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Use of Biomarkers in Earthworms to Detect Use and Abuse of Field Applications of a Model Organophosphate Pesticide

L. H. Booth, S. Hodge, K. O'Halloran

CENTOX (Centre for Environmental Toxicology), Landcare Research, Post Office Box 69, Lincoln 8152, New Zealand

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Sublethal biomarkers are now used routinely in environmental monitoring to examine the toxicity of chemicals to nontarget species and have been successfully applied in a wide range of species including birds (Johnston 1995), fish (Adams et al. 1990), and invertebrates (Lagadic et al. 1994). Here we evaluate two enzyme biomarkers, cholinesterase (ChE) and glutathione S-transferase (GST), in the common pasture earthworm *Aporrectodea caliginosa*, as tools for environmental monitoring of soil contamination.

Cholinesterases are a group of enzymes in the central nervous system that are inhibited by organophosphate and carbamate pesticides. These enzymes have been routinely used as a biomarker in vertebrate and invertebrate species to diagnose organophosphate poisoning (Edwards and Fisher 1991; Fairbrother et al. 1991). Glutathione S-transferase is a family of enzymes involved in detoxification of xenobiotics including pesticides. In insects, GST activity is induced by a number of chemicals including organophosphates (Lagadic et al. 1993), and in earthworms, GST activity has been induced in *Pheretima posthuma* by the pesticides lindane, endosulfan, and aldrin (Hans et al. 1993).

Chlorpyrifos is an organophosphate insecticide commonly used in Canterbury, New Zealand, to control grass grub and porina caterpillar. Cholinesterase and GST have both been evaluated as biomarkers in *A. caliginosa* under controlled laboratory conditions, where both biomarkers were significantly affected by a predetermined concentration of chlorpyrifos (28 mg/kg) (Booth et al. 1998). Cholinesterase activity was inhibited by up to 90% compared with controls, while GST activity was induced by 148% compared with controls after 14 days exposure.

As laboratory data are often used to predict the potential impacts of pesticides on earthworm populations in agricultural land and to establish "safe" values for pesticides in terrestrial ecosystems, it is important to validate laboratory data with field studies. To determine the potential effects of chlorpyrifos on ChE activity in the field, ChE activity was evaluated in the laboratory after exposure to a laboratory simulated field rate of chlorpyrifos (4 mg/kg). Cholinesterase activity was significantly inhibited by 35% compared with the control at this concentration (*unpub. data*). In subsequent field exposures, ChE activity was investigated using mesocosms, and in natural earthworm populations. However, no inhibition of ChE activity occurred in response to

Correspondence to: L. H. Booth

chlorpyrifos application at recommended field rates for pasture (Booth et al. 2000). However, there was also an absence of effects on earthworm mortality or abundance, which indicates that chlorpyrifos was not toxic to this earthworm species at these application rates. In order to examine more closely the links between laboratory and field data, a field experiment was conducted at both normal and artificially high application rates to correlate with the concentrations used in the laboratory experiments. These field results were compared with those obtained in controlled laboratory experiments, and findings are discussed with regard to the use of ChE activity in this species as a biomarker of chlorpyrifos exposure.

MATERIALS AND METHODS

The pesticide tested in this experiment was the organophosphate chlorpyrifos (Lorsban 40EC, DowElanco NZ, New Plymouth, NZ). Water was used as a control. Pesticide residues in soil samples were analysed by gas chromatography with mass spectroscopy by contract to LincLab. Chlorpyrifos residues were determined by solvent extraction, followed by a solid phase cleanup, and analysis by Capillary Column Gas Chromatography Mass Spectrometry (GCMS). This method is based on AOAC method 29.013. Recovery for chlorpyrifos in soil was found to be quantitative (90%).

The field site consisted of an established pasture dominated by perennial rye grass (*Lolium perenne*) with some white clover (*Trifolium repens*) lying on a Templeton silt loam with an average carbon content of 3.5% $\pm$ 0.06 SE of dry weight and a nitrogen content of 0.29% $\pm$ 0.004 SE; n = 6 in both cases. There was no history of agrochemical use on the plot for at least 2 yr. The pasture was grazed by sheep prior to the experiments being performed, which produced a sward height at the beginning of the trials of approximately 50 mm.

To allow comparison of the results from laboratory and field experiments, the predicted environmental concentration (PEC) of chlorpyrifos in the soil after application at recommended field application rate was determined. This concentration could then be compared with the concentrations used in the laboratory experiments. Chlorpyrifos typically does not penetrate the soil below 13 mm (Kuhr and Tashiro 1978), therefore when chlorpyrifos is applied at the recommended application rate of 0.8 kg a.i./ha, the PEC or the maximum concentration of chlorpyrifos that would be found in the top 13 mm of soil is 6.15 g/m^3 which equates to 4 mg/kg. Previous laboratory exposures were conducted at a sublethal concentration (28 mg/kg) determined in preliminary experiments (Booth et al. 1998). This concentration is seven fold higher than the PEC. Therefore in the present experiment, chlorpyrifos was applied at the recommended rate for pasture given by the manufacturer and also at a high rate (7 x field rate) equivalent to that used in the laboratory experiments. For both application rates, the pesticide was applied in 200 L of water / ha using a conventional spray boom at a pressure of 300 kPa. Each of the three spray treatments (field rate, high rate, and water control) was replicated five times. The 15 plots were arranged in a 5 x 3 Latin square design. Each plot was 10 x 10 m and there were 4-m margins between rows, between columns, and around the boundary. These plots were used for both the mesocosm and the natural population experiments.

Samples were taken from the top 10 cm of soil from representative mesocosms and plots at 7, 14, and 28 d after spraying to monitor degradation of the pesticides in soil over time. To assess if there were any systematic differences in soil properties across the experimental area, a sample was taken from the top 10 cm of soil from each plot on each sampling occasions. Soil pH, moisture content, combustible organic content, and chlorpyrifos concentration of the soil were measured.

Mesocosms consisted of 250-mm lengths of 4-mm-thick polyurethane culvert pipe with a 200-mm diameter. The base of each pipe was covered with stainless steel mesh (pore size 537µm, Mico Wakefield, Christchurch, NZ) fixed in position using a galvanized steel screw-tightened band. Two mesocosms were sunk into the centre 1 m^2 of each plot, leaving 50 mm of pipe above the soil surface. The mesocosms were filled to ground level with a natural Templeton silt loam soil collected close to the field sight. Seven days prior to the plots being sprayed with pesticides, 20 juvenile *A. caliginosa* were added to each pipe and a covering of loose wheat straw placed on the soil surface. All mesocosms were covered with stainless steel mesh (as above) held in position with adhesive tape. Before pesticide application, the mesh lids were removed to allow the spray to reach the soil surface unimpeded. After spraying, the lids were replaced and sealed with fresh tape. One pipe from each plot was collected 7 d after spraying and the remaining mesocosms collected 14 d after spraying. The mesocosms were returned to the laboratory and the soil was sorted by hand. Missing worms were assumed to be dead. To establish the efficiency of this hand sorting, 12 samples were sorted a second time. This showed that the first sorting recovered 92.4 ± 1.5% SE of the worms. Retrieved worms were frozen at -80°C for subsequent enzyme biomarker analysis. Earthworms from each pipe were divided into two groups and assayed for ChE or GST activity.

To examine responses in wild populations, two 20-cm sided cubes of soil were taken from each plot using a spade at 7, 14, and 28 d after spraying, returned to the laboratory and sorted by hand. Earthworms of the species *A. caliginosa* were categorized into age class (juvenile or adult), weighed, and juveniles were frozen at -80°C for subsequent enzyme analysis.

Frozen samples of earthworms were defrosted on ice. The samples for determination of ChE activity were homogenized in ice-cold phosphate buffer containing 0.02 M potassium dihydrogen phosphate (BDH), pH 7.5. After a 60 min settling period on ice, the crude homogenate was used for analysis. Cholinesterase activity was determined using a method based on that described by Ellman et al. (1961), adapted for use in earthworms and minimized for microtitre plates. In summary, acetylthiocholine iodide (15 mM final concentration) was added to the reaction mixture containing homogenate and 5,5',-dithio-bis-2-nitrobenzoic acid (DTNB). Acetylthiocholine iodide is hydrolysed by cholinesterase and releases thiocholine, which reacts with DTNB to produce a yellow anion, which was detected spectrophotometrically at 405 nm. Cholinesterase activity is expressed as nmoles acetylthiocholine hydrolysed/min/mg protein.

For GST activity, earthworm samples were homogenized in ice-cold phosphate buffer containing 0.02 M potassium dihydrogen phosphate plus 2 mM glutathione (reduced form, GSH) (Sigma), pH 7.0. Analysis was conducted using the supernatant following

centrifugation of homogenate at 15,000 rpm for 5 min. Glutathione S-transferase activity was determined by the method of Habig et al. (1974), adapted for earthworms and minimized for microtitre plates. The substrate 1-chloro-2,4-dinitrobenzene (CDNB) was added to the reaction mixture containing GSH and homogenate. The GST catalyses the conjugation of CDNB to glutathione producing S-(2,4-dinitrophenyl)glutathione, and enzyme activity was monitored spectrophotometrically at 340 nm. Activity is expressed as nmoles GSH conjugated/min/mg protein.

Samples were analysed for protein content using the Bradford method (Bradford 1976), with bovine serum albumin (BSA, Sigma Chemical Co., St Louis, MO, USA) as a standard.

Data were analysed by repeated measure analysis of variance to test for treatment and day effects. Comparisons of means were made using the Bonferroni method.

RESULTS AND DISCUSSION

There were no differences between plots allocated to different pesticide treatments with regard to soil water content, organic content, or pH ($F_{2,36} = 0.09$, $P = 0.92$ [water content]; $F_{2,36} = 0.95$, $P = 0.40$ [pH]; $F_{2,36} = 1.5$, $P = 0.21$ [organic content]). The organic content of the soil did not change between sampling dates, being consistently around 8.5% of dry weight ($F_{2,36} = 3.02$, $P = 0.60$). Water content remained at around 25 to 27% on the first two sampling occasions but dropped significantly, by approximately 4%, on the last sampling occasion ($F_{2,36} = 65.42$, $P < 0.0001$). The soil pH was approximately 6.0, and did not change over the 28-d experiment.

Pesticide residue analysis, which commenced at the time the first earthworm samples were taken (at 7 d) showed no degradation of chlorpyrifos over the 7-28 d sampling period indicating that this pesticide is immobile and reasonably persistent in soil (Cox 1995). Soil residues in the field rate plots ranged from 0 - 0.09 mg/kg, with a mean of 0.05 ± 0.02 mg/kg, while residues in the high rate plots ranged from 0.23 - 1.32 mg/kg (mean of 0.74 ± 0.21 mg/kg). Residue analysis of the mesocosm samples also showed no degradation of pesticide over the sampling time. Chlorpyrifos concentrations of 0.32 - 1.18 mg/kg (mean 0.73 ± 0.19 mg/kg were found in the field rate plots, while in the high rate plots, concentrations ranged from 1.4 - 6.4 mg/kg (mean 3.72 ± 1 mg/kg).

There was no effect of pesticide treatment on earthworm mortality in the mesocosms after 14 days ($F_{2,12} = 0.75$, $P = 0.50$). In the natural populations , the overall density of *Aporrectodea caliginosa* did not vary significantly with time ($F_{2,132} = 0.025$, $P = 0.98$), being approximately 500/m^2 on all sampling occasions. There were no differences in the abundance of adult and juvenile earthworms or their biomass due to pesticide exposure. These results are in agreement with previous field trials on chlorpyrifos (Culy and Berry 1995; Kring 1969; Thompson and Sans 1974).

Glutathione S-transferase activity in the mesocosm earthworms was not affected by either rate of pesticide exposure ($F_{2,22} = 1.18$, $P = 0.33$) or by time ($F_{1,22} = 0.10$, $P = 0.75$) (Table 1) and similar results were found in the natural earthworm populations (Table 2). Therefore this enzyme is considered to be not particularly useful as a

Table 1. Effect of exposure to chlorpyrifos in mesocosms on GST activity (nmoles GSH conjugated/min/mg protein) in laboratory-reared juvenile *A. caliginosa.* (mean ± SE, n = 5).

Exposure treatment	Day 7	Day 14
Control	139.5 ± 24.5	117 ± 7.5
Field rate	115.4 ± 9.3	156.3 ± 27.5
High rate	125.1 ± 11.15	92.7 ± 23.1

Table 2. Effect of exposure to chlorpyrifos on GST activity (nmoles GSH conjugated/min/mg protein) in natural populations of juvenile *A. caliginosa.* (mean ± SE, n = 5).

Exposure	Day 7	Day 14	Day 28
Control	206.5 ± 20.3	217.5 ± 18.2	215.2 ± 25.5
Field rate	196.8 ± 16.5	214 ± 23	202 ± 18.2
High rate	180.3 ± 12.1	210.1 ± 18.9	178.7 ± 13

biomarker of organophosphate exposure in this earthworm species at the application rates used here.

While there was no effect of time on ChE activity, earthworms exposed to chlorpyrifos showed significant inhibition of activity in both the mesocosms ($F_{2,22} = 3.59$, $P < 0.05$) (Figure 1) and in the natural earthworm populations ($F_{2,12} = 8.72$, $P < 0.005$) (Figure 2). Cholinesterase activity was inhibited by up to 31% compared to control activity in earthworms exposed to the high rate of pesticide in the mesocosms. Juvenile earthworms sampled from the natural populations exhibited up to 45% enzyme inhibition compared to control activity and showed no sign of recovery by day 28. These results are comparable to the inhibition that occurred under laboratory conditions, where ChE activity in juvenile laboratory-bred *A. caliginosa* was inhibited by up to 90% at 28 mg/kg (Booth et al. 2000). At the normal application rate in the field there was no effect on ChE activity, a result which is consistent with a previous field experiment using this system (Booth et al. 2000).

This research highlights the similarities and discrepancies between effects shown in the laboratory and those found in the field situation. Although ChE activity is inhibited at the simulated field rate in the laboratory (*unpub. data*), no effects were observed in field earthworms exposed to the equivalent rate. Similarly, the biomarker response measured in earthworms under laboratory conditions due to the high application rate was greater than that experienced in the field. This may be due, in part, to the differences in environmental influences over chlopyrifos degradation in the soil. However, the results also highlight that inhibition of cholinesterase in *A. caliginosa* is a far more sensitive indicator of pesticide exposure than is mortality or reduction in population density. This biomarker therefore has potential to detect a sublethal biochemical response, which is not only more sensitive than estimating earthworm densities in the field, it is

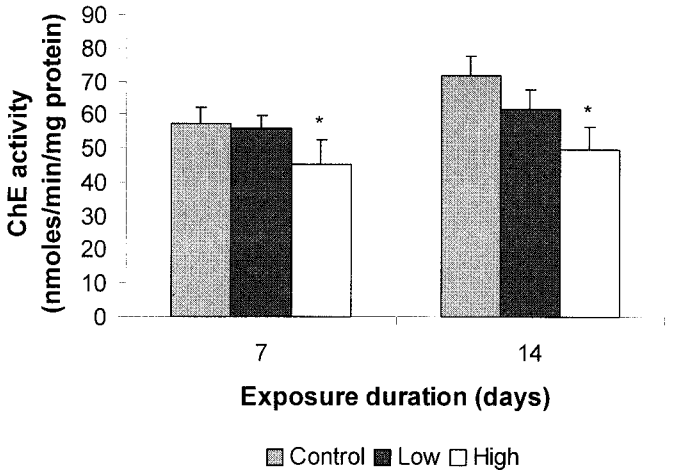

Figure 1. Effect of exposure to chlorpyrifos in mesocosms on ChE activity in laboratory-reared juvenile *A. caliginosa* (mean ± SE, n = 5).
*, $P < 0.05$

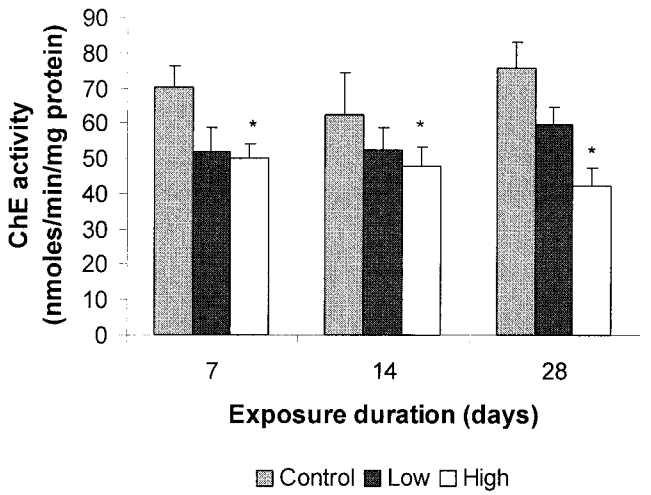

Figure 2. Effect of exposure to chlorpyrifos on ChE activity in natural populations of *A. caliginosa* (mean ± SE, n = 5).
* $P < 0.05$

also more reliable for decision making . Field densities can vary for many reasons and are difficult to assess correctly, while biochemical inhibition is easier both to measure and to link to the presence of a pollutant. However, due to the lack of effects at recommended field application rates of this pesticide, the toxicological significance of this response is limited.

In conclusion, inhibition of ChE activity in *A. caliginosa* is a sensitive, rapid, and lasting response to chlorpyrifos exposure when compared with mortality or abundance. However, due to the lack of effects at recommended field application rates, it is of limited use for detecting ecotoxiological effects of exposure to chlorpyifos in the field. We therefore propose that this biomarker may only be useful for detecting excessive overuse/abuse of this organophosphate, particularly as recovery of ChE activity to control levels appears to be retarded.

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