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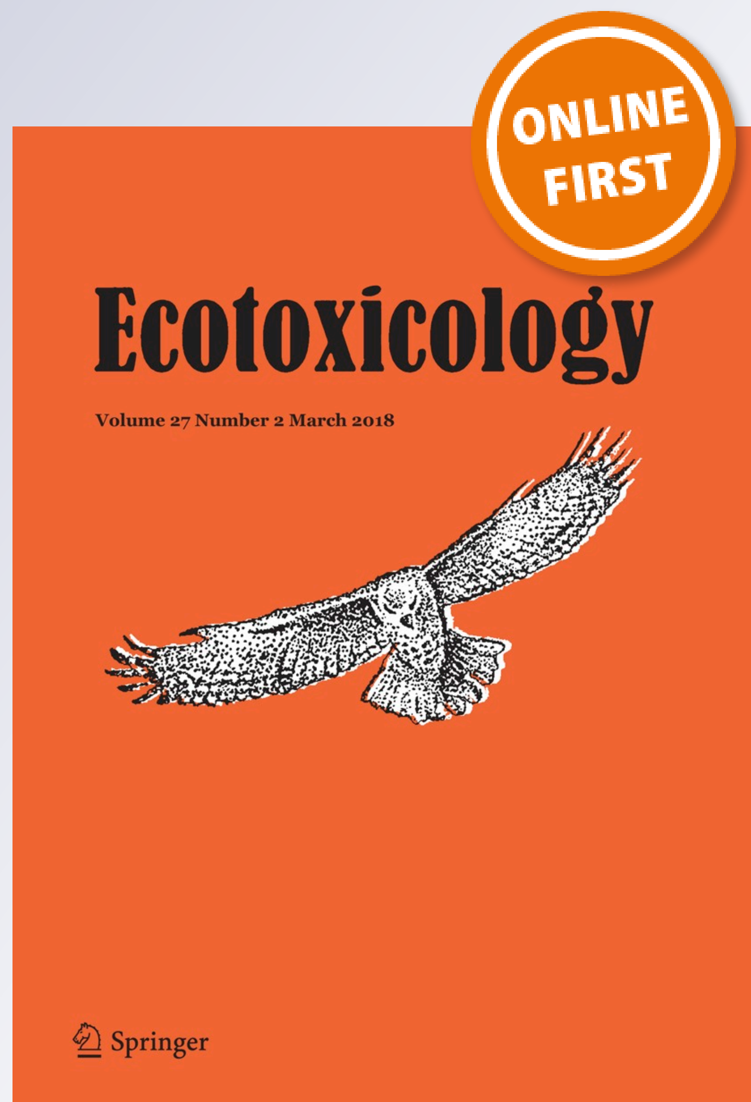

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Response of digestive enzymes and esterases of ecotoxicological concern in earthworms exposed to chlorpyrifos-treated soils

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Abstract

Assessment of organophosphorus (OP) pesticide exposure in non-target organisms rarely involves non-neural molecular targets. Here we performed a 30-d microcosm experiment with *Lumbricus terrestris* to determine whether the activity of digestive enzymes (phosphatase, β-glucosidase, carboxylesterase and lipase) was sensitive to chlorpyrifos (5 mg kg^{-1} wet soil). Likewise, acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) activities were measured in the wall muscle and gastrointestinal tissues as indicators of OP exposure. Chlorpyrifos inhibited the acid phosphatase (34% of controls), carboxylesterase (25.6%) and lipase activities (31%) in the gastrointestinal content. However, in the gastrointestinal tissue, only the carboxylesterase and lipase activities were significantly depressed (42–67% carboxylesterase inhibition in the foregut and crop/gizzard, and 15% lipase inhibition in the foregut). Chlorpyrifos inhibited the activity of both cholinesterases in the gastrointestinal tissues, whereas the AChE activity was affected in the wall muscle. These results suggested chlorpyrifos was widely distributed throughout the earthworm body after 30 d of incubation. Interestingly, we found muscle carboxylesterase activity strongly inhibited (92% of control) compared with that detected in the gastrointestinal tissues of the same OP-exposed individuals. This finding was explained by the occurrence of pesticide-resistant esterases in the gastrointestinal tissues, which were evidenced by zymography. Our results suggest that digestive processes of *L. terrestris* may be altered by chlorpyrifos, as a consequence of the inhibitory action of the insecticide on some digestive enzymes.

Keywords *Lumbricus terrestris* · Digestive enzymes · Zymography · Cholinesterases · Carboxylesterases · Lipases

Introduction

Inhibition of cholinesterase and carboxylesterase activities is used to assess exposure to organophosphorus (OP) pesticides in non-target organisms (Wheelock et al. 2008; Nunes 2010). These enzymes take part in the mode of toxic action and detoxification of OP pesticides. For example, the acute toxicity of OPs is due to the inhibition of acetylcholinesterase (AChE, EC 3.1.1.7) activity; a key enzyme regulating the neural transmission (Fukuto 1990). Likewise, the active site of butyrylcholinesterase (BChE, EC 3.1.1.8) and carboxylesterase (EC 3.1.1.1) bind OP molecules, therefore leading to their inactivation (Masson and Lockridge 2010; Sogorb and Vilanova 2010). However, this detoxification mechanism is efficient as long as OP compounds bioactivate previously to their oxon metabolites (oxygen analogs). In this chemical configuration, the OPs display a higher affinity for the active sites of cholinesterases and carboxylesterases compared with their parent chemicals (Chambers et al. 2010).

Searching new molecular targets of OP toxicity is a topic of growing concern (Quistad and Casida 2000). For example, digestive enzymes of pest species have been proposed as suitable molecular targets for biopesticide development (Rodrigues Macedo and Machado Freire 2011). Recently, some studies have reported significant changes in the activity of digestive enzymes of pest species exposed to OP and neonicotinoid pesticides (Stygar et al. 2013; Kalita et al. 2016). Similarly, Sanchez-Hernandez

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et al. (2014) found that the gastrointestinal carboxylesterase activity of the earthworm *Lumbricus terrestris* was sensitive to inhibition by chlorpyrifos-oxon, which resulted in a reduced uptake rate of this toxic OP metabolite through the gut epithelium. Taken together, these studies suggest that OP pesticides may disrupt the digestive processes through changes in the digestive enzyme activities. In this context, *L. terrestris* is an ideal model to investigate digestive toxicology because of its large body size that facilitates the sampling of gut tissue and luminal content at different segments of the alimentary canal. Furthermore, the structure and function of the digestive canal of this species are well described (Drake and Horn 2007). For example, the maximum glycolytic enzyme activities are generally found in the first segment of the gut (foregut), although this pattern of activity distribution may change with the earthworm species and the enzyme (Zhang et al. 1993; Lattaud et al. 1998; Garvín et al. 2000; Nozaki et al. 2013). Therefore, determination of digestive enzyme activities in different segments of the earthworm digestive tract is recommended in digestive toxicology. Moreover, *L. terrestris* is an anecic earthworm that feeds on plant debris that collects from the soil surface and drags into their burrows (Römbke et al. 2005; Griffith et al. 2013), although it also ingests soil mixed with organic matter (Brown et al. 2000). This feeding behavior suggests that pesticide exposure in this earthworm takes place mainly by ingestion of contaminated plants and soil. In addition, earthworms need the enzymatic support from microbial symbionts that inhabit its gastrointestinal lumen, and from those in the ingested soil, to face the digestive processes (Drake and Horn 2007). Therefore, pesticides may also disrupt the earthworm digestion and nutrient assimilation processes by direct interaction with the microbial exoenzyme cocktail, or indirectly through toxic action on the microbiota.

To date, the impact of OP pesticides on earthworm digestive enzymes has not been investigated. Among OPs of current usage, chlorpyrifos (*O,O*-diethyl *O*-3,5,6-trichloro-2-pyridyl phosphorothioate, CAS No. 2921-88-2) is one of the most used OPs pesticides in agriculture. Some comprehensive reviews have examined exhaustively its environmental fate and reactivity (Racke 1993; Giesy and Solomon 2014), toxicology (Barron and Woodburn 1995), and biodegradation in soils (Singh 2008; John and Shaike 2015). Chlorpyrifos is a highly lipophilic chemical (log $K_{OW} = 4.70$, Yu et al. 2006), and it has a high sorption partition coefficient between soil organic carbon and the soil solution (log $K_{OC} = 3.70$–4.13, Mackay et al. 2006). The values of these partitive properties suggest that the main route for chlorpyrifos uptake by earthworms should be the gastrointestinal tract (Yu et al. 2006).

This study seeks, therefore, to provide the first evidence for a digestive enzyme disruption in earthworms exposed to OP pesticides. The main aim was to determine whether the activity of phosphatases (acid and alkaline), β-glucosidase, lipase and carboxylesterase changed after earthworm exposure to a single and sublethal treatment with chlorpyrifos (5 mg kg^{-1} wet soil). A second aim was to determine whether these enzymatic changes depended on the segment of the gastrointestinal tract (i.e., crop/gizzard and foregut) or the location of the enzyme activity (i.e., tissue and lumen). To check whether chlorpyrifos was widely distributed throughout the earthworm body, and bioactivated to its toxic metabolite chlorpyrifos-oxon, the activity of cholinesterases and carboxylesterase was measured in both the wall muscle and the digestive tissues. It is our intention that results in this study contribute to increase the understanding of OP toxicology on non-neural secondary targets such as digestive enzymes.

Materials and methods

Earthworms and soil spiking

Earthworms were purchased from a local commercial supplier (Decathlon®, Toledo, Spain), and acclimatized in the laboratory (15 °C and permanent darkness) using plastic containers (345 × 325 × 150 mm). Earthworms were fed weekly with litter, which was deployed on the soil surface. The soil used for earthworm acclimatization and microcosm assay was loamy in texture, classified as Anthrosol (IUSS Working Group WRB 2015), and was collected from an abandoned agricultural area in Toledo (Spain). The physicochemical properties of the soil (<2 mm, $n = 8$) were: pH $= 8.12 \pm 0.04$, electrical conductivity $= 230.0 \pm 8.6$ µS cm^{-1}, total organic carbon (TOC) $= 7.95 \pm 0.61$ mg C g^{-1} dry mass. The TOC content was measured according to the dichromate redox colorimetric method by Skjemstad and Baldock (2008).

The soil was spiked with an aqueous solution of chlorpyrifos made from a commercial formulation (Cuspide® 48E, 48% w/w chlorpyrifos, Comercial Química Massí, S. A., Barcelona, Spain) to yield a final concentration of 5 mg chlorpyrifos kg^{-1} wet soil. To this, the soil was extended in a tray to form a 2-cm soil layer and the pesticide solution was sprayed on the soil surface. Afterward, the soil was stirred using a spatula to distribute the pesticide in the bulk soil. Control soils received the same volume of water.

Experimental design

The soils were divided into eight replicates (400 g wet mass) per treatment (control and chlorpyrifos-treated soil) and placed in 500-ml cylindrical plastic vessels. The moisture was adjusted up to 40% of maximum water

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holding capacity (13.4 ± 0.63% H_2O content for controls and 13.9 ± 1.04% H_2O content for chlorpyrifos-treated soils). These test vessels were kept for 24 h at 15 °C and darkness for equilibration before releasing the earthworms.

Sixteen adult *L. terrestris* were assigned to both experimental groups: control soils (5.73 ± 0.76 g body weight, $n = 8$) and chlorpyrifos-treated soils (5.51 ± 0.47 g, $n = 8$). Therefore, each test vessel contained a single earthworm to have an individual density of 11.2 ± 1.24 g earthworm kg^{-1} wet soil ($n = 16$). The vessels were placed again at 15 °C and permanent darkness for 30 d. Although vessels were covered with holed plastic lids to minimize water evaporation and allow air exchange, we checked water losing by weighting vessels and was corrected adding water. Periodically, we added litter (2 g) as food on the soil surface. After 30 d, earthworms were removed and weighted, and a soil sample was taken from each replicate to check pH and chlorpyrifos concentration.

Tissue homogenization

Earthworms were cooled in the fridge (4 °C) to facilitate tissue dissection. Dissection was started in the mid-dorsal line by a longitudinal incision. The crop/gizzard segment and the foregut (between the gizzard and the clitellum) were open to collect carefully the luminal content from both segments, avoiding the scraping of the gut epithelium. We made a pooled sample of the luminal content taken from both gastrointestinal segments because of the small amount of sample found in the crop/gizzard segment of most earthworms. Although the tissues were rinsed with water, the foregut tissue contained fragments of chloragogeneous tissue. This is a hematopoietic tissue that covers the gut (Fischer 1993) and it is considered the functional analog of the vertebrate liver or invertebrate hepatopancreas (Engelmann et al. 2011). The wall muscle (from clitellum towards anus) was also removed and rinsed with distilled water to eliminate soil particles and tissue debris.

The luminal content was immediately frozen at −20 °C, whereas the tissues were homogenized (1:10, w/v) in ice-cold 20 mM Tris–HCl buffer (pH = 7.6), containing 1 mM EDTA, using a glass-PTFE Potter-Elvehjem tissue grinder connected to a Heidolph type ST1 homogenizer. The homogenates were centrifuged at 9000×*g* at 4 °C for 20 min, and the post-mitochondrial fraction was aliquoted and stored at –80 °C.

Digestive enzyme activities

The activity of acid phosphatase (EC 3.1.3.2), alkaline phosphatase (EC 3.1.3.1), β-glucosidase (EC 3.2.1.21), lipase (EC 3.1.1.3) and carboxylesterase was determined in the gastrointestinal tissues (crop/gizzard and foregut) and luminal content. Although carboxylesterase activity is used as a biomarker of OP exposure (Wheelock et al. 2008), we also included it in the battery of digestive enzymes because of its physiological role in lipid digestion (Ross and Edelmann 2012; Ríos et al. 2014).

Determination of phosphatase and β-glucosidase activities followed the spectrophotometric method by Nozaki et al. (2013), adapted to 96-well bottom flat microplates. Briefly, homogenates (10 µl for both acid and alkaline phosphatase and 20 µl for β-glucosidase) were incubated in 15 mM Tris–HCl (pH = 6.5 for acid phosphatase, pH = 9.5 for alkaline phosphatase, and pH = 7.3 for β-glucosidase) containing 5 mM (final concentration) of 4-nitrophenyl phosphate for phosphatase activities and 4-nitrophenyl-D-glucanopyranoside for β-glucosidase activity. The hydrolytic reaction was stopped after 10 min (acid phosphatase) and 20 min (alkaline phosphatase and β-glucosidase) of incubation at room temperature (22 °C) by addition of 50 µl of 0.1 M NaOH, and the absorbance of the 4-nitrophenol formed was read at 405 nm.

Lipase activity was determined according to Gupta et al. (2002) using 4-nitrophenyl palmitate (4-NPP) as the substrate. Hydrolysis rate of 4-NPP (1.6 mM, final concentration) was performed in an incubation medium composed of 230 µl of 50 mM Tris-HCl (pH = 8.0) containing 0.4% (w/v) Triton X-100 and 0.1% (w/v) Arabic gum, and 20 µl of tissue homogenate (or 50 µl of gut content). The formation of 4-nitrophenolate was monitored for 10 min at 22 °C at 412 nm and quantified using an external curve produced with 4-nitrophenol.

Carboxylesterase activity was measured using 1-naphthyl butyrate (1-NB) as the substrate and following the discontinuous assay by Thompson (1999). The reaction mixture contained 0.1 M Tris–HCl (pH = 7.4), 2 mM 1-NB and the sample, and was incubated for 10 min at 22 °C and continuous agitation. The hydrolysis of 1-NB was stopped by adding 50 µl of a solution made dissolving 0.1% Fast Red ITR in 2.5% (w/v) SDS and 2.5% Triton X-100. Microplates were left in the dark for 30 min for color development. The specific carboxylesterase activity was calculated using an external curve made with 1-naphthol (Sigma-Aldrich. Madrid, Spain). Kinetic assays were run in triplicate and read using an Asys HiTech UVM340 plate reader (Asys HiTech GmbH, Eugendorf, Austria). Blanks (i.e., sample-free reaction media) were included in the assays to discount non-enzymatic generation of the reaction products.

Digestive enzyme activities were also measured in the luminal content following the same procedures as described above. However, the enzyme assays were performed using the water extracts of the luminal content. These extracts were prepared as follows: 0.2 g wet luminal content were mixed for 30 min with 4.0 ml distilled water using an orbital

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shaker (Elmi® Intelli-mixer RM-2L, 25 rpm). Each tube was then shaken manually before immediate removal of 1.25-ml of the suspension with a Handystep® Brand repeating pipette. Aliquots (50 µl for carboxylesterase activity, or 100 µl for phosphatases, β-glucosidase and lipase activities) of the aqueous suspension of luminal content were poured into 96-well flat bottom microplates containing the corresponding buffer solution and substrate. The enzyme assays proceeded as described above for tissue. Specific activity was expressed on the basis of total organic carbon content of the luminal material (Skjemstad and Baldock 2008).

Biomarkers of chlorpyrifos exposure

The activity of AChE, BChE, and carboxylesterase was determined as indicators of chlorpyrifos exposure. The cholinesterase activities were measured according to the method by Ellman et al. (1961), adapted to the 96-well microplate format by Wheelock et al. (2005). Reaction medium (200 µl) was composed of 0.1 M Na phosphate buffer (pH = 8.0), 320 µM 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), 3 mM acetylthiocholine iodide (AChE activity) or butyrylthiocholine iodide (BChE activity), and the sample. Kinetics were read for 10 min (1-min intervals) at 412 nm and room temperature (22 °C). The specific cholinesterase activity was expressed as nmol of product min^{-1} mg^{-1} of total protein. To this, a calibration curve produced with DTNB and serial concentrations of reduced glutathione (6.25–100 µM) allowed quantification of the product formed during hydrolysis of substrates (Eyer et al. 2003), and protein concentrations were determined using the Bradford method (Bradford 1976) with bovine serum albumin as the standard. Carboxylesterase activity was determined using the ester substrate 1-NB as described above for the gastrointestinal tissues.

Native PAGE electrophoresis

Non-denaturing polyacrylamide gel electrophoresis (native-PAGE) was used to compare the profiles of carboxylesterase isoforms between tissue and treatments. 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. The in-gel staining esterase activity was performed according to the method by Manchenko (2002). Gels were incubated for 10 min (continuous agitation) with a staining solution (filtered immediately before use) composed of 3 mM 1-NB and 0.5 mg ml^{-1} Fast Blue RR in 0.1 M Na-phosphate buffer (pH = 7.2). Stained gels were scanned using a Gel Doc™ EZ Imager system (Bio-Rad Laboratories, Hercules, CA, USA) and the protein bands were identified using the Image Lab software system (version 3.0.1, Bio-Rad Laboratories). A standard of native proteins (NativeMark™ Protein Standard, Invitrogen) covering molecular weights from 20 to 480 kDa was used to compare the relative migration and estimate the apparent native molecular mass of carboxylesterase isozymes.

In vitro inhibition of lipase activity

We found acid phosphatase, β-glucosidase and lipase activities significantly depressed in the luminal content of chlorpyrifos-exposed earthworms, so we tested whether such an inhibition was caused by a direct interaction between the enzyme and the pesticides. Preliminary assays incubating tissue homogenates or water suspensions of the luminal content with 200 µM (final concentration) chlorpyrifos-oxon showed, however, that only the lipase activity was directly inhibited by this pesticide. Therefore, we tested whether this inhibitory response was dose-dependent. Samples were incubated in the presence of serial concentrations of chlorpyrifos-oxon (3.8×10^{-9} to 3.0×10^{-4} M, final concentration) for 30 min at 20 °C. The residual enzyme activity was measured as described above, and the percentages of residual lipase activity were plotted against the logarithmic molar concentration of chlorpyrifos to obtain the pesticide concentration that caused 50% inhibition of the initial enzyme activity (IC_{50}). These dose-dependent curves were adjusted to the exponential kinetic model proposed by Estevez and Vilanova (2009), which assumes two sensitives and one resistant component of enzyme activity. For fixed times of inhibition (30 min), the kinetic model is expressed as follows:

$$E = E1_0 \times e^{-k_{i1} \times t \times I} + E2_0 \times e^{-k_{i2} \times t \times I} + E_R$$

where $E1_0$ and $E2_0$ are the sensitive enzyme activities (initial enzyme activity) expressed in percentage of the total enzyme activity, E_R is the resistant fraction of enzyme activity, k_{i1} and k_{i2} are the inhibition rate constants, t is the inhibition time (min), and I is the molar concentration of chlorpyrifos-oxon. The IC_{50} values at 30 min were also calculated for each enzymatic component according to the equation (Estevez and Vilanova 2009):

$$IC_{50} = \frac{Ln(2)}{k_i \times 30}$$

Chlorpyrifos residue analysis

Nominal concentration of chlorpyrifos was checked by extraction of the pesticide from soil using QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) method,

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as adapted for soil samples by Asensio-Ramos et al. (2010), and subsequent quantification by high-pressure liquid chromatography system (Agilent 1200 Series) as described in Sanchez-Hernandez et al. (2017). The concentration of the pesticide was also measured at the end of the incubation time (30 d) to determine the persistence of chlorpyrifos during our microcosm experiment.

Statistical analysis

The non-parametric Mann-Whitney *U* test was used to detect significant differences in the ranges of enzyme activities between the chlorpyrifos-exposed and control earthworm groups. A *post hoc* analysis of the powerful of the statistical test outcomes was performed according to Mayr et al. (2007) and using the free software GPower (www.gpower.hhu.de).

Results and discussion

Response of digestive enzyme activities to chlorpyrifos

Tissue acid phosphatase, alkaline phosphatase and β-glucosidase activities did not change after chlorpyrifos exposure (Fig. 1), except the lipase activity that decreased in the foregut tissue ($p = 0.025$, $r = 0.57$) (Fig. 2a). Moreover, acid phosphatase ($p = 0.001$, $r = 0.97$) and lipase ($p = 0.021$, $r = 0.72$) activities decreased significantly in the luminal content of the pesticide-exposed earthworms (Figs. 1a and 2a). Although we found the luminal β-glucosidase activity also decreased in the earthworms exposed to chlorpyrifos, the magnitude of such response was not statistically significant compared with controls ($p = 0.080$, $r = 0.41$, Fig. 1c). Similarly, the luminal carboxylesterase activity was lower in the pesticide-exposed earthworms (0.123 ± 0.034 μmol h^{-1} g^{-1} organic C, $n = 8$) than in the control earthworms (0.165 ± 0.064 μmol h^{-1} g^{-1} organic C, $n = 8$), although no statistically significant ($p = 0.062$). Conversely, alkaline phosphatase activity increased in the luminal content of chlorpyrifos-exposed earthworms compared with control earthworms ($p = 0.041$, $r = 0.42$) (Fig. 1b). These findings suggest that digestive enzymes in the luminal content were more sensitive to chlorpyrifos than those located in the gastrointestinal tissues. On the other hand, test soils had a basic pH after 30-d incubation with earthworms ($pH = 8.04 \pm 0.03$ for chlorpyrifos-free soils and 8.10 ± 0.06 for chrlorpyrifos-treated soils, mean ± SD, $n = 8$), which would facilitate chlorpyrifos decomposition generating chlorpyrifos-oxon. Although this metabolite was not detected in the chromatographic analysis of our soils, we postulated that the presence of chlorpyrifos-oxon in the

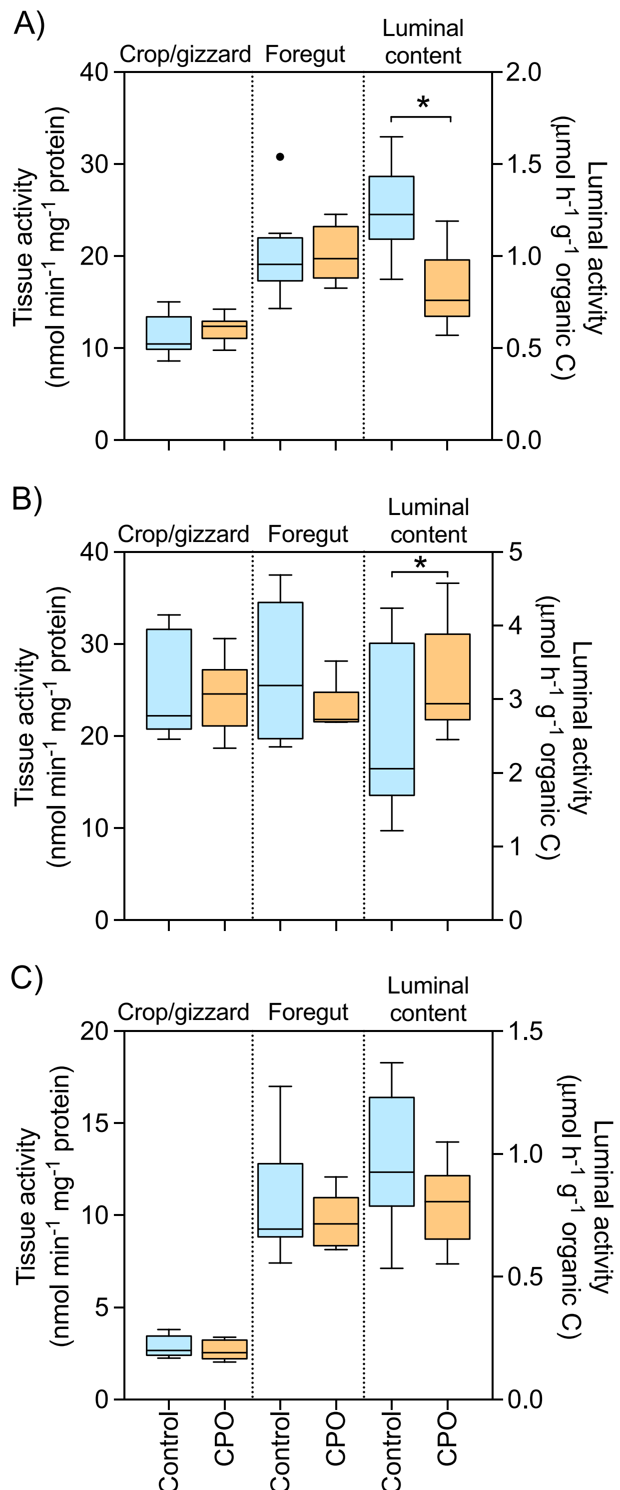

Fig. 1 Response of acid phosphatase (**a**), alkaline phosphatase (**b**) and β-glucosidase (**c**) activities measured in the gastrointestinal tissue (crop/gizzard and foregut) and luminal content of earthworms ($n = 8$) incubated for 30 d in chlorpyrifos-free (control) and chlorpyrifos-treated soils (CPO, 5 mg kg^{-1} wet soil). Tukey box plots indicate the median, the 25th and 75th percentiles (box edges), the range (whiskers) and outliers (black dots). Significant differences between treatments are indicated by asterisks ($p < 0.05$, Mann-Whitney test)

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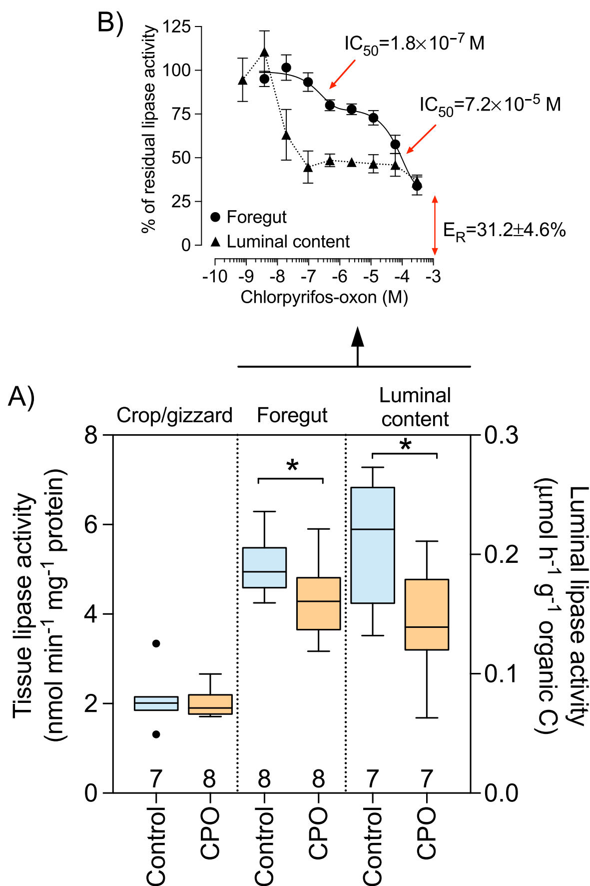

Fig. 2 **a** Response of lipase activity in the gastrointestinal tissue (crop/gizzard and foregut) and luminal content of earthworms incubated for 30 d in chlorpyrifos-free (control) and chlorpyrifos-treated soils (CPO, 5 mg kg^{-1} wet soil). Tukey box plots as shown in Fig. 1, and sample sizes indicated at the bottom. **b** *In vitro* dose-dependent curves in the presence of serial concentrations of chlorpyrifos-oxon (3.8×10^{-9}–3.0×10^{-4} M). IC_{50} median inhibitor concentration, ER enzyme resistant fraction to pesticide inhibition

ingested soils by earthworms could inhibit digestive enzyme activities in the luminal environment of earthworm gut. Moreover, the presence of serine hydrolases such as carboxylesterases and lipases in the luminal content of earthworms could reduce chlorpyrifos-oxon bioavailability to microbes as well as to limit its uptake through the gut epithelium. This assumption is supported by the study of Sanchez-Hernandez et al. (2014), who showed that the luminal carboxylesterase activity of the earthworm *Aporrectodea caliginosa* acted as a bioscavenger for chlorpyrifos-oxon, reducing its gastrointestinal uptake.

We incubated the gastrointestinal tissue homogenates and the aqueous extracts of the luminal content with serial concentrations of chlorpyrifos-oxon to provide evidence for a direct interaction between lipase activity and this pesticide. We found a dose-dependent relationship between chlorpyrifos-oxon and the foregut lipase activity, which was significantly fitted ($r^2 = 0.95$, $p = 0.002$) to a two-phase exponential decay kinetic (Fig. 2b). This model allowed us to distinguish two chlorpyrifos-sensitive lipase fractions ($IC_{50} = 72.3$ µM and $IC_{50} = 182$ nM), and a resistant fraction (31.2% of residual lipase activity). Such a dose-dependent relationship was not observed, however, for the lipase activity measured in the crop/gizzard tissue. In this case, percentages of enzyme inhibition varying between 30 and 48% of controls were registered at high pesticide concentrations (6.0×10^{-5} and 1.5×10^{-3} M). Likewise, luminal lipase activity was more sensitive than that measured in the tissue (foregut or crop/gizzard), with percentages of enzyme inhibition of 60% at chlorpyrifos-oxon concentrations as low as 10^{-7} M. Despite this high sensitivity, the response of luminal lipase activity to the pesticide could not be fitted to the non-lineal model (Fig. 2b). Taken together, these results suggest that chlorpyrifos could interact with earthworm digestive enzymes in two plausible ways. First, the decreased acid phosphatase and β-glucosidase activities in the luminal content of pesticide-exposed earthworms could be due to an indirect effect of chlorpyrifos via inhibition of microbial activity. In fact, some studies have shown that chlorpyrifos inhibits soil microbial proliferation (Pozo et al. 1995; Kadian et al. 2012; John and Shaike 2015). Second, inhibition of lipase activity took place via direct interaction between chlorpyrifos-oxon and the enzyme. This result is not surprising because lipases from other organisms such as mammals are sensitive to inhibition by a wide range of OP compounds (Quistad et al 2006). However, our results are the first to provide solid evidence through both *in vivo* and *in vitro* experiments that earthworm lipase activity is sensitive to inhibition by chlorpyrifos, being a potential non-neural biomarker of OP exposure.

The fact that earthworm digestive enzyme activities were sensitive to chlorpyrifos, suggests that the digestive function could be committed in a scenario of soils polluted by OP pesticides. However, earthworm weight varied between 5.73 ± 0.76 g ($t = 0$ d) and 5.05 ± 0.45 g ($t = 30$ d) in the control group, and between 5.51 ± 0.47 g ($t = 0$ d) and 4.87 ± 0.59 g ($t = 30$d) in the chlorpyrifos-treated group. Therefore, no significant changes in the body weight were detected between treatments at both sampling times ($p > 0.28$). Nonetheless, there was a slight, but statistically significant, decrease in the body weight (12% compared with $t = 0$d) recorded at the end of the incubation time for both groups ($p = 0.017$ for the control group, and $p = 0.036$ for the chlorpyrifos-exposed group), which could not be attributed to the pesticide toxicity.

In the current study, the inhibition of the hydrolytic activity of serine hydrolases (carboxylesterase and lipase) by chlorpyrifos suggest that OPs could disrupt the earthworm digestive function, which could have direct implications at individual level. This topic has been a matter of concern in insect physiology. In the last decade, some

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studies have documented digestive enzymes of insect larvae as non-neurotoxic molecular targets of pesticides. For example, the digestive enzymes amylase, cellulose and lipase of the silkworm *Philosamia ricini* (Lepidoptera) were significantly inhibited after 24 h of exposure to spiked fresh leaves with 1.5 and 2.0 mg l^{-1} chlorpyrifos (Kalita et al. 2016). Likewise, the systemic neonicotinoid imidacloprid caused a significant decrease of trypsin, chymotrypsin and aminopeptidase activities is successive generations of *Cameraria ohridella* larvae collected from pesticide-treated trees (Stygar et al. 2013). The acid and alkaline phosphatase activities of *Spodoptera litura* (Lepidotera) larvae were sensitive to inhibition by chemical extracts from *Citrillus colocynthis* (Cucurbitaceae) that displayed insecticidal properties (Ponsankar et al. 2017). Taken together, these studies leave open the possibility of investigating digestive enzymes of insects as molecular targets for pest control (Rodrigues Macedo and Machado Freire 2011). In a comparable approach, our results encourage future studies to elucidate at what extend OP pesticides, and others, may disrupt digestive processes of non-target organisms with consequences for individual growth and development.

Response of cholinesterases and carboxylesterases to chlorpyrifos exposure

Cholinesterase and carboxylesterase activities were measured in the wall muscle and gastrointestinal tissues as biomarkers of OP exposure. We found a significant inhibition of both esterase activities in all tissues. These findings confirmed, therefore, the pesticide distribution in the earthworm body and the bioactivation of chlorpyrifos into chlorpyrifos-oxon, which displays a high affinity for the active site of these enzymes (Chambers et al. 2010). Both AChE and BChE activities were significantly inhibited (26–42% of controls, respectively) in the gastrointestinal tissues of chlorpyrifos-exposed earthworms (Fig. 3a, b). A 37% inhibition of muscle AChE activity was also found in the earthworms incubated in the chlorpyrifos-contaminated soils ($p = 0.002$, $r = 0.69$) (Fig. 3c). Butyrylcholinesterase activity was not measured in the muscle tissue because this cholinesterase is absented in *L. terrestris* (Rault et al. 2007). Despite soil chlorpyrifos concentrations decreased from 5.95 ± 1.06 mg active ingredient kg^{-1} wet soil (corresponding to 6.90 ± 1.18 mg kg^{-1} dry soil, mean ± SD) at $t = 0$ d to 1.05 ± 0.12 mg chlorpyrifos kg^{-1} wet soil (or 1.28 ± 0.14 mg kg^{-1} dry soil) at the end of the incubation period (30 d), inhibition of cholinesterase activity still persisted. These results support previous data from studies that demonstrated the recovery rate of both AChE and carboxylesterase activities following acute OP exposure takes several weeks to fully reverse their normal variation of

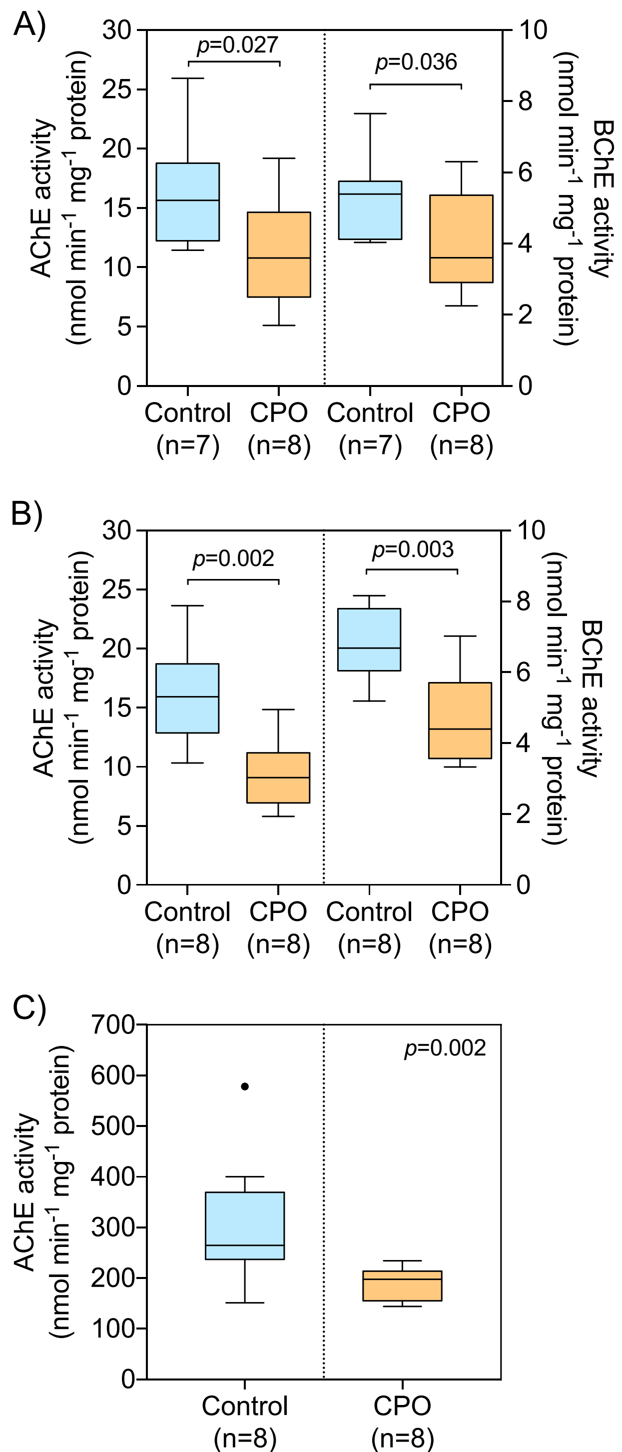

Fig. 3 Variation of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) activities in the crop/gizzard (**a**), foregut (**b**) and wall muscle (**c**) of earthworms ($n = 8$) incubated for 30 d in chlorpyrifos-free (control) and chlorpyrifos-treated soils (CPO, 5 mg kg^{-1} wet soil). Tukey box plots as shown in Fig. 1, with sample sizes indicated at the bottom and *p*-values (Mann-Whitney test) indicated at the top

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activity (Rault et al. 2008; Velki and Hackenberger 2013; Muangphra et al. 2015).

Chlorpyrifos had a higher impact on carboxylesterase activity of all target tissues than that on cholinesterase activities. Compared with controls, the percentages of carboxylesterase inhibition were 92, 67 and 42% for muscle, foregut and crop/gizzard, respectively (Fig. 4a). These marked differences in the enzyme inhibition could lead us to conclude that muscle carboxylesterase activity was more sensitive to chlorpyrifos, or even that the earthworm skin was the primary route of pesticide uptake. However, zymograms revealed a group of carboxylesterase isozymes with estimated molecular weights ranging between 20 and 66 kDa, which were present in all three tissues (wall muscle, crop/gizzard, and foregut) and were strongly inhibited by chlorpyrifos (Fig. 4b). But, the unexpected finding was to find a group of heavier molecular weight esterases (66–480 kDa) that only were present in the gastrointestinal tissues and showed resistance to chlorpyrifos inhibition. The absence of these isozymes in the muscle would explain the apparent higher inhibition degree of muscle carboxylesterase activity detected by enzyme assay only. These results suggest, therefore, the need of including zymographic analysis of post-electrophoresed polyacrylamide gels as a complementary tool in the use of carboxylesterases as biomarkers of susceptibility and exposure to OP pesticides.

Conclusions

Current results show that a sublethal and environmentally realistic concentration of chlorpyrifos in soil led to a significant decrease of digestive enzyme activities of *L. terrestris*. In the case of acid phosphatase and β-glucosidase activities, this effect was caused probably by the toxic action of the pesticide on gut symbiont microbiota. However, in the case of lipase activity, the inhibition response was attributed to a direct interaction between chlorpyrifos-oxon and the enzyme as in vitro outcomes revealed. These results add to others already published on the impact of chlorpyrifos on the

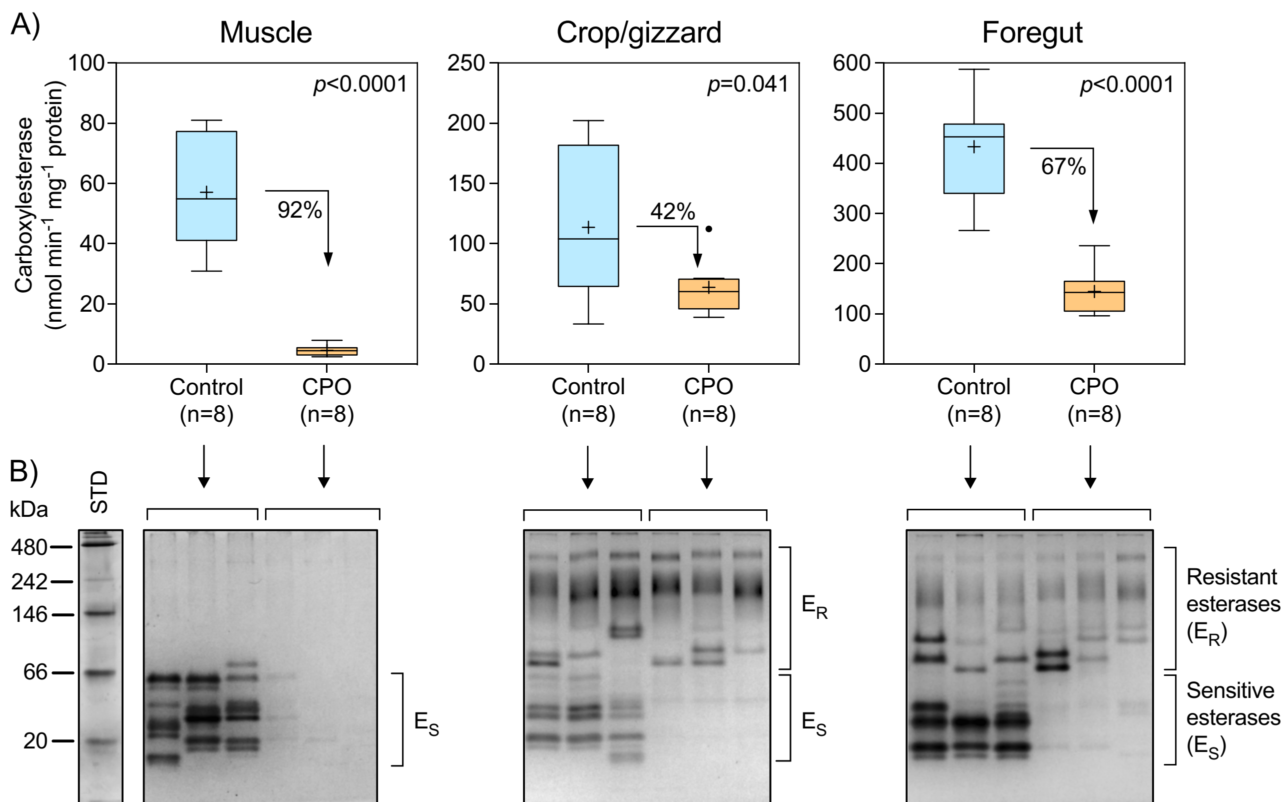

Fig. 4 **a** Variation of carboxylesterase activity in the wall muscle and gastrointestinal tissues of earthworms ($n = 8$) incubated for 30d in chlorpyrifos-free (control) and chlorpyrifos-treated soils (CPO, 5 mg kg^{-1} wet soil). Tukey box plots as shown in Fig. 1, with *p*-values (Mann-Whitney test) indicated at the top, and inhibition percentages of the mean enzyme activity (means showed by "+") indicated with arrows. **b** Electrophoresis gels showing the pattern of carboxylesterase isozymes of tissue homogenates (3 samples per treatment) after incubation of the post-electrophoresed gels in the presence of 1-naphthyl butyrate and Fast Blue RR. Protein load was 11.7–20.0 μg (wall muscle), 13.9–23.6 μg (crop/gizzard) and 10.0–16.0 μg (foregut). Protein standard contained soybean trypsin inhibitor (20 kDA), bovine serum albumin (66 kDa), lactate dehydrogenase (146 kDa), B-phycoerythrin (242 kDa), and apoferritin (480 kDa), and was visualized with Coomassie brilliant blue (CBB) staining

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gastrointestinal carboxylesterase activity of *L. terrestris* (Sanchez-Hernandez et al. 2014), suggesting that digestive toxicology of OP pesticides in these soil organisms may have direct implications in the digestion of lipid materials. Moreover, among the digestive enzymes analyzed in this study, the lipase activity may be used as a biomarker of OP exposure because of its high sensitivity and dose-dependent response to OP exposure. Current results also suggest that determination of carboxylesterase inhibition as sensitive biomarkers of OP exposure should be assisted by zymography of post-electrophoresed polyacrylamide gels, because sensitive of the multiple isozymes do not always correspond to inhibition degree detected in pesticide-exposed earthworms, leading to erroneous conclusions about the route of pesticide uptake.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors, according to the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes (Official Journal of the European Union L 276/33, 20-10-2010).

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