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Lysosomal neutral red retention time as a biomarker of organophosphate exposure in the earthworm *Aporrectodea caliginosa*: laboratory and semi-field experiments

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Aporrectodea caliginosa is the most common endogeic (topsoil) earthworm in New Zealand and, because of its habitat, is potentially vulnerable to surface-applied pesticides. Lysosomal damage to earthworms, which can be visualized by the use of the neutral red retention assay (NRRA) has been evaluated in this species as a biomarker of organophosphate exposure. Earthworms were exposed in the laboratory to sub-lethal concentrations of chlorpyrifos and diazinon. In a semi-field experiment, earthworms were placed in mesocosms in a field sprayed with these pesticides at the rate recommended for a vegetable crop. In the laboratory, the neutral red retention time (NRRT) was significantly reduced following exposure to both pesticides compared with controls. In the semi-field experiment, earthworm NRRT was significantly reduced by both pesticides. These experiments have shown that the NRRA is very sensitive to exposure to chlorpyrifos and diazinon even at field rates. It therefore shows promise as a potential biomarker of contamination of soil by organophosphates.

Keywords: earthworms, biomarker, neutral red retention time, laboratory, semi-field.

Abbreviations: NRRT, neutral red retention time; PAHs, polycyclic aromatic hydrocarbons.

Introduction

The use of agrochemicals and other environmental contaminants has the potential to damage agro-ecosystems and wildland resources in New Zealand. Sensitive biomarkers for environmental monitoring are being developed in the earthworm *Aporrectodea caliginosa* Savigny that will enable detection of deleterious environmental contamination. *Aporrectodea caliginosa* was chosen as an indicator species in New Zealand because of its widespread occurrence in arable and pasture lands. This species inhabits that topsoil and hence is potentially vulnerable to surface applied pesticides, which makes it an ideal candidate for assessment of agro-ecosystem contamination.

Pollutants can impact on an organism in a variety of ways, and often the earliest changes can be detected at the cellular or subcellular level. Lysosomes are organelles which are primarily responsible for digestive processes, and are involved in the uptake and sequestration of xenobiotics (Moore 1990). The use of neutral red is a well-established technique for evaluating cell toxicity (Borenfreund and Puerner 1984) and has been used to test the cytotoxicity of a wide range of compounds including pesticides, solvents, and pharmaceuticals (Babich and Borenfreund 1990). Neutral red is a membrane-permeable dye that diffuses

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through the cellular membranes into the acidic lysosomal compartment where it is protonated to become a membrane impermeant cation (Lowe *et al*. 1992). This allows the levels of dye in the lysosome to reach higher concentrations than in the cytosol. The lysosomes maintain the low pH via proton pumps in the lysosomsal membrane, and if the efficiency of these pumps is reduced, the neutral red dye is deprotonized, allowing it to diffuse out into the cytosol, colouring it red (Seglen 1983). The assay therefore makes use of the fact that lysosomes in healthy, unstressed cells will retain the neutral red dye for long periods after uptake. In contrast, in stressed cells, dye will leak from the lysosomes into the cytoplasm more rapidly. The neutral red retention time (NRRT) assay has been used in earthworms to detect the biological impact of a variety of compounds including heavy metals and polycyclic aromatic hydrocarbons (PAHs), and has also been used for the detection of contaminants at contaminated sites (Svendsen *et al*. 1996, Weeks and Svendsen 1996, Eason *et al*. 1999). Eason *et al*. (1999) also exposed *Eisenia andrei* to the organophosphate chlorpyrifos and showed a significant reduction in NRRT. This paper builds on these findings using the common pasture worm *Aporrectodea caliginosa*.

The aim of this research was to determine in the laboratory the effects of the organophosphorous insecticides chlorpyrifos and diazinon at sub-lethal doses on the earthworm lysosomal membrane NRRT in *A. caliginosa* (Savigny), and to evaluate this biomarker (NRRA) in a controlled field experiment.

Materials and methods

Pesticides

The pesticides used were the organophosphates chlorpyrifos (Lorsban 40EC, DowElanco Limited NZ, New Plymouth, NZ) and diazinon (Basudin 600EW, Ciba-Geigy NZ Limited, Avondale, NZ). Both pesticides are commonly used on pasture and arable land in the Canterbury region of New Zealand, mainly to control grass grub (*Costelytra zealandica*) and porina caterpillars (*Wisean cervinata*).

Earthworms

A laboratory colony of *A. caliginosa* was derived from adult earthworms collected in Canterbury, New Zealand. Templeton silt loam (3.8% organic matter) collected from the Selwyn District, Canterbury, was dried at 30°C for 24 h to kill any extant earthworms and other macro-invertebrates. The soil was then rehydrated with distilled water to produce a moisture content of 25–30% by mass. Dry grass-meal was added at the rate of 15 g per kg dry soil to provide food for the earthworms. The pH of the reconstituted soil was 6.5–7. Adult earthworms were maintained in this soil in 1-litre glass jars. The soil was changed once every 4 weeks and cocoons were removed and maintained on wet filter paper until hatching. Juvenile earthworms were then placed in 10-litre plastic buckets containing the previously described soil, until required for experimentation. All earthworms were maintained at 20°C in the dark.

Laboratory experiments

Laboratory tests were adapted from the Earthworm Acute Toxicity Tests (OECD 1984) using Templeton silt loam as described above and adjusted to 25–30% moisture with either distilled water or an aqueous solution of pesticide. In two separate experiments (one for each pesticide), earthworms were exposed to 500 g of pesticide-contaminated soil in 500 ml glass jars. Pesticide was prepared as an aqueous solution, added to soil up to a moisture content of 25% and mixed thoroughly to ensure a homogeneous mixture. Sub-lethal concentrations of pesticide, viz 60 mg diazinon per kg soil and 28 mg chlorpyrifos per kg soil respectively, were used. These values had been determined in a previous experiment (Booth *et al*. 1998). For each treatment there were four replicate jars, each with five adult earthworms and the NRRT in these earthworms was compared with controls containing no pesticide. Treatments were randomly allocated to jars. The start of the experiment was staggered by placing earthworms in one replicate jar from each treatment on each of the first four days of the week. The jars were maintained in an incubator at 20°C and received constant light. After 7 days' exposure, the earthworms were removed and the NRRT for each individual earthworm was determined.

Semi-field experiment

The semi-field experiment was conducted in December (summer) 1998 at a site consisting of a paddock of a commercially grown variety of dwarf green beans (*Phaseolus vulgaris* L.). Chlorpyrifos and diazinon were applied at the recommended rate for a vegetable crop given by the manufacturers instructions: 1.3 l in 200 l of water per ha for diazinon and 0.5 l in 200 l of water per ha for chlorpyrifos. Pesticides were applied using a conventional spray boom at a pressure of 300 kPa.

The paddock was divided into 18 plots of 10 × 10 m, with a 4 m boundary between them and around the boundary. Each of the three treatments (control, chlorpyrifos, and diazinon) were replicated six times and were arranged in two square blocks of nine plots. Each treatment (see below) was randomly allocated to each plot in each block. Adult laboratory-bred earthworms were placed into mesocosms consisting of 250 mm lengths of 4 mm thick polyurethane culvert pipe with a 200 mm bore. The base of each pipe was covered with a 300 mm × 300 mm square of stainless steel mesh (pore size 537 m; Mico Wakefield, Christchurch, NZ) that was bent around the pipe and fixed in position using a galvanized steel 'jubilee clip' (Paykel Ltd, Christchurch, NZ). The mesh was trimmed and the edges sealed with adhesive tape (the mesh was removed before spraying and replaced afterwards).

One pipe was sunk into the centre 1 m^2 of each plot, leaving 50 mm of pipe above the soil surface. The pipes were filled to ground level with a pre-prepared laboratory soil mixture to enable cross reference to laboratory assays. Five adult *A. caliginosa* were added to each pipe. The pipes were covered with a circle (205 mm diameter) of stainless steel mesh held in position with adhesive tape. Before pesticide application, the mesh lids were removed from the pipes to allow the spray to reach the soil surface unimpeded. After spraying, the lids were replaced and sealed with fresh tape.

Due to the number of samples, removal of pipes after 7 days was staggered over 3 days, so that on each day, two randomly chosen pipes for each treatment were removed for sampling. The pipes were returned to the laboratory, and the soil sorted by hand and retrieved earthworms analysed for NRRT.

NRRT biomarker assay

A neutral red working solution (80 mg ml^{-1}) was prepared from a stock solution by diluting in physiological Ringer (Lockwood 1963). Coelomic fluid was collected from the earthworm by inserting a 26 gauge needle containing 50 µl of physiological Ringer into the coelomic cavity posterior to the clitellum and allowing it to fill by intracoelomic pressure and a gentle drawing action on the syringe. Twenty µl of coelomic fluid was placed onto a slide, and 20 µl of dye solution was added, and the two mixed and then covered with a cover slip. Microscope slides were kept in a humidity chamber at room temperature when not under observation. Each slide was scanned at 5 min intervals and the number of stained and unstained cells counted at each scan. This was continued until 50 % of the cells showed leakage of the lysosomal membrane, or for 60 min, whichever event occurred first.

Statistics

NRRT data were treated as censored survival data because recording stopped at 60 min. A simple survival model (the exponential) was fitted to data from each jar, giving estimates of a parameter and its variance for each replicate. These were then fed into a normal-based experimental design model with two variance components: the within-replicate variation, taken as the variances estimated in the survival analyses, and a between-unit component requiring estimation. Models were fitted using maximum likelihood, and likelihood ratio tests were used to compare different models. Replicates with all earthworms with retention times greater than 60 min were removed from the analyses as parameters could not be estimated. Three models were fitted for each data set: the null model, assuming all three groups would give the same result; a model allowing the control to differ from the two treatments, and a model allowing all three groups to differ independently of each other. These were compared using likelihood ratio tests.

Results

Effect of chlorpyrifos and diazinon on NRRT in the laboratory exposures

Exposure to chlorpyrifos significantly reduced the NRRT from a mean of 52 min for controls to a mean of 21 min for exposed earthworms ($\chi^2_1 = 11.65$, $P < 0.005$) (figure 1). Diazinon also significantly reduced NRRT compared with controls ($\chi^2_1 = 8.74$, $P < 0.005$) (figure 2). There was no evidence of any differences in responses between sampling times for either pesticide ($\chi^2_1 = 0.34$, $P = 0.56$ [chlorpyrifos], $\chi^2_1 = 1.02$, $P = 0.32$ [diazinon]).

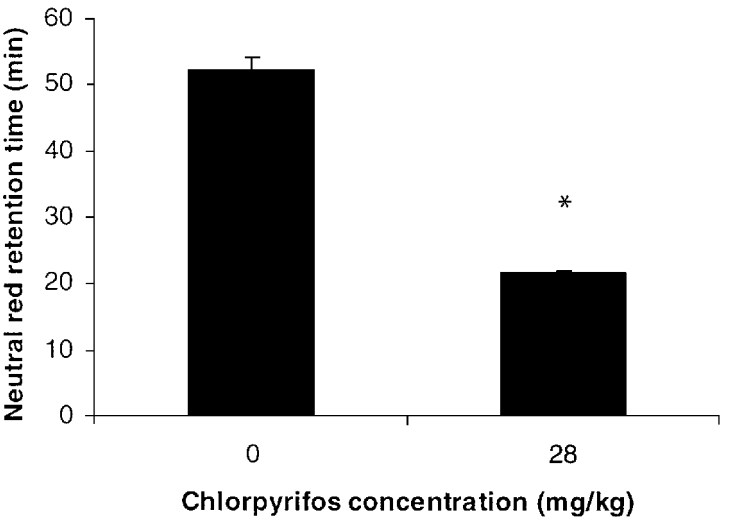

Figure 1. Neutral red retention time in *Aporrectodea caliginosa* exposed to a sub-lethal dose of chlorpyrifos (mean ± SE). * Values differ significantly from control ($P < 0.005$).

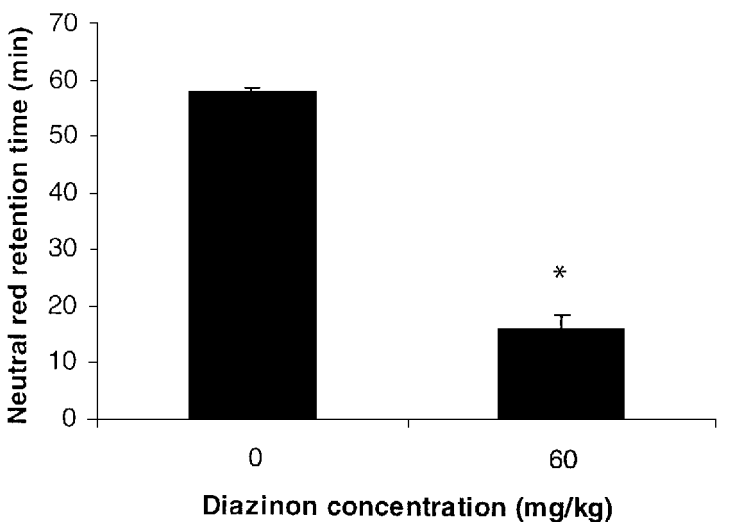

Figure 2. Neutral red retention time in *Aporrectodea caliginosa* exposed to a sub-lethal dose of diazinon (mean ± SE).* Values differ significantly from control ($P < 0.005$).

Effect of chlorpyrifos and diazinon on NRRT in the semi field exposures

Exposure to both pesticides significantly reduced NRRT compared with controls ($\chi^2_2 = 9.63$, $P < 0.005$), but there were no significant differences between the two pesticides ($\chi^2_2 = 0.48$, $P = 0.49$) (figure 3), and there was no temporal effect ($\chi^2_2 = 0.98$, $P = 0.61$).

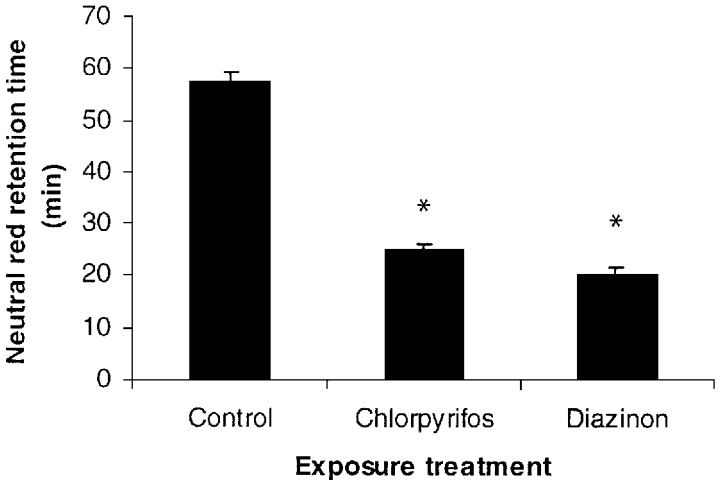

Figure 3. Neutral red retention time in *Aporrectodea caliginosa* exposed to chlorpyrifos and diazinon in the semi-field experiment (mean ± SE). * Values differ significantly from control ($P < 0.005$).

Discussion

Lysosomal stability (measured by the neutral red retention time) as a potential biomarker of pesticide contamination was investigated in the common earthworm species *Aporrectodea caliginosa*, following exposure to two widely used organophosphate insecticides, chlorpyrifos and diazinon. Both pesticides reduced NRRT in the laboratory exposures and this response appeared to be similar for the two pesticides. These results confirm the findings of Eason *et al*. (1999) which also demonstrated that this assay was sensitive to exposure to organophosphorous pesticides. In the semi-field experiment, NRRT was significantly reduced at the normal field application rate for both pesticides. To allow direct comparison of the laboratory and field results, the maximum concentration of pesticide to which earthworms were likely to be exposed in the semi-field experiment was calculated based on the amount of pesticide applied per hectare and the assumption that the pesticide would not penetrate the soil below 1.3 cm (Kuhr and Tashiro 1978). These calculations resulted in maximum concentrations of chlorpyrifos and diazinon of approximately 1 and 4 mg kg^{-1}, respectively, which is 28- and 15-fold lower than their respective sub-lethal concentrations in laboratory exposures. These concentrations would be found in the top 1.3 cm of the soil only, as neither of these pesticides are very mobile in the soil. The exposure of the earthworms in the laboratory experiments is continuous, while this would not be the case in the semi-field experiment as earthworms can move away from the pesticide. This indicates that this assay is very sensitive to these pesticides and detects a response in the earthworms at concentrations far below the highest sub-lethal concentration for each pesticide. Due to this sensitivity, this biomarker could be developed for use in a field situation for detecting environmental contamination by pesticides. The NRRT assay also appears to be sensitive to non-chemical stressors such as hypothermia, osmotic shock, and dietary depletion in marine organisms (Moore 1985). In order to apply this biomarker in the field, further research is needed to assess the effects of environmental factors (e.g. moisture content, temperature, soil type, and soil pH) on the NRRT response in earthworms.

Although the NRRT assay has considerable potential as a biomarker of organophosphate exposure, in order to be useful as an early warning indicator of an adverse impact on earthworm populations it needs to be linked to ecologically relevant endpoints such as growth and fecundity. Previous field studies conducted with chlorpyrifos and diazinon have reported that these pesticides are not toxic in the short-term to earthworm populations in the field (Booth *et al*. 2000), but long-term impacts, such as effects on fecundity are currently being determined.

In conclusion, the neutral red retention assay shows considerable potential as a biomarker of soil contamination by chlorpyrifos and diazinon and is being further developed as a biomarker for organophosphate impacts in agroecosystems.

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