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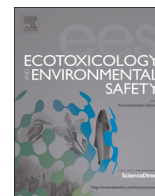
Ecotoxicology

Earthworm-induced carboxylesterase activity in soil: Assessing the potential for detoxification and monitoring organophosphorus pesticides

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A B S T R A C T

Soil enzyme activities are attracting widespread interest due to its potential use in contaminant breakdown, and as indicators of soil deterioration. However, given the multiple environmental and methodological factors affecting their activity levels, assessment of soil pollution using these biochemical endpoints is still complex. Taking advantage of the well-known stimulatory effect of earthworms on soil microbes, and their associated enzyme activities, we explored some toxicological features of carboxylesterases (CbEs) in soils inoculated with *Lumbricus terrestris*. A microplate-scale spectrophotometric assay using soil–water suspensions was first optimized, in which kinetic assay parameters (K_m, V_{max}, dilution of soil homogenate, and duration of soil homogenization) were established for further CbE determinations. Optimal conditions included a soil-to-water ratio of 1:50 (w/v), 30-min of shaking, and 2.5 mM of substrate concentration. As expected, CbE activity increased significantly in soils treated with *L. terrestris*. This bioturbed soil was used for exploring the role of CbE activity as a bioscavenger for organophosphorus (OP) pesticides. Soil treated with two formulations of chlorpyrifos revealed that CbE activity was a significant molecular sink for this pesticide, reducing its impact on soil microbial activity as shown by the unchanged dehydrogenase activity. Dose-dependent curves were adjusted to an exponential kinetic model, and the median ecological dose (ED_{50}) for both pesticide formulations was calculated. ED_{50} values decreased as the time of pesticide exposure increased (14d-ED_{50}s $=20.4$–26.7 mg kg^{-1}, and 28d-ED_{50}s$=1.8$–2.3 mg kg^{-1}), which suggested that chlorpyrifos was progressively transformed into its highly toxic metabolite chlorpyrifos-oxon, but simultaneously was inactivated by CbEs. These results were confirmed by in vitro assays that showed chlorpyrifos-oxon was a more potent CbE inhibitor ($IC_{50}=35.5$–4.67 nM) than chlorpyrifos (0.41–0.84 μM). The results showed that earthworm-induced CbE activity is an efficient bioscavengers for OP pesticides, acting as a soil safeguarding system. Moreover, the simple dose-response curves against OP exposure suggest that this enzyme – combined with other enzyme activities (e.g., dehydrogenase) – may be a suitable biomarker of pesticide exposure.

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1. Introduction

There is a growing recognition that pesticides represent a serious threat to those soil components with a beneficial effect on crops such as microorganisms and related enzymes (Johnsen et al., 2001; Gianfreda and Rao, 2008; Hussain et al., 2009). For example, some studies have shown that dehydrogenase activity – an indicator of soil microbial activity (Moore and Russell, 1972) – is inhibited by organophosphorus (OP) pesticides (Pandey and Singh, 2006; Bishnu et al., 2011; Kadian et al., 2012; Riah et al., 2014; Marín-Benito et al., 2014); a class of agrochemicals commonly used in the agriculture (Abhilash and Singh, 2009; Zhang et al., 2011). Acute toxicity of OP pesticides is highly dependent on their oxon-analog metabolites, which display a toxic potency two-three orders of magnitude higher than their parent compounds (Chambers et al., 2010). This enhanced toxicity is explained by a greater affinity of the 'oxon' metabolites for the active site of serine hydrolases such as acetylcholinesterase (Casida and Quistad, 2005), i.e., a critical enzyme in the regulation of synaptic transmission at the nervous system (Fukuto, 1990; Lionetto et al., 2011). Carboxylesterases (CbEs) are another group of serine hydrolases with a high affinity for oxon metabolites of OPs. Indeed, the

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irreversible inhibition between CbE activity and OPs is considered a stoichiometric mechanism of detoxification in many organisms (Sogorb and Vilanova, 2002; Wheelock et al., 2008; Colovic et al., 2013). Thus, sensitivity of CbE activity to inhibition by OPs, and the number of CbE molecules able to bind OPs determine the efficacy of these esterases as a detoxification system (Maxwell, 1992; Chanda et al., 1997).

In soils, extracellular enzymes catalyse multiple biochemical processes that govern nutrient cycles, and microorganisms and plant roots are their main sources (Tabatabai and Dick, 2002). Many of these extracellular enzymes constitute a promising bio-technological strategy to degrade organic pollutants (Burns et al., 2013). Thus, oxidoreductases (e.g., laccases, lignin peroxidases and Mn-dependent peroxidases) and hydrolases (e.g., proteases, cellulases or chitinases) have gained a growing concern as efficient catalysts of environmental contaminants (reviewed in Gianfreda and Rao (2004)). Likewise, extracellular enzyme activities have been used as indicators of soil contamination, and the concept of "median ecological dose" (ED_{50}) arises as a quantitative measurement for this purpose. This parameter, defined by Babich et al. (1983) as "the concentration of a toxicant that inhibits a micro-organism-mediated ecological process by 50%", has been widely used to asses metal toxicity on soil enzyme activities and other microbial processes such as denitrification, ATP content or C mineralization (Speir et al., 1999a; Moreno et al., 2002; Hinojosa et al., 2008; Tejada et al., 2008; Gao et al., 2009). However, as far as we know, there are no studies on the use of this toxicological endpoint in pesticide-contaminated soils.

Past studies have documented the presence of CbE activity in the soil, although their potential interaction with OPs has been little investigated (Satyanarayana and Getzin, 1973; Cacco and Maggioni, 1976; Wittmann et al., 2004). More recently, some studies suggest that soil CbE activity may provide a detoxification system comparable to that described for animals, and earthworms (e.g., Lumbricus terrestris) could contribute to maximize this environmental service (Sanchez-Hernandez et al., 2014). In this study, L. terrestris was selected as a model organism to promote soil extracellular enzymes because it exerts a strong impact on soil structure and chemico-biological properties. This worm ingests soil to obtain nutrients and also feeds on litter and plant debris, dragging them into burrows, thus incorporating organic matter into the soil and creating hotspots of microbial activity (Jouquet et al., 2006; Griffith et al., 2013). It also produces casts (feces) and middens (small mounds of mineral soil, casts and organic material at the burrow entrance), which stimulate microbial proliferation at the soil surface (Edwards, 2004). Thus, feeding, burrowing and casting of L. terrestris indirectly stimulate soil enzyme activities associated with soil organic matter decomposition and nutrient cycling (Tao et al., 2009; Kizilkaya et al., 2010; Dempsey et al., 2013). Therefore, and taking into account the stimulatory effect of L. terrestris on soil microbial activity, a microcosm trial was performed to increase the understanding of soil CbEs as detoxifiying enzymes. The main aim of this study was to examine whether the enhanced CbE activity resulting from earthworm activity provides a molecular sink for OP pesticides, reducing their impact on soil microbial activity. Such an assessment was performed through the calculation of ED_{50} values and the measurement of dehydrogenase activity, the latter being very sensitive to OP exposure (Riah et al., 2014). Carboxylesterase assays were performed in soil–water suspensions using a microplate format protocol. Therefore, a second aim of this study was to optimize a micro-scale spectrophotometric assay, calculating the kinetic parameters K_m and V_{max} that guaranteed the measurement of enzyme activity at saturating conditions.

2. Materials and methods

2.1. Soil and earthworms

An agricultural soil collected from Montes de Toledo (Toledo, Spain) was used for the experimental trials. This soil type was also used in a previous study (Sanchez-Hernandez et al., 2014); it had a maximum water holding capacity (WHC) of 0.30 ± 0.03 g H_2O g^{-1} dry soil, and a particle size distribution as follows: 10.7% clay, 10.8% silt, 54.5% coarse sand and 23.7% fine sand. The physicochemical properties of soil sample (< 2 mm, $n=6$) used in this study were: pH$=7.17 \pm 0.05$, electrical conductivity (EC)$=169 \pm 43$ μS cm^{-1}, and total organic carbon (TOC)$=27.7 \pm 4.0$ g C kg^{-1}. Adult specimens of L. terrestris were purchased from a local supplier (Poisson Fenag, Madrid, Spain) and acclimatized for one month in temperature-controlled chambers (15 °C and darkness), using the same soil than that used in the microcosm trials. Maintenance of earthworms in the laboratory followed the recommendations by Lowe and Butt (2005).

2.2. Microcosm experiment

Groups of three earthworms (3.95 ± 0.75 g, mean $\pm$ SD, $n=18$) were released into 6 plastic containers ($14.5 \times 14 \times 12$ cm) each filled with 1 kg of wet soil (< 5 mm). Earthworms were weekly fed with 10 g of leaf (Morus alba) litter per container, which were added on the soil surface. Controls consisted of earthworm-free soils ($n=6$) and earthworm-free soils containing 10 g of leaf litter ($n=6$). In this latter group, leaf litter was mixed with the soil using a spoon. Soils were wetted at 50% of maximum WHC, and water loss was measured by periodically weighting the containers, and moisture was corrected by adding distilled water. All containers were kept in an acclimatized chamber (15 °C and dark) for 10 weeks. This incubation period was found as optimum in a previous study aimed to explore the impact of L. terrestris on soil CbE activity (Sanchez-Hernandez et al., 2014). After 10 weeks, the earthworms were removed and the soils were stored at -80 °C until biochemical analysis (Wallenius et al., 2010).

2.3. Sample preparation for enzyme assays

Enzyme activities were measured in both soil–water suspensions and buffered soil extracts. The former were prepared according to Popova and Deng (2010), with slight modifications: one gram of wet soil and 50 ml of distilled water were added to Falcon™ tubes, which were then shaken for 30 min at room temperature (≈ 20 °C) in an orbital shaker (Elmi® Intelli-mixer RM-2L, 50 rpm). Each tube was then shaken manually before immediate (< 30 s) removal of 1.25-ml of the suspension with a Handystep® Brand repeating pipette (Fig. 1). Aliquots (50-μl) of the suspension were poured into 96-well flat bottom microplates containing the corresponding buffer solution and substrate. Although the aims on this study were focused with the use of soil–water suspensions as the source of extracellular CbE activity, this type of sample provided a turbid reaction medium, so we also measured the enzyme activity using buffered soil extracts for comparisons. These buffered extracts were obtained by mixing 1 g wet soil with 5 ml of 50 mM Tris–HCl buffer (pH=7.5) containing 1% (w/v) Triton X-100 and 1 mM EDTA according to Fornasier and Margon (2007), with some modifications by Sanchez-Hernandez et al. (2014).

2.4. Carboxylesterase activity

Carboxylesterase (EC 3.1.1.1) activity was measured by a stopped assay using 96-well microplates (Thompson, 1999), and using
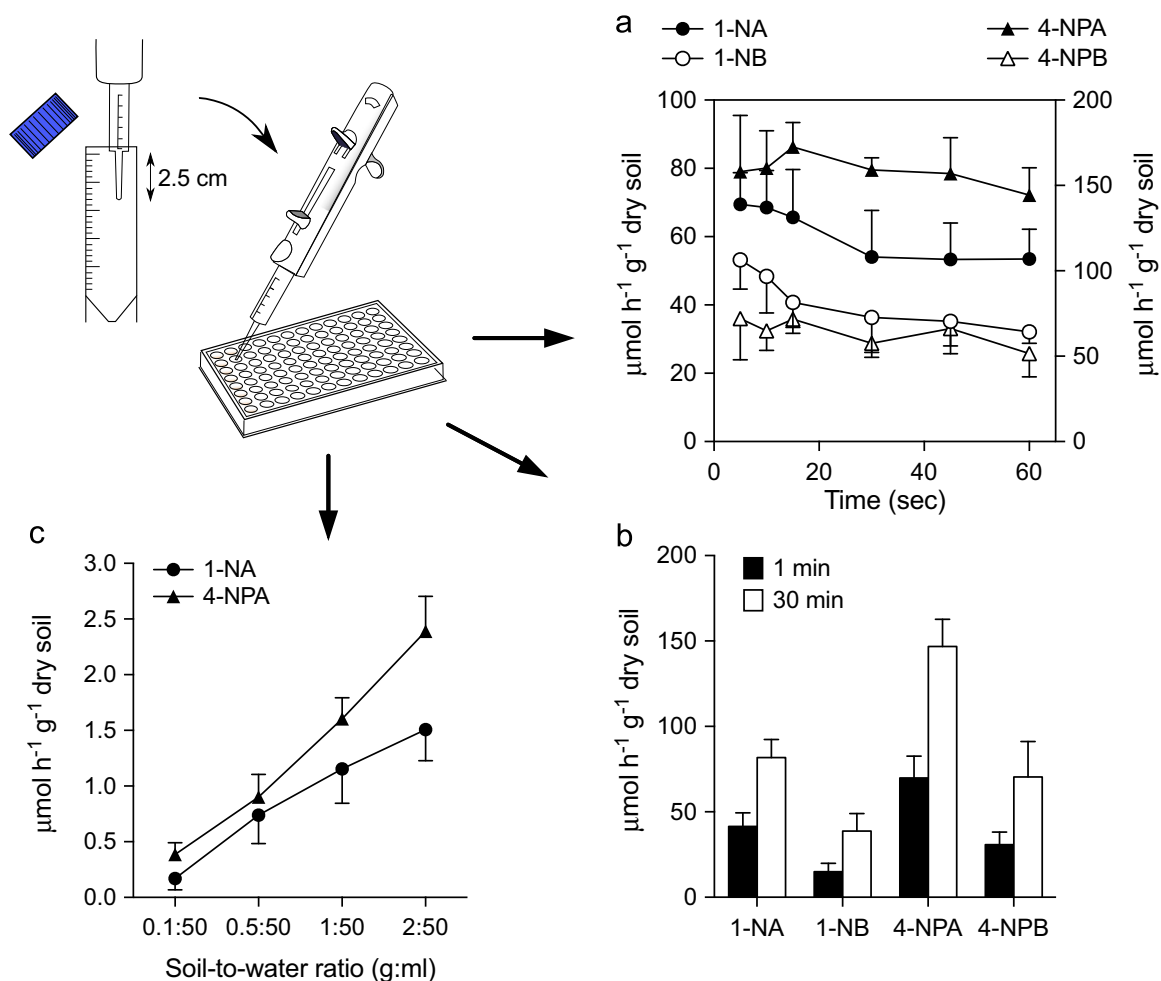
Fig. 1. Optimization of a microplate-scale assay for carboxylesterase (CbE) activity. Variation in CbE activity with: (a) time for soil particles to settle out, (b) time of shaking and (c) soil-to-water ratio. Data are the mean and standard deviation for six (graph c) and four replicates (graphs a and b). The esterase activity was measured using the substrates 1-naphthyl acetate (1-NA), 1-naphthyl butyrate (1-NB), 4-nitrophenyl acetate (4-NPA) and 4-nitrophenyl butyrate (4-NPB).

the substrates 1-naphthyl acetate (1-NA), 1-naphthyl butyrate (1-NB), 4-nitrophenyl acetate (4-NPA) and 4-nitrophenyl butyrate (4-NPB). Initially, we used a battery of substrates because of the occurrence of multiple CbE isoforms in animal tissues (Wheelock et al., 2008) and in the soil (Sanchez-Hernandez et al., 2014). The reaction mixture consisted of 140 µl of Tris–HCl 0.1 M (pH=7.4), 50 µl of sample (soil–water suspension or buffered soil extract) and 10 µl of each substrate added separately (final concentration 2.5 mM). The plates were shaken for 15 min at 20 °C in a thermostatically controlled orbital shaker (Elmi® Skyline DTS-2, 800 rpm). The product of the reaction was measured in an Asys HiTech UVM340 microplate reader (Asys HiTech Gmbh, Eugendorf, Austria). Controls (substrate-free) and blanks (soil-free) were used to correct background absorbance and non-enzymatic hydrolysis of the substrates, respectively. The kinetic parameters K_m and V_{max} were calculated using both soil–water suspensions and buffered soil extracts.

2.5. Dehydrogenase activity

Dehydrogenase activity was measured according to von Mersi and Schinner (1991) using iodonitrotetrazolium chloride as the substrate. The formation of iodonitrotetrazolium formazan (INTF) was determined spectrophotometrically at 464 nm, and the results were expressed as µmol INTF h^{-1} g^{-1} dry soil.

2.6. Effect of chlorpyrifos on soil carboxylesterase activity

Three toxicity trials were performed to explore the potential role of CbE as a molecular sink for OPs, using chlorpyrifos as a model insecticide. The first experiment (Trial I: chlorpyrifos formulation effect) examined the impact of two chlorpyrifos formulations on CbE activity: the granular formulation Dursban® 5G (5% w/w chlorpyrifos) and the emulsifiable formulation Cuspide® 48E (48% w/v chlorpyrifos). These two formulations were selected because they are the most commonly available formulations for chlorpyrifos, and both show marked differences in soil persistence (Racke, 1993). Both pesticides were obtained from a local supplier (Toledo, Spain). This experiment considered, therefore, a three-factor design: formulation (Dursban® 5G and Cuspide® 48E), pesticide concentration (0, 5, 10, 20 and 40 mg active ingredient kg^{-1} wet soil), and time of exposure (14 and 28 d). Cuspide® 48E was dissolved in water to yield nominal concentrations, whereas Dursban® 5G was added directly to the soil. Four replicates (40 g wet soil) were placed in Petri dishes, and incubated in an acclimatized chamber (continuous dark and 25 °C). Soil samples were used for determining both CbE and dehydrogenase activities. Moreover, ED_{50} values were estimated by the kinetic model described below.

The second experiment (Trial II: chlorpyrifos-oxon toxicity) consisted of spiking soil with serial concentrations (0, 0.5, 5 and 50 mg kg^{-1} wet soil) of chlorpyrifos-oxon (O,O-diethyl O-3,5,6-

trichloro-2-pyridyl phosphate, > 98% purity, Dr. Ehrenstorfer, Augsburg, Germany), which is the most toxic metabolite of chlorpyrifos. This exposure set up also used soil samples (40 g wet soil/dish) placed in Petri dishes, and kept at 25 °C and darkness. Chlorpyrifos-oxon was dissolved in ethanol and applied onto the soil to yield the nominal concentrations. Control (pesticide free) soils received an equivalent volume of ethanol. Soils were gently mixed using a spatula and the solvent was evaporated by placing the dishes inside a fume hood for 30 min. Soil samples were collected 2 and 10 d after pesticide treatment for measuring enzyme activities.

The last experiment (Trial III: in vitro inhibition kinetics) was performed to compare the inhibitory potency of chlorpyrifos (O,O-diethyl O-(3,5,6-trichloro-2-pyridinyl) phosphorothioate, > 98% purity, Dr. Ehrenstorfer, Augsburg, Germany) and chlorpyrifos-oxon on CbE activity. Soil–water suspensions (100 µl) were incubated in the presence of 10 µl of chlorpyrifos (1×10^{-3}–5×10^{-9} M) or chlorpyrifos-oxon (2×10^{-3}–1×10^{-9} M), in 96-well microplates, which were shaken at 800 rpm for 30 min and 20 °C. Controls comprised 100 µl of the sample and the corresponding volume of distilled water or dimethyl sulfoxide. The latter was included to check enzyme inhibition because both pesticides were initially dissolved in this solvent. However, dimethyl sulfoxide had no effect on CbE activity at a concentration of 10% in the reaction medium. On completion of the 30-min inhibition period, 130 µl of 0.1 M Tris–HCl (pH=7.4) and 10 µl of 1-NA (or 1-NB) were added and residual CbE activity was determined as described above.

2.7. Pesticide residue analysis

Pesticide residues were extracted from soil using QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) method, as adapted for soil samples by Asensio-Ramos et al. (2010). Chlorpyrifos concentrations were determined in a high-pressure liquid chromatography system (Agilent 1200 Series) equipped with a diode-array detector (Sanchez-Hernandez et al., 2014).

2.8. Data analysis

Non-parametric statistics (Kruskal–Wallis test followed by Mann–Whitney's test) was used to assess the effect of earthworms on CbE activity, and to examine the impact of pesticides on both CbE and dehydrogenase activities. The Jonckheere–Terpstra trend test was also used to examine whether the median values of CbE activity of the pesticide-treated soils decreased linearly with increasing pesticide concentration. Moreover, the response of soil CbE activity to Dursban® 5G and Cuspide® 48E treatments was adjusted to the exponential kinetic model proposed by Estevez and Vilanova (2009) for in vitro kinetic assays, which assumes one sensitive and one resistant component of esterase activity. For fixed times of inhibition, the kinetic model is expressed as follows:

$$E = E_0 \times e^{-(b \times I)} + E_R \quad [b = k_i \times t]$$

where E_0 (initial enzyme activity) represents the sensitive component of the enzyme activity, E_R is the resistant fraction of CbE activity, I is the pesticide concentration, k_i is the second-order rate constant of the inhibition process, and t is the fixed time of exposure (e.g., 14 or 28 d). We used this kinetic model to calculate the pesticide concentration that inhibited enzyme activity 50% of its initial, which would correspond to the ED_{50} value proposed by Babich et al. (1983):

$$ED_{50} = \frac{\ln(2)}{k_i \times t}$$

Data obtained from the Trial III (in vitro inhibition kinetics)

were fitted to the non-linear regression model:

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

where y is the percentage of residual CbE activity relative to controls, min is the y response to the highest pesticide concentration, max is the y response to the lowest pesticide concentration, x is the logarithm of pesticide concentration, the Hillslope coefficient describes the steepness of the dose–response curve, and IC_{50} is the median concentration of chlorpyrifos (or chlorpyrifos-oxon) that causes a 50% reduction of the initial CbE activity (Motulsky and Christopoulos 2004). Statistical analysis was performed using the SPSS® Statistics software (v. 21, IBM® Software, USA), whereas plots and non-linear regression analysis were done using the SigmaPlot® software (v. 11, Systat Software, USA).

3. Results

3.1. Optimization of a high-throughput spectrophotometric assay for CbE activity

Carboxylesterase activity was first optimized for soil–water suspensions from earthworm-treated soils. Three variables were assessed in the method: (i) time after soil homogenization for removal of aliquots, (ii) duration of homogenization, and (iii) soil-to-water ratio. Fig. 1a shows the changes in CbE activity in aliquots serially removed from the soil–water suspension for 60 s, with the tubes in a vertical position. Hydrolysis of naphthyl esters decreased slightly over time, whereas the enzyme activity towards the nitrophenyl esters remained unchanged. Homogenization of the samples for 30 min yielded a two-fold increase in CbE activity, relative to 1 min of homogenization (Fig. 1b). Increasing the soil-to-water ratio yielded higher CbE activities, but the inter-replicate variability also increased (Fig. 1c). The higher contents of soil particles and organic matter in the high soil-to-water ratio probably interfered absorbance readings. The procedure adopted for subsequent assays included use of a soil-to-water ratio of 1:50 (w/v), homogenization for 30 min, and almost immediate removal (between 15 and 60 s) of aliquots from the tubes. This method yielded an adequate level of enzyme activity, as well as minimized inter-assay variations.

Variation in CbE activity related to substrate concentration was also included in the optimization procedure. Carboxylesterase activity showed a Michaelis–Menten kinetic (Fig. 2a and 2b), which was comparable between both type of samples (soil–water suspension and buffered soil extract). However, naphthyl-ester hydrolysis (1-NA and 1-NB) decreased at high substrate concentrations (> 2.5 mM) in the buffered soil extracts; these values were therefore disregarded for K_m and V_{max} calculations (Fig. 2b). The maximum hydrolysis rates followed the sequence 4-NPA > 4-NPB > 1-NA > 1-NB, whereas CbE showed a lower affinity for 4-NPA ($K_m = 1.02$–2.02 mM) than for the other substrates. Moreover, V_{max} in soil–water suspensions was 6- to 16-times higher than in buffered soil extracts, although K_m values were similar in both types of sample. The catalytic efficiency, calculated as the V_{max}/K_m ratio, was higher for the hydrolysis of 4-carbon chain esters in soil–water suspensions (Table 1). Taken these results together, a final substrate concentration of 2.5 mM in the reaction medium was selected as optimal for the subsequent enzyme assays.

Using these assay conditions, soils treated with L. terretris had a higher CbE activity ($P < 0.05$, Mann–Whitney U test) than control soils and soils inoculated with leaf litter (Fig. 2c). Furthermore, CbE
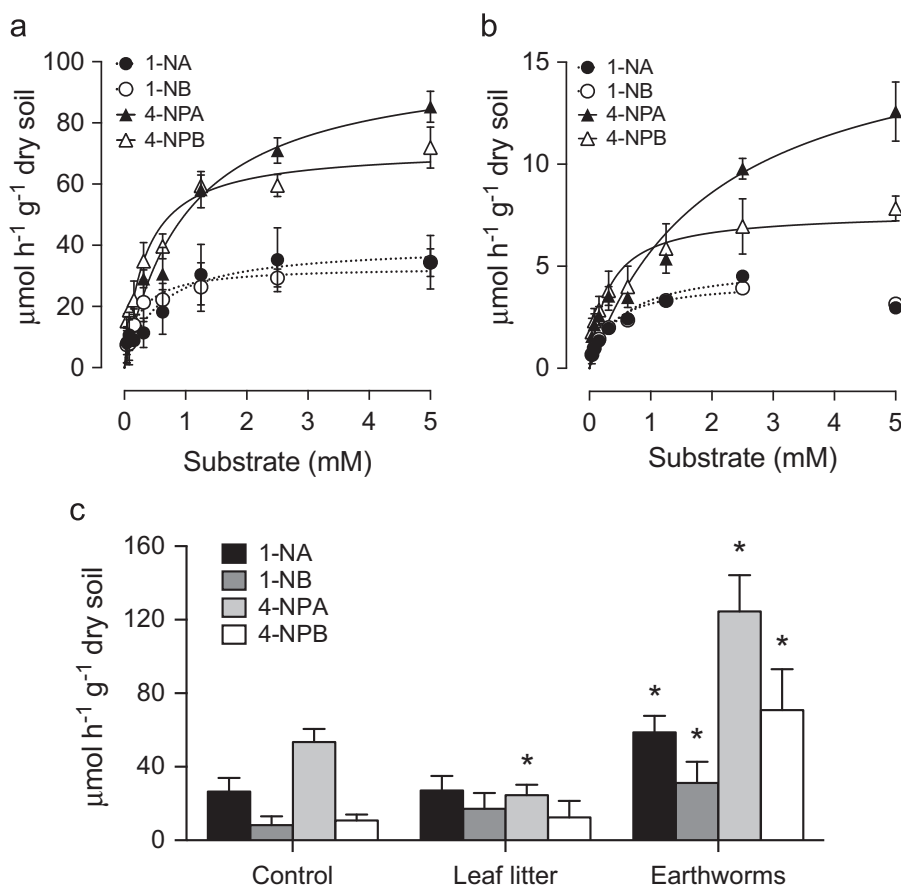
Fig. 2. Effect of substrate concentration on carboxylesterase (CbE) activity in earthworm-treated soils, using soil–water suspensions (graph a) and buffered soil extracts (graph b). Each point corresponds to the mean ($\pm$ SD) of three individual determinations. Graph (c): mean ($\pm$ SD, $n=6$) CbE activity in earthworm-free soils (control), leaf litter-inoculated soils (leaf litter) and earthworm-treated soils (earthworms). Esterase activity was measured using multiple naphthyl and nitrophenyl esters (see Fig. 1) in soil–water suspensions. Asterisks denote significant differences compared with control soils ($P < 0.05$, Mann–Whitney U test).

Table 1
Kinetic parameters (mean $\pm$ SE) for hydrolysis of naphthyl and nitrophenyl esters using soil–water suspensions and buffered (Tris/Triton/EDTA buffer) soil extracts.

Sample type	Substrate	V_{max} (μmol h^{-1} g^{-1} dry soil)	K_m (mM)	V_{max}/K_m (ml h^{-1} g^{-1})	P values	R^2
Soil–water suspension	4-nitrophenyl acetate	101.4 ± 7.48	1.02 ± 0.20	100	< 0.0001	0.98
	4-nitrophenyl butyrate	71.66 ± 4.87	0.33 ± 0.08	217	< 0.0001	0.94
	1-naphthyl acetate	42.05 ± 3.29	0.61 ± 0.17	69.3	< 0.0001	0.92
	1-naphthyl butyrate	35.00 ± 1.67	0.26 ± 0.05	135	< 0.0001	0.95
Buffered soil extract	4-nitrophenyl acetate	17.33 ± 3.40	2.02 ± 0.88	8.58	0.0002	0.92
	4-nitrophenyl butyrate	7.65 ± 0.63	0.30 ± 0.09	25.1	0.0003	0.90
	1-naphthyl acetate	5.06 ± 0.52	0.52 ± 0.14	9.73	0.0002	0.95
	1-naphthyl butyrate	4.24 ± 0.30	0.34 ± 0.07	12.3	< 0.0001	0.96

activity was about 10 times higher in soil–water suspensions than in buffered soil extracts (1-NA: 3.67 ± 1.20; 1-NB: 3.68 ± 1.53; 4-NPA: 14.9 ± 1.00; 4-NPB: 6.03 ± 1.15 μmol h^{-1} g^{-1} dry soil, mean $\pm$ SD, $n=6$).

3.2. Effect of chlorpyrifos on carboxylesterase and dehydrogenase activities

Trial I (chlorpyrifos formulation effect): Both chlorpyrifos formulations caused a similar response in both CbE and dehydrogenase activities (Fig. 3). Treatment with Cuspide® 48E had a significant effect on CbE activity at both sampling times (14d-1NA: $H_{(4)}=15.0$, $P=0.005$; 28d-1NA: $H_{(4)}=11.7$, $P=0.02$; 14d-1NB: $H_{(4)}=17.04$, $P=0.002$; 28d-1NB: $H_{(4)}=10.31$, $P=0.035$). Likewise,

treatment with Dursban® 5G caused a significant inhibition of CbE activity at $t=14$ d (1-NA: $H_{(4)}=12.8$, $P=0.012$; 1-NB: $H_{(4)}=14.7$, $P=0.005$), and $t=28$ d (1-NB: $H_{(4)}=14.3$, $P=0.006$), except for the hydrolysis of 1-NA at $t=28$ d ($H_{(4)}=7.43$, $P=0.11$). Dose-response relationships yielded significant fits ($R^2 \geq 0.92$, $P < 0.005$) to a decay kinetic model, which enabled calculation of ED_{50} values (Table 2). The 28 d-ED_{50} values for CbE activity using 1-NB were 10 times lower than the 14 d-ED_{50} values, irrespective the chlorpyrifos formulation. Nevertheless, CbE activity towards 1-NA was only fitted to this model for the Cuspide® 48E-spiked soil incubated for 14 d (Fig. 3a). Dehydrogenase activity was not found inhibited in the soils treated with both chlorpyrifos formulations compared with controls. Conversely, there was a significant increase (Mann–Whitney U test, $P < 0.05$) of this enzyme activity in some
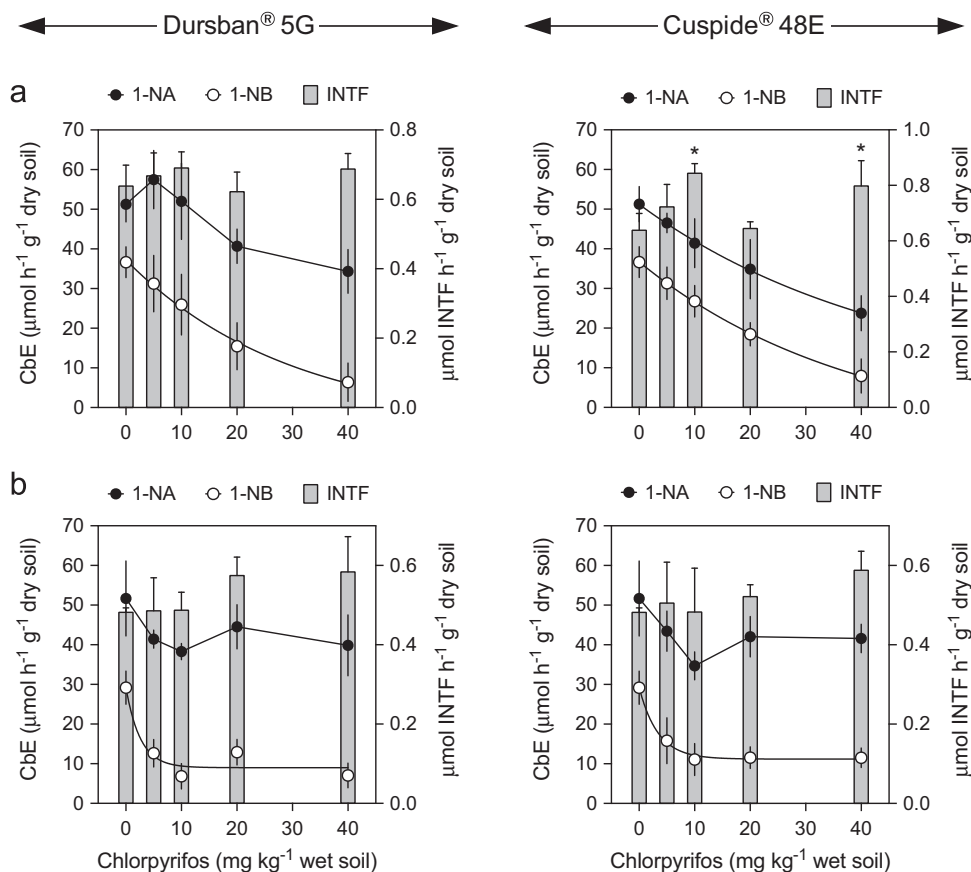
Fig. 3. Effect of Dursban® 5G and Cuspide® 48E on soil carboxylesterase (CbE) activity and dehydrogenase activity (gray bars). Enzyme activities were measured after 14 days (graphs a) and 28 days (graphs b) of pesticide treatment. Data are the mean and standard deviation ($n=4$). Mean values were fitted to a decay exponential model, which enabled calculation of the median ecological dose (see Table 2). *$P < 0.05$, Mann–Whitney U test. Esterase activity was measured using 1-naphthyl acetate (1-NA) and 1-naphthyl butyrate (1-NB).

Table 2
Inhibition kinetic parameters (mean $\pm$ SE) and statistics for carboxylesterase activity measured in chlorpyrifos-treated soils.

Formulation	Time (d)	Substrate	E_0	E_R	P	R^2	ED_{50}[a]
Dursban® 5G	14	1-NB	42.0 ± 5.2	–	0.004	0.995	20.4
	28	1-NB	20.2 ± 4.3	9.0 ± 2.3	0.079	0.921	1.8
Cuspide® 48E	14	1-NA	53.3 ± 8.0	–	0.0012	0.998	34.7
	14	1-NB	45.0 ± 2.0	–	0.0002	0.999	26.7
	28	1-NB	18.1 ± 1.0	11.2 ± 0.6	0.006	0.993	2.3

E_0=initial esterase activity; E_R=resistant esterase activity; 1-NB=1-naphthyl butyrate; 1-NA=1-naphthyl acetate; ED_{50}=median ecological dose (mg kg^{-1}).

[a] ED_{50} values for 1-naphthyl acetate in 14-d Dursban® 5G-spiked soils was not possible to calculate because of lack of data fitting.

pesticide-treated soils but without display any dose-dependent relationship (Fig. 3). Although the nominal concentrations of both chlorpyrifos formulations were the same, accurate determination of OP concentration was, however, required because of differences in formulation (granular *vs* emulsifiable). There was a good agreement between nominal and measured chlorpyrifos concentrations, although soil spiking with Dursban® 5G led to a higher inter-assay variation (coefficients of variation [CV] ranging between 12% and 64%) than with the emulsifiable concentrate (CV=3.6–30%) (Fig. 4).

Trial II (chlorpyrifos-oxon toxicity): Fig. 5a shows the dose-dependent relationships between soil CbE activity and chlorpyrifos-oxon concentrations. The Kruskal–Wallis test on pesticide concentrations showed that the enzyme activity was not affected by the insecticide after 2 days of treatment ($H_{(3)}=6.33$, $P=0.09$ for 1-NA, and $H_{(3)}=7.17$, $P=0.06$ for 1-NB), although the mean CbE activity in the soils treated

with 50 mg kg^{-1} was significantly ($P < 0.05$) lower than controls. Likewise, a tendency to decrease the CbE activity as the pesticide concentration increased was found significant (Jonckheeree trend test, $P < 0.05$). At longer exposure time ($t=10$ d), chlorpyrifos caused a significant decrease of CbE activity ($H_{(3)}=11.2$, $P=0.011$ for 1-NA, and $H_{(3)}=10.14$, $P=0.017$ for 1-NB), which was dose-dependent ($P < 0.01$, Jonckheeree's trend test). The measurement of CbE activity in the buffered soil extracts from chlorpyrifos-oxon-treated soils confirmed the decrease of enzyme activity caused by the pesticide. Nevertheless, the impact of chlorpyrifos-oxon exposure on the 1-NB hydrolysis was more pronounced in buffered soil extracts than in soil–water suspensions (the highest concentrations caused inhibition percentages between 85% and 94% of controls) (insets in Fig. 5a). Chlorpyrifos-oxon had a significant effect on dehydrogenase activity after 2 d of exposure ($H_{(3)}=8.73$, $P=0.03$), but not at 10 d ($H_{(3)}=2.54$, $P=0.47$). Nevertheless, only the 50-mg kg^{-1} treatment decreased significantly
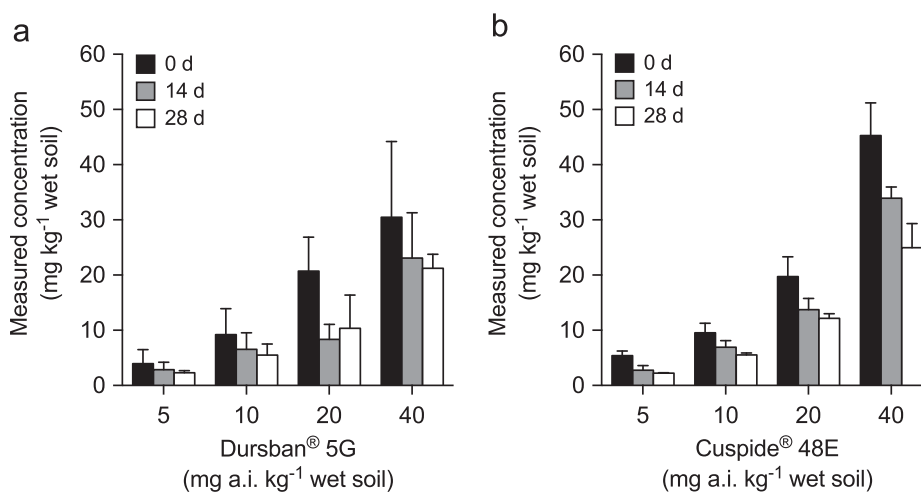
Fig. 4. Concentrations (mean $\pm$ SD, $n=4$) of chlorpyrifos measured in soils after 0, 14 and 28 days of treatment with Dursban® 5G (graph a) and Cuspide® 48E (graph b) in comparison with nominal concentrations (5, 10, 20 and 40 mg active ingredient kg^{-1} wet soil).
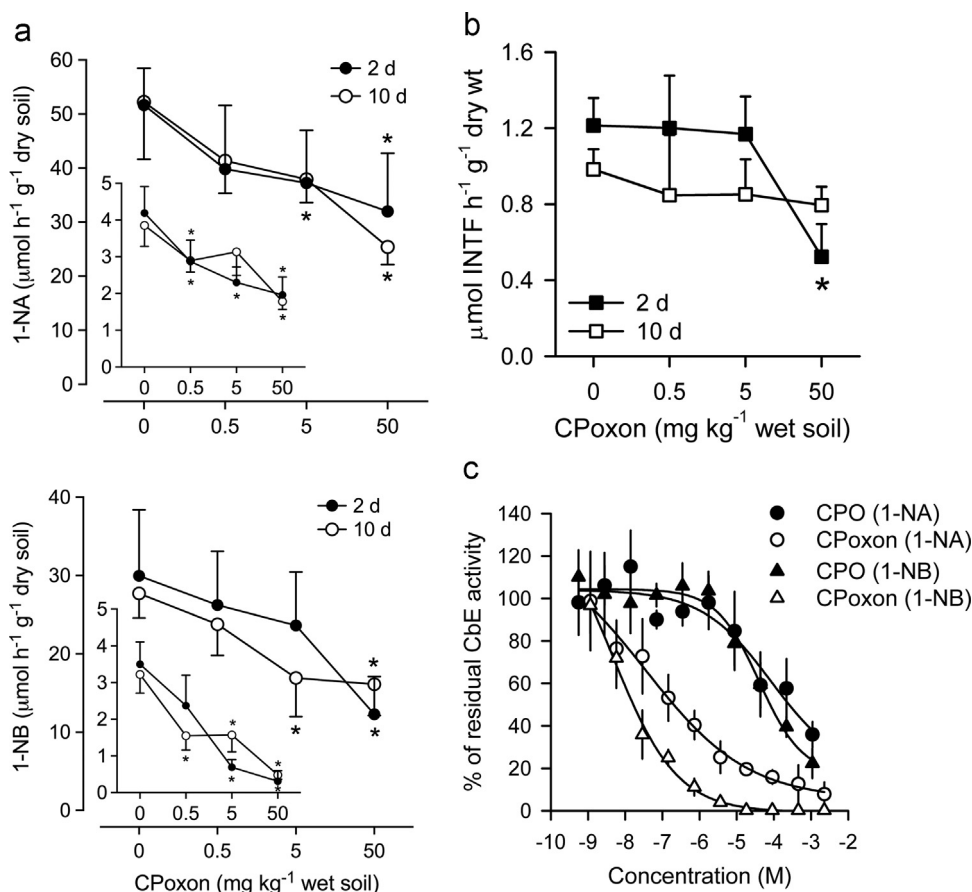

Fig. 5. Panel (a): Variation of carboxylesterase (CbE) activity towards 1-naphthyl acetate (1-NA) and 1-naphthyl butyrate (1-NB) (mean $\pm$ SD, $n=4$) in earthworm-treated soils after 2 and 10 days of spiking with chlorpyrifos-oxon (CPoxon). Enzyme activity was determined using soil–water suspensions and buffered soil extracts (inset graphs). Asterisks denote significant differences compared with controls ($P < 0.05$, Mann–Whitney U test). Graph (b): Dehydrogenase activity (mean $\pm$ SD, $n=4$) in earthworm-treated soils spiked with CPoxon and sampled 2 and 10 days after treatment. Asterisk denotes significant differences compared with control ($P < 0.05$, Mann–Whitney U test). Graph (c): In vitro inhibition of CbE activity after 30-min incubation of soil–water suspensions in the presence of chlorpyrifos (CPO) and CPoxon. Data are the mean and standard deviations of three independent assays.

($P=0.02$) this oxidoreductase activity compared with controls (Fig. 5b).

Trial III (in vitro inhibition kinetic): Inhibition kinetics of CbE activity against chlorpyrifos and chlorpyrifos-oxon followed a sigmoidal model ($R^2 > 0.91$, $P \leq 0.001$) for both pesticides and substrates (1-NA and 1-NB) (Fig. 5c). Chlorpyrifos-oxon was a more potent inhibitor (30 min-IC$_{50}$ values$=35.5$ and 4.6 nM for 1-NA and 1-NB, respectively) than chlorpyrifos (30 min-IC$_{50}=84.6$ and 40.6 μM for 1-NA and 1-NB, respectively).

4. Discussion

4.1. Use of soil–water suspensions for carboxylesterase activity

In this study, we optimized a microplate-scale spectrophotometric assay using soil–water suspensions for determining CbE activity. This sample preparation was preferred because it yielded higher hydrolytic activity than buffered soil extracts. Indeed, many studies use soil suspensions that are usually prepared using deionized water or 50 mM acetate buffer, often at a soil-to-liquid ratio of 1:100 (w/v), and following a homogenization procedure that involves continuous magnetic stirring (15–30 min) (Popova and Deng, 2010; Turner, 2010; Deng et al., 2013) in combination with use of a blender (Bell et al., 2013) or a Brinkmann™ Polytron (Sinsabaugh et al., 2000; Saiya-Cork et al., 2002). In our study, the standardized procedure that provided the most reliable data was as follows: (1) after homogenization of the soil for 30 min, the tube was vigorously shaken by hand; (2) the tube was placed in a vertical position before immediate (< 60 s) removal of 1.25 ml of suspension. The standard design of the tip helps removal of the sample at the same depth (approx. 2.5 cm) (Fig. 1); (3) one or two aliquots were discharged in the tube, and the desired volume (i.e., 50 µl) was immediately poured in the wells (i.e. four or five replicates); (4) the tip was washed once or twice with distilled water between samples and steps 1 to 3 repeated (or the tip is changed between samples).

Determination of K_m and V_{max} is an essential step for optimizing enzyme kinetic assays at saturating substrate concentrations, because it enables more accurate detection of differences in enzyme activity between soils or treatments (German et al., 2011). Moreover, the kinetic behavior of enzymes may differ between the different soil fractions in which the enzyme is present (Gianfreda et al., 2012). In this study, catalytic efficiency of CbE activity was much higher in soil–water suspensions than in buffered soil extracts. The marked difference was attributed to maximum reaction velocity (V_{max}) because substrate affinity (K_m) was similar in both types of sample. The high V_{max} in soil–water suspensions probably reflected an increase in enzyme concentration. Indeed, the multiple forms in which enzymes may be dispersed in soil (e.g., intracellular in living and resting cells, free in soil solution or extracellularly associated with organomineral complex of soil; Nannipieri et al. (2002)) contribute to the total activity measured in the kinetic assay. As a result, V_{max} values are expected to be higher than those measured in buffered soil extracts. Assuming that a large fraction of CbE activity is extracellularly associated with organomineral complex of soil, K_m values indicate that substrate affinity did not vary significantly when CbE was extracted using the Tris/Triton X-100/EDTA buffer solution.

4.2. Soil carboxylesterases: potential bioscavengers for organophosphorus insecticides

Soil incubated for 10 weeks with L. terrestris ensured high levels of CbE activity. This was an expected result as earthworms stimulate soil enzyme activities (Tao et al., 2009; Dempsey et al., 2013; Jusselme et al., 2013; Sanchez-Hernandez et al., 2014). Data from pesticide-spiking trials suggested that CbE provides a detoxification system in OP-contaminated soils. This esterase was very sensitive to chlorpyrifos, and the hydrolytic activity decreased in a dose-dependent manner with pesticide concentration. This inhibition-based scavenging mechanism may, therefore, reduce pesticide bioavailability and toxicity. Indeed, dehydrogenase activity was not negatively affected by either Dursban® 5G or Cuspide® 48E treatments; although many studies report an inhibition of this enzyme activity by OP pesticides such as chlorpyrifos, methyl parathion, profenofos, quinalphos, ethion or diazinon (Pandey and Singh, 2006; Bishnu et al., 2011; Kadian et al., 2012; Marín-Benito et al., 2014; Riah et al., 2014). However, the metabolite chlorpyrifos-oxon caused a significant decrease in dehydrogenase activity at high concentrations (50 mg kg^{-1}), probably because the detoxification capacity of CbEs was saturated, although other potential factors such as pesticide bioaccesibility should not be excluded. In animals, the greatest sensitivity of CbE activity to inhibition by OPs compared to other serine esterases (e.g., brain AChE activity) has led to postulate CbE as an efficient stoichiometric detoxification system (Maxwell, 1992; Wheelock et al., 2008). Our findings suggest that a comparable detoxification mechanism could be attributed to soil CbE activity. This would explain why 50 mg kg^{-1} chlorpyrifos-oxon inhibited dehydrogenase activity after 2 d of exposure, simultaneously with high CbE inhibition. At this level of pesticide exposure, the number of CbE molecules was not sufficient to block chlorpyrifos-oxon toxicity. Nevertheless, soils treated with Dursban® 5G or Cuspide® 48E did not display inhibition of dehydrogenase activity. This unexpected result may be due to the nature of the chemical interaction between the OP and CbE activity. The affinity of OPs for the active site of esterases is high with the "oxon" form of the pesticide. In this chemical configuration, the OP has a coordinate covalent bond between the phosphorus atom and an oxygen atom (Chambers et al., 2010). However, most OP pesticides (such as chlorpyrifos) have a sulfur atom bound to the phosphorus, and they are weak esterase inhibitors in that configuration. This difference in the strength of esterase inhibition between oxon metabolites and the parent compounds was observed in our study (Fig. 4c). The IC$_{50}$ values clearly showed that chlorpyrifos-oxon was a more potent CbE inhibitor (IC$_{50}$s in the range of nM) than chlorpyrifos. In vitro outcomes also provided some explanation to results obtained from the Dursban® 5 G- and Cuspide® 48E-treated soils. In these soils, gradual activation of chlorpyrifos by microorganisms or abiotic factors led to inhibition of CbE activity, which was even more pronounced at longer exposure times ($t = 28$ d). However, the dehydrogenase activity did not change, probably because CbE activity inactivated chlorpyrifos-oxon on formation.

Current results also indicated that decrease of CbE activity was due to a direct interaction between chlorpyrifos-oxon and the active site of the esterase, instead of a decreased microbial activity caused by the pesticide. Many studies have examined pesticide effects on soil enzyme activities through field surveys or microcosm experiments (Floch et al., 2011; Riah et al., 2014). In those, soil enzyme activities display complex responses to pesticide exposure because of direct and indirect effects that occur simultaneously (Gianfreda and Rao, 2008). It is therefore difficult to assess the adverse effects of pesticides on soil biochemical processes. However, our findings show that soil CbE activity displays simple dose-dependent responses, fitted to a decay exponential model, to chlorpyrifos exposure. With this inhibition kinetic model, it was possible to estimate ED$_{50}$ values, which decreased with time of exposure (Table 2). Time-dependent variations of ED$_{50}$ values in metal-contaminated soils have been documented by others (Babich et al., 1983; Moreno et al., 2002), who suggested that increased ED$_{50}$ values was a consequence of metal tolerance developed by some microbial populations. In our study, assuming a direct interaction between CbE activity and the OP pesticide, the decrease of ED$_{50}$ with time was attributed to a progressive activation of chlorpyrifos to chlorpyrifos-oxon. Although several authors have proposed logistic (Haanstra et al., 1985) and Michaelis–Menten inhibition kinetic models (Speir et al., 1999b) for calculating ED$_{50}$ values in soil enzyme studies, we chose the exponential decay model proposed by Estevez and Vilanova (2009) because it assumes the existence of a sensitive and a resistant esterase, which is in line with present data and previous studies

(Sanchez-Hernandez et al., 2014). The ED$_{50}$ values estimated in our study (1.8–34.7 mg kg^{-1}) fell within the range of chlorpyrifos concentrations normally reported in agricultural soils after soil-surface applications of an emulsifiable formulation (2.0–58.8 mg kg^{-1}), or after soil-incorporated applications of a granular formulation (0.2–48.7 mg kg^{-1}) (reviewed in Racke (1993)). These results suggest that concentrations of chlorpyrifos detected in pesticide-treated lands may inhibit CbE activity at levels that its detoxification capacity would be saturated. This hypothetical scenario could be real in those situations in which residues of chlorpyrifos persist long-term in soil (e.g., repeated treatments of pesticides). Although still premature, the measurement of CbE activity, in combination with determination of other indicators of microbial activity, may provide valuable ecotoxicological information during the environmental risk assessment of OP impact on the soil system at short-term scale.

Acknowledgments

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