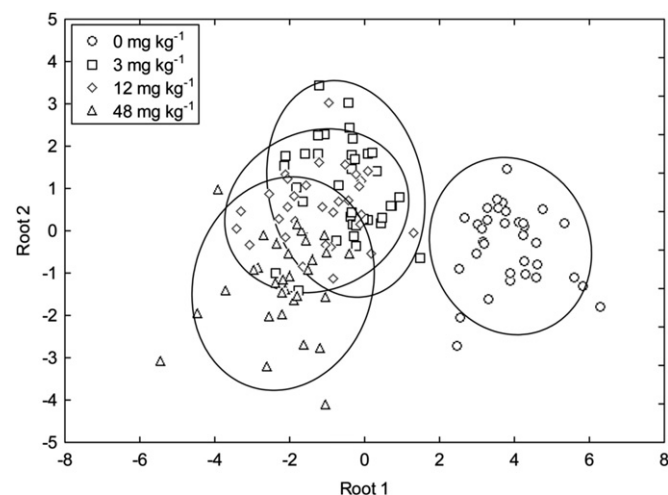


Fig. 4. Representation of the chlorpyrifos treatments (0, 3, 12 and  $48 \text{ mg kg}^{-1} \text{ dw}$ ) in the first factorial discriminant analysis plane considering the response of all esterase activities of *Lumbricus terrestris* following exposure to chlorpyrifos-spiked soils. Ellipses represent 90% confidence ranges.

*L. terrestris*, which suggested that free active chlorpyrifos-oxon was still present in the earthworm tissues (supplementary methodological issues). Moreover, we used a granular formulation of chlorpyrifos, and contaminated soil or particles from the pesticide formulation could still be present in the earthworm gut lumen during the recovery period. In consequence, these factors could contribute to further esterase inhibition or perturbing the normal reestablishment of enzyme activity.

Most of the field studies with earthworms have used ChE inhibition solely to assess OP exposure (Booth et al., 2000; Reinecke and Reinecke, 2007; Denoyelle et al., 2007). Although these studies reported inhibition of ChE activity in earthworms exposed to environmentally realistic pesticide concentrations, the high inter-individual variability of ChE activity in non-exposed individuals suggests that there is a need to include new biomarkers to more accurately evaluate the impact of anti-ChE pesticides on wildlife. Inhibition of CE activity by OPs appears to be a promising biomarker of pesticide exposure. A comprehensive examination on the interaction of CE activity with anti-ChE and synthetic pyrethroid agrochemicals in a wide range of species has been recently published by Wheelock et al. (2008). In that work, numerous cases were cited in which CE inhibition served as a more sensitive biomarker of exposure to anti-ChE pesticides than the inhibition of ChE activity, which is further supported by the results of the current study. However, CE activity of *L. terrestris* also displays a high interindividual variability. The presence of multiple CE isozymes could account for this high variation of normal CE activity (Sanchez-Hernandez and Wheelock, 2009). The current state of the literature on CEs involves the use of colorimetric substrates that are inexpensive, do not require high-grade optics and are readily available (e.g.,  $\alpha$ -NA or 4-NPA). However, it has been repeatedly demonstrated that results from these studies cannot be extrapolated to other substrates of interest. For example, correlation profiles performed on 4-NPA and pyrethroid hydrolysis activity in human liver microsomes showed little correlation between the hydrolytic profiles ( $r^2 = 0.29$  for a fenvalerate analog), suggesting that different isozymes are involved in the hydrolysis of the two substrates (Wheelock et al., 2003). Other studies have shown that the IC50 of diazinon-oxon and chlorpyrifos-oxon varied with the substrate used in the assay (Wheelock et al., 2005). Accordingly, the results in this study regarding the observed substrate specificities of chlorpyrifos-mediated CE inhibition have a strong precedent in the literature. Of particular interest is the observation that 4-NPV was a more sensitive reporter of OP-mediated inhibition of CE activity than 4-NPA or  $\alpha$ -NA, an observation in line with previous in vitro data (Sanchez-Hernandez and Wheelock, 2009). Current results suggest that this particular chlorpyrifos-sensitive CE isozyme(s) prefers substrates with greater lipophilicity (i.e. longer alkyl chain). Carboxylesterase outcomes suggest that biomarker studies should employ a battery of CE substrates that represent a range of physiochemical properties because the isozyme profiles and substrate specificities are unknown in most environmental indicator organisms.

We used chlorpyrifos concentrations varying between the PEC value and  $16 \times \text{PEC}$ ; a range of concentrations in the same order of magnitude than that ( $0.3$  and  $32 \text{ mg kg}^{-1} \text{ dw}$ ) found in soils after agricultural applications (Racke, 1993; Murray et al., 2001). In addition, the half-life of chlorpyrifos varies between 1 and 2 weeks when applied to the soil surface or between 4–8 weeks for soil-incorporated applications (Racke, 1993). These field half-lives can even increase when chlorpyrifos is applied at extremely high concentrations ( $1000 \text{ mg kg}^{-1} \text{ dw}$ ), for example as a termiticide (Racke, 1993; Murray et al., 2001), or after consecutive agricultural applications. In consequence, we believed that inhibition and recovery responses of esterases in our earthworms could be

reproduced in the field because agricultural applications of this OP can lead to residue levels in soil comparable to those used in this study.

#### 4.2. Oxime-induced reactivation of ChE activity

In the last decade, oximes have become a complementary tool in the assessment of wildlife exposure to OP pesticides. Significant 2-PAM-induced reactivation of plasma ChE activity has been reported in birds (Parsons et al., 2000; Maul and Farris, 2005; Fildes et al., 2006) and reptiles (Sanchez-Hernandez et al., 2004) exposed to OP pesticides. In these field studies, low levels of plasma ChE activity that were not statistically different from the controls still evidenced a significant increase of enzyme activity after 2-PAM treatment. One of the main advantages of this methodology is its high sensitivity and specificity to detect individuals with OP-mediated ChE inhibition. In invertebrates, this index of OP exposure has been used with marine and freshwater crabs (Monserrat and Bianchini, 2000; Vioque-Fernández et al., 2007), mussels (Escartín and Porte, 1997; Binelli et al., 2006) and terrestrial snails (Laguette et al., 2009). However, sometimes no reactivation of OP-inhibited ChE activity is observed. This phenomenon can be most likely explained by the dealkylation reaction of the ChE-OP complex, leading to ChE aging (Blake, 2004). In this situation, reactivation of the phosphorylated ChE activity is not possible either spontaneously or chemically. In a previous in vitro study, we examined the efficiency of 2-PAM to reactivate phosphorylated ChE activity from *E. fetida* and *L. terrestris* (Rodríguez and Sanchez-Hernandez, 2007). We found that 2-PAM was able to reactivate the OP-inhibited ChE of both species, although the reactivation potency of the oxime decreased as the time increased since OP exposure. The lack of reactivation potency of oximes with the time elapsed since OP exposure is probably due to ChE aging. The in vitro AT50s of chlorpyrifos-oxon-inhibited ChE activity were 8 d for *L. terrestris* and 2.8 d for *E. fetida*. Current results reproduced these in vitro observations with *L. terrestris*. We found that both oximes were able to reactivate the phosphorylated ChE activity during the first week following chlorpyrifos exposure, and the in vivo AT50 value was 7 d for the group exposed to the highest chlorpyrifos concentration.

Some field studies have shown that the ChE activity of *Aporrectodea caliginosa* (Booth et al., 2000; Reinecke and Reinecke, 2007) and *A. chlorotica* (Denoyelle et al., 2007) shows a high interindividual variation, which could cause difficulties in the identification of earthworms with ChE inhibited by OPs. We have reported a substantial variability in both ChE and CE activities in non-exposed *L. terrestris* which indicates that multiple biological endpoints should be applied when assessing agrochemical exposure in these soil organisms. In addition, the use of chemical reactivation techniques with oximes is a complementary methodology to recognize OP-exposed individuals, at least, those with a short ( $\leq 1$  week) history of acute OP exposure. Hence, this methodology would enable the detection of multiple episodes of OP acute exposure during field biomonitoring.

## 5. Conclusions

Muscle ChE activity of *L. terrestris* showed a slow recovery to full normal activity after acute exposure to chlorpyrifos-spiked soils, and ChE activity remained strongly inhibited in the group exposed to the highest chlorpyrifos concentration ( $48 \text{ mg kg}^{-1}$ ). Chemical reactivation of phosphorylated ChE activity using the oximes 2-PAM or OBx appears to be a workable methodology for identifying OP-exposed earthworms in the field, although its effectiveness decreases with time following acute OP exposure. Although CE

activity was also inhibited by chlorpyrifos, a concentration-response curve was not observed as for the ChE response. The use of 4-NPV as a reporter of activity evidenced a more sensitive and extended response of CE activity. Based upon these results, it would be advisable to use both ChE and CE activities, in combination with oxime reactivation methods, to assess OP exposure in earthworms sampled in the agroecosystem. Moreover, such a strategy could be appropriate in soil toxicity testing when earthworm biomarkers are included as either indirect measurements of pesticide bioavailability or sublethal endpoints.

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## Appendix. Supplementary information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.envpol.2010.02.009.

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