SETAC PRESS

Environmental Toxicology and Chemistry, Vol. 20, No. 11, pp. 2494–2502, 2001

Printed in the USA
0730-7268/01 $9.00 + .00

# A COMPARISON OF BIOMARKER RESPONSES IN THE EARTHWORM *APORRECTODEA CALIGINOSA* TO THE ORGANOPHOSPHORUS INSECTICIDES DIAZINON AND CHLORPYRIFOS

LYNN H. BOOTH* and KATHRYN O'HALLORAN
CENTOX (Centre for Environmental Toxicology), Landcare Research, P.O. Box 69, Lincoln, Canterbury, New Zealand

(*Received* 20 *October* 2000; *Accepted* 20 *March* 2001)

**Abstract**—Three biomarkers in *Aporrectodea caliginosa* Savigny were evaluated for their ability to detect exposure to organophosphorus insecticides, and these physiological responses were related to effects on growth and life-table parameters. Adult and juvenile earthworms were exposed to a laboratory-simulated field rate (low concentration) and a higher sublethal concentration of diazinon and chlorpyrifos. After a four-week exposure, juveniles were evaluated for cholinesterase activity, glutathione *S*-transferase activity, and growth, and adults were evaluated for the lysosomal neutral red retention assay (NRRA) and growth. Cholinesterase activity and the NRRA were more sensitive than growth in each age group for detecting exposure to the pesticides. Life-table parameters were evaluated in earthworms exposed as juveniles and as adults. Maturation was less sensitive to pesticides than was cocoon production. Growth and cocoon production in earthworms exposed as juveniles appeared to be more sensitive to organophosphorus insecticides than earthworms exposed as adults. Life-table responses in juveniles may, therefore, be more predictive of long-term impacts of organophosphorus insecticide applications on populations than responses in adults. Biomarker responses occurred at similar or lower concentrations than those causing an adverse effect on cocoon production and cocoon viability, indicating their usefulness in risk assessment for predicting ecologically relevant assessment end points.

**Keywords**—Earthworms Biomarkers Fecundity Growth

## INTRODUCTION

The application of man-made chemicals to the environment has resulted in the need for development of methods to assess, monitor, and mitigate their impacts. Until recently, the most common end point measured when evaluating toxicity of chemicals to earthworms was mortality. While mortality (measured as the LC50, or the concentration that is lethal to 50% of individuals) values are widely used, they can provide only a measure of short-term acute toxicity and are not always useful for predicting the ecological consequences of exposure to a particular chemical, e.g., as seen with reproduction, where effects are observed at concentrations well below the LC50 value [1]. The use of biomarkers in environmental monitoring is now becoming a routine method for examining toxicity of chemicals [2]. Biomarkers can provide information on the potential adverse impacts of contaminants and can act as early warning signals of impending environmental damage.

*Eisenia fetida* Savigny is the standard test earthworm recommended for use in terrestrial ecotoxicology testing [3], but its occurrence is limited to sites rich in organic matter, such as dung heaps, and hence it is not an ideal species for extrapolation of laboratory data to field conditions. *Aporrectodea caliginosa* Savigny is widespread throughout both Northern and Southern Hemisphere countries and is the most common earthworm in New Zealand. It is found in arable and pasture land [4,5], and due to its habitat in the topsoil, it is vulnerable to surface-applied pesticides. This increases its ecological relevance compared with *E. fetida,* which makes it an ideal candidate for assessment of the potential impact of agrochemicals in the field.

* To whom correspondence may be addressed (boothl@landcare.cri.nz).

The biomarkers evaluated here are cholinesterase (ChE) activity, which has been used to detect organophosphorus insecticide exposure in a variety of species [6–8]; glutathione *S*-transferase (GST) activity, which is involved in the detoxification of xenobiotics [9]; and the lysosomal neutral red retention assay (NRRA), which gives a measure of membrane stability by measuring the retention time of neutral red (NRRT) within lysosomes. The NRRA makes use of the fact that lysosomes in healthy, unstressed cells will retain the neutral red dye for long periods after uptake, which results in a high NRRT. In contrast, in stressed cells, dye will leak from the lysosomes into the cytoplasm more rapidly, leading to a lower NRRT value. This assay has been used as a biomarker of heavy metal and polyaromatic hydrocarbon contamination [10,11]. However, for these biomarkers to be applied in a useful manner for predicting the impacts of contaminants in the field, they need to be linked to long-term effects on the organism.

To evaluate long-term effects of chemicals on earthworms, growth and assessment of life-table parameters have proved to be sensitive parameters. Life-table parameters include effects on maturation of juveniles, cocoon production, and cocoon viability. Cocoon production can be adversely affected by exposure to contaminants at concentrations that do not affect adult mortality. This means that, even though population densities of earthworms may not be immediately affected by a chemical exposure, adverse reproductive consequences may subsequently result in a reduction in population density. In addition, cocoon viability or hatching success are more sensitive to pesticide exposure than is mortality [12].

To date, most earthworm toxicity testing has been conducted using adults. However, juveniles are often more sensitive to toxic chemicals than are adults [13]. Furthermore, contaminants can affect growth and sexual development in

juvenile earthworms at concentrations less than those affecting adults [14,15]. Consequently, the use of toxicity data obtained exclusively from adult specimens to evaluate ecotoxicological risk may underestimate the effects of pollutants on soil invertebrate populations.

The aim of this research was to compare the effects of diazinon and chlorpyrifos on the three biomarkers ChE, GST, and the NRRA and to evaluate their potential usefulness in a field situation by relating the biomarker response to long-term effects on the organism, such as growth and fecundity.

## MATERIALS AND METHODS

### *Pesticides*

The pesticides tested in these experiments were the organophosphorus insecticides diazinon (Basudin 600EW [60% a.i.], Novartis, Auckland, NZ) and chlorpyrifos (Lorsban 40EC [40% a.i.], Dow Elanco, New Plymouth, 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*). Tap water was used as a control.

### *Invertebrates*

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

### *Preparation of soil*

Laboratory tests were adapted from the Organization for Economic Cooperation and Development (OECD), Paris, France, guideline 207 [3] using the natural soil described above in place of artificial soil. Earthworms were exposed to a high sublethal concentration of diazinon (60 mg/kg) or chlorpyrifos (28 mg/kg), as determined in previous experiments (data not shown), and to a lower (laboratory-simulated field rate) concentration for each pesticide and were compared with water controls containing no pesticide. The low concentrations were based on estimates of the amount of pesticide that earthworms would be exposed to in the field following application of the pesticides at manufacturers' recommended rates. The calculations assumed that the pesticide would not penetrate the soil below 13 mm [16]; therefore, when diazinon is applied at the maximum field rate of 4 L of Basudin® (600EW, Novartis) per hectare, the maximum concentration in the soil would be 18.46 g a.i./$m^3$, which equates to 12 mg/kg (dry wt). When chlorpyrifos is applied at the field rate of 2 L of Lorsban (40EC, Dow Elanco) per hectare, the maximum concentration in the soil would be 6.15 g a.i./$m^3$, which equates to 4 mg/kg. The desired amount of pesticide was thoroughly mixed into soil as an aqueous solution to give the working concentration of pesticide, and soil was placed into glass jars. During all experiments, jars were maintained in an incubator at 20°C with constant light. Moisture content was checked weekly and maintained at 25% by adjusting the weight of the jar against the weight known from the previous week prior to sampling.

### *Assessment of biomarkers and life-table parameters*

A series of four experiments was conducted to evaluate the responses of juvenile and adult earthworms to diazinon and chlorpyrifos. Those experiments were biomarkers and growth in juveniles, biomarkers and growth in adults, life-table parameters in juveniles, and life-table parameters in adults.

### *Assessment of biomarkers and growth in juveniles*

Cholinesterase and GST activities were measured only in juveniles. In two separate experiments (one for diazinon and one for chlorpyrifos), 500 g of prepared soil was placed into 500-ml jars. Ten juvenile *A. caliginosa* (one–three months old) were added to each of eight replicate jars for each pesticide treatment/exposure period, and these were compared with controls at each time point. After one-, two-, and four-week exposures, earthworms were removed from the relevant jars for that time point and frozen for subsequent ChE and GST activity analysis. Earthworms that were maintained for four weeks were weighed weekly to determine the effect of each pesticide on growth.

### *Enzyme biomarker assays*

Enzyme activity and protein concentrations were determined using a Reader 340 ATTC (SLT Instruments, Salzberg/Grodig, Austria) with 96-well microtiter plates (Corning, NY, USA) and the software 2000 package (SLT Instruments, Salzberg/Grodig, Austria). Frozen samples of earthworms were defrosted slowly on ice. The samples for determination of ChE activity were prepared by homogenization using an ultraturrax with a 5-mm element. Ten earthworms were homogenized in 4 ml of ice-cold phosphate buffer containing 0.02 M potassium phosphate (BDH, NZ), pH 7.5. After a 60-min settling period on ice, the crude homogenate was used for analysis. For GST activity, earthworm samples were homogenized in ice-cold phosphate buffer containing 0.02 M potassium phosphate plus 2 mM glutathione (reduced form, GSH) (Sigma Chemical, St. Louis, MO, USA), pH 7.0. Analysis was conducted using the supernatant following centrifugation of homogenate at 10,000 *g* for 5 min.

Cholinesterase activity was determined using a method based on that described by Ellman et al. [17] adapted for use in earthworms and minimized for microtiter plates. In summary, 15 mM acetylthiocholine iodide (final concentration) (Sigma) was added to the reaction mixture containing homogenate, 0.02 M potassium phosphate buffer, and 0.5 mM 5,5′-dithio-bis-2-nitrobenzoic acid (final concentration) (Sigma). Acetylthiocholine iodide is hydrolyzed by cholinesterase and releases thiocholine, which reacts with 5,5′-dithio-bis-2-nitrobenzoic acid to produce a yellow anion, which is detected spectrophotometrically at 405 nm. Cholinesterase activity is expressed as nmoles acetylthiocholine hydrolyzed/min/mg protein.

Glutathione *S*-transferase activity was determined by the method of Habig et al. [18], adapted for earthworms and minimized for microtiter plates. The substrate 1 mM 1-chloro-2,4-dinitrobenzene (final concentration) (Sigma) in ethanol (0.05% final concentration) was added to the reaction mixture con-

taining GSH and homogenate. The GST catalyzes the conjugation of 1-chloro-2,4-dinitrobenzene to glutathione, producing *S*-(2,4-dinitrophenyl) glutathione, and enzyme activity is monitored spectrophotometrically at 340 nm. Activity is expressed as nmoles GSH conjugated/min/mg protein.

Samples were analyzed for protein content using the Bradford method [19], with bovine serum albumin (BSA, Sigma) as a standard.

*Assessment of biomarkers and growth in adults*

The NRRA was measured only in adults. In two separate experiments (one for diazinon and one for chlorpyrifos), 1 kg of prepared soil was placed into 1-liter jars. Ten adult *A. caliginosa* were weighed and added to each of four replicate jars/treatment. The start of the experiment was staggered so that earthworms were placed in one replicate from each treatment on each of the first 4 d of the week. Earthworms were weighed weekly to determine the effect of pesticide on growth, and after four weeks, the NRRT for each earthworm was determined.

*Neutral red retention time assays*

A neutral red working solution of 80 mg/ml was prepared in earthworm physiological ringer solution [20]. Celomic fluid was collected from the earthworm by inserting a 25-g needle containing 50 μl of ringer into the celomic cavity posterior to the clitellum and allowing it to fill by intracelomic pressure and a gentle drawing action on the syringe. The celomic fluid was then placed onto a clean glass slide and mixed with an equal volume of neutral red solution before a cover slip was placed on top. Slides were scanned for 2 min at 5-min intervals and the number of stained and unstained cells counted. Microscope slides were kept in a humidity chamber when not under observation. The cells were counted until 50% of the cells were red or for 60 min. This time was recorded as the neutral red retention time.

*Effects on life-table parameters in juveniles*

Juvenile earthworms three months old and weighing 200 to 300 mg were exposed to pesticide-contaminated soil (Templeton silt loam) for 12 weeks and compared with controls. To each of four replicate jars for each treatment, 500 g of prepared soil and 10 earthworms were added. Three sets of four jars for each treatment were prepared so that the earthworms could be removed from the soil and placed into fresh contaminated soil monthly to allow 12 weeks of continuous exposure. The pesticide concentrations in each set of jars were analyzed prior to addition of earthworms to ensure the concentrations of each set of jars were the same. After 2, 4, 6, 8, 10, and 12 weeks of exposure, earthworms were weighed and maturity was determined from the presence of a fully developed clitellum. Soil was wet sieved at 4, 8, and 12 weeks to remove cocoons. Pesticide concentrations and pH were monitored to ensure conditions throughout the experiment were strictly controlled. Cocoons were placed into 90-mm petri dishes with wet filter paper and kept under controlled conditions until hatching (four–six weeks) and cocoon viability was calculated.

*Effects on life-table parameters in adults*

Adult earthworms of 500 to 600 mg were exposed to pesticide-contaminated soil for four weeks (exposure period) and compared with controls. To each of four replicate jars for each treatment, 1 kg of prepared soil and 10 earthworms were added. Earthworms were weighed weekly and, after four weeks, adult earthworms were removed from the pesticide-contaminated soil. To monitor recovery, adults were then placed in fresh soil (no pesticide) and maintained until growth and cocoon production were similar in all treatments. Earthworms were weighed every two weeks, and every four weeks, the soil was wet sieved to remove cocoons. Cocoons were placed into 90-mm petri dishes with wet filter paper and rinsed with fresh water every second day to prevent growth of mold on the cocoons. The cocoons were kept under controlled conditions until hatching (four–six weeks), and cocoon viability was calculated.

*Statistics*

Cholinesterase and GST activities were analyzed using ANOVA in SYSTAT (SPSS, Chicago, IL, USA) with treatment and day as factors, with post hoc comparisons of treatment means made using Tukey's test. Data were log transformed to normalize prior to analysis.

Data from the NRRA were treated as censored tolerance data because recording stopped at 60 min. A simple exponential model 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, i.e., 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. Three models were fitted for each data set, including the null model, assuming all three groups give the same results; a model allowing the control to differ from the two treatments; and a model allowing all three groups to differ. These were compared using likelihood ratio tests.

Growth and fecundity data were analyzed by repeated measures ANOVA in SYSTAT and the Huyn–Feldt epsilon was used to calculate *p*- values. Post hoc comparisons of treatment means were made using the Bonferroni adjustment to calculate *p*-values. Growth data were log transformed to normalize prior to analysis, and maturation data were transformed by arcsin (square root [x]) to normalize prior to analysis.

To determine the concentrations of each pesticide causing adverse effects on biomarker response, growth, and fecundity, data were analyzed using ANOVA with a post hoc Dunnett's test at a significance of $p < 0.05$.

Cocoon production by earthworms exposed to pesticide-treated soil for four weeks as juveniles or as adults was compared using calculated control means for each age group. The remaining treatments were then divided by the control mean, giving a measure of inhibition of cocoon production (relative to the mean for the control). Data were transformed by arcsin (square root[*x*]) prior to analysis.

Cocoon viability was analyzed by repeated measures ANOVA in S-PLUS 2000 (Data Analysis Products, MathSoft, Seattle, WA, USA). Pairwise comparisons of treatment means were made using the Bonferroni adjustment to calculate *p*-values.

## RESULTS

*Assessment of biomarkers and growth in juveniles*

Cholinesterase activity was inhibited by exposure to 60 and 12 mg/kg diazinon compared with controls ($F_{2,27} = 21.99$, *p*

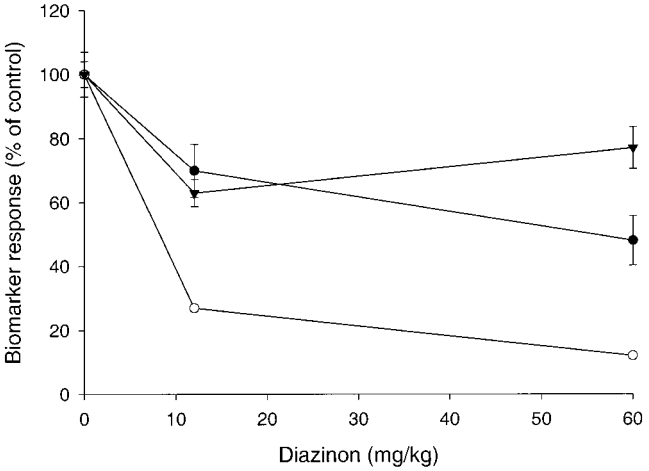


Fig. 1. Chlorinesterase (ChE) activity, glutathione *S*-transferase (GST) activity, and growth in juvenile *Aporrectodea caliginosa* after exposure to diazinon for four weeks (mean ± standard error). ○, ChE activity; ▼, GST activity; ●, growth.


Fig. 3. The neutral red retention assay (NRRA) and growth in adult *Aporrectodea caliginosa* after exposure to diazinon for four weeks (mean ± standard error). ○, NRRA; ●, growth.

$< 0.0005$), but there was no difference between activity at the two concentrations (Fig. 1). After four weeks of exposure, enzyme activity was inhibited by up to 90% compared with control activity (43.3 nmoles/min/mg protein) in earthworms exposed to 60 mg/kg diazinon (5.2 nmoles/min/mg protein) and by up to 75% compared with controls at 12 mg/kg (11.6 nmoles/min/mg protein). Diazinon exposure significantly inhibited GST activity in juvenile earthworms exposed to both 12 mg/kg (30.9 nmoles/min/mg protein) and 60 mg/kg (nmoles/min/mg protein) compared with controls (49.4 nmoles/min/mg protein) ($F_{2,27} = 540$, $p < 0.05$) (Fig. 1). Earthworms also experienced a concentration-dependent retardation of growth at both concentrations of diazinon ($F_{8,76} = 7.37$, $p < 0.0005$) (Fig. 1).

Chlorpyrifos acted similarly to diazinon, with dose-dependent inhibition of ChE activity of 70% (8.4 nmoles/min/mg protein) and 35% (18.9 nmoles/min/mg protein) for earthworms exposed to 28 and 4 mg/kg, respectively, compared with controls (24.8 nmoles/min/mg protein) ($F_{4,45} = 54.79$, $p < 0.0001$) (Fig. 2). This effect increased with time ($F_{2,45} = 25.03$, $p < 0.0001$), but this was not dependent on pesticide concentration. Chlorpyrifos had no effect on GST activity at either 4 mg/kg (98 nmoles/min/mg protein) and 28 mg/kg (84 nmoles/min/mg protein) compared with controls (101.4 nmoles/min/mg protein) ($F_{4,45} = 1.30$, $p = 0.28$) (Fig. 2). However, 28 mg/kg of chlorpyrifos had a significant effect on earthworm growth ($F_{2,21} = 7.11$, $p < 0.001$), but earthworms exposed to 4 mg/kg of chlorpyrifos were not significantly different from the control (Fig. 2).

### *Assessment of biomarkers and growth in adults*

The NRRT was significantly reduced in adult earthworms exposed to diazinon from 56 min in the control to 20 and 14 min, respectively, for 12 and 60 mg/kg diazinon ($\chi^2_1 = 26.41$, $p < 0.001$) (Fig. 3). Chlorpyrifos had a similar effect, with a reduction from 54 min in the controls to 26 and 18 min for 4 and 28 mg/kg chlorpyrifos, respectively ($\chi^2_1 = 20.36$, $p < 0.001$) (Fig. 4). Growth appeared to be reduced in a concentration-dependent manner by exposure to both diazinon ($F_{8,36} = 2.90$, $p < 0.05$) (Fig. 3) and chlorpyrifos ($F_{8,36} = 3.98$, $p < 0.005$) (Fig. 4). However, due to a high degree of measured

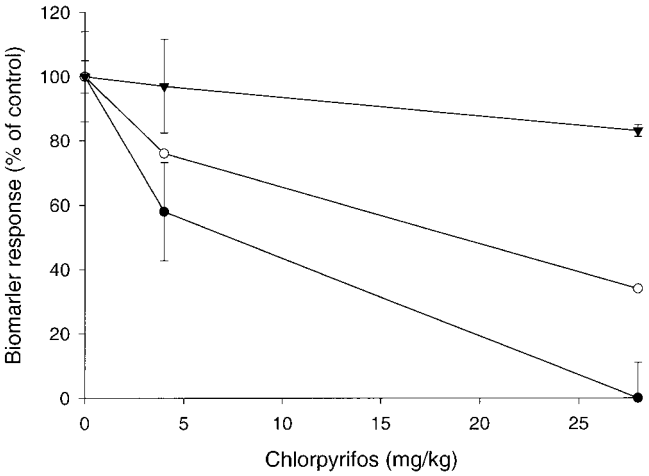


Fig. 2. Chlorinesterase (ChE) activity, glutathione *S*-transferase (GST) activity, and growth in juvenile *Aporrectodea caliginosa* after exposure to chlorpyrifos for four weeks (mean ± standard error). ○, ChE activity; ▼, GST activity; ●, growth.

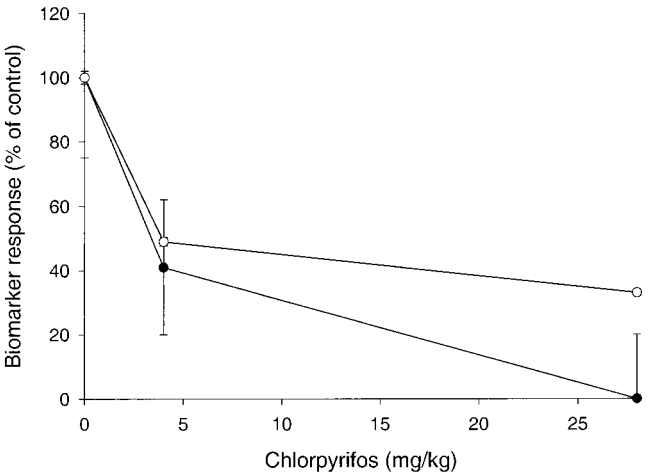


Fig. 4. The neutral red retention (NRRA) and growth in adult *Aporrectodea caliginosa* after exposure to chlorpyrifos for four weeks (mean ± standard error). ○, NRRA; ●, growth.

Table 1. Growth of juvenile *Aporrectodea caliginosa* exposed to diazinon and chlorpyrifos for 12 weeks (mean ± standard error)

| | Mean weight per earthworm (mg)[a] | | | |
|---|---|---|---|---|
| Treatment | Week 0 | Week 4 | Week 8 | Week 12 |
| Control | 257 ± 8 | 659 ± 78 | 599 ± 136 | 632 ± 73 |
| 12 mg/kg diazinon | 237 ± 40 | 512 ± 73A | 576 ± 68 | 576 ± 83 |
| 60 mg/kg diazinon | 247 ± 40 | 427 ± 58A | 462 ± 87A | 441 ± 114A |
| 4 mg/kg chlorpyrifos | 270 ± 17 | 562 ± 78 | 554 ± 86 | 394 ± 78A |
| 28 mg/kg chlorpyrifos | 256 ± 14 | 504 ± 34A | 468 ± 120A | 475 ± 44A |

[a] Significant differences ($p < 0.05$) between treatment and control are indicated for each week. Values with the same letter were significantly different from the control but not significantly different from each other.

variability in this end point, significant differences were only evident at the sublethal concentrations.

*Effects on life-table parameters in juveniles*

Pesticide exposure had a significant effect on growth in juvenile earthworms, though these effects varied with time ($F_{24,174} = 4.54$, $p = 0.0005$) (Table 1). After four and six weeks of exposure, growth differed from the control in all treatments except for 4 mg/kg chlorpyrifos ($p < 0.05$). However, after 10 weeks, growth was significantly lower at this field application rate of chlorpyrifos ($p < 0.05$). Growth in earthworms exposed to 12 mg/kg diazinon had recovered to control levels by eight weeks, while growth in earthworms exposed at the sublethal concentrations of diazinon and chlorpyrifos was slowed compared with the controls at all time points measured ($p < 0.05$).

Maturation of juvenile earthworms was influenced by exposure to pesticide, and this was also dependent on time ($F_{16,40} = 2.73$, $p < 0.01$) (Table 2). After four weeks of exposure, maturation was significantly different from the control at the sublethal concentration of both pesticides ($p < 0.001$), but not at the simulated field rates. Control earthworms matured more quickly than earthworms exposed to pesticide, but there was no significant difference between any treatments by week 10, and by week 12, all earthworms from all treatments were mature.

Cocoon production was significantly reduced by pesticide exposure ($F_{4,10} = 7.98$, $p < 0.005$) relative to the controls (Table 3). Cocoon production increased with time ($p < 0.0001$), and the difference in cocoon production in pesticide-exposed earthworms relative to controls also appeared to increase with time (as evidenced by square-root transformation of data), indicating that, on the original scale, treatment differences increased from week 4 to week 12. However, this effect was not significant ($F_{8,20} = 1.81$, $p = 0.135$). Overall cocoon production differed from the controls for all treatments except for 4 mg/kg chlorpyrifos ($p < 0.05$ [12 mg/kg diazinon], $p < 0.01$ [60 mg/kg diazinon, 28 mg/kg chlorpyrifos]).

Cocoon viability in juveniles exposed to pesticide was not evaluated due to very low hatching even in controls, likely due to growth of mold on cocoons. However, cocoon viability was able to be evaluated in cocoons produced in the adult exposure experiment by using improved procedures.

*Effects on life-table parameters in adults*

Growth in adult earthworms was significantly influenced by exposure to pesticides ($\chi^2_{14} = 88.61$, $p < 0.001$), with significant effects on growth in earthworms exposed to 60 mg/kg diazinon and 28 mg/kg chlorpyrifos after four weeks (Table 4). After a four-week recovery period, earthworm growth had returned to control levels in all pesticide-treated groups ($p > 0.05$ for all comparisons).

Cocoon production was significantly reduced by exposure to both pesticides ($F_{4,15} = 21.79$, $p < 0.001$) (Table 5). Earthworms produced fewer cocoons than the controls for all treatments except 4 mg/kg chlorpyrifos ($p < 0.05$ [12 mg/kg diazinon], $p < 0.0001$ [60 mg/kg diazinon], $p < 0.0005$ [28 mg/kg chlorpyrifos]). After a four-week recovery period, cocoon production was still significantly less than the control for all treatments except for 12 mg/kg diazinon ($p < 0.001$ [60 mg/kg diazinon], $p < 0.01$ [4 mg/kg chlorpyrifos], $p < 0.05$ [28 mg/kg chlorpyrifos]). However, after an eight-week recovery period, cocoon production of pesticide-exposed earthworms had returned to control levels.

Although cocoon viability could not be assessed during the four-week exposure period due to low success in controls and treatments, after four weeks of recovery, viability of cocoons from earthworms exposed to pesticides was significantly lower

Table 2. Maturation of juvenile *Aporrectodea caliginosa* exposed to diazinon and chlorpyrifos for 12 weeks (mean ± standard error)

| | Number of mature earthworms (%)[a] | | | | |
|---|---|---|---|---|---|
| Exposure treatment | Week 4 | Week 6 | Week 8 | Week 10 | Week 12 |
| Control | 74 ± 6 | 99 ± 1 | 100 ± 0 | 100 ± 0 | 100 ± 0 |
| 12 mg/kg diazinon | 31 ± 6 | 81 ± 6 | 86 ± 5 | 94 ± 2 | 100 ± 0 |
| 60 mg/kg diazinon | 4 ± 2A | 26 ± 8A | 37 ± 7A | 69 ± 7 | 100 ± 0 |
| 4 mg/kg chlorpyrifos | 34 ± 5 | 79 ± 7 | 90 ± 4 | 94 ± 3 | 100 ± 0 |
| 28 mg/kg chlorpyrifos | 17 ± 7A | 62 ± 8 | 77 ± 8 | 87 ± 6 | 100 ± 0 |

[a] Significant differences ($p < 0.05$) between treatment and control are indicated for each week. Values with the same letter were significantly different from the control but not significantly different from each other.

Table 3. Cocoon production by adult *Aporrectodea caliginosa* derived from juveniles exposed to diazinon and chlorpyrifos for 12 weeks (mean ± standard error)

| | Time (weeks) | | | |
|---|---|---|---|---|
| Treatment | Week 4 | Week 8 | Week 12 | Overall[a] |
| Control | 0.6 ± 0.09 | 5.6 ± 0.98 | 5.7 ± 1.42 | 4.0 ± 0.83 |
| 12 mg/kg diazinon | 0 ± 0 | 1.27 ± 0.59 | 0.93 ± 0.43 | 0.73 ± 0.34A |
| 60 mg/kg diazinon | 0.10 ± 0.07 | 0.97 ± 0.56 | 0.37 ± 0.17 | 0.50 ± 0.29A |
| 4 mg/kg chlorpyrifos | 0.33 ± 0.27 | 3.1 ± 0.71 | 2.4 ± 0.54 | 1.9 ± 0.51 |
| 28 mg/kg chlorpyrifos | 0 ± 0 | 0.3 ± 0.12 | 0.6 ± 0.12 | 0.3 ± 0.08A |

[a] Overall significant differences ($p < 0.05$) between treatment and control are indicated. Values with the same letter were significantly different from the control but not significantly different from each other.

than the controls ($p < 0.05$ [12 mg/kg diazinon], $p < 0.01$ [60 mg/kg diazinon], $p < 0.005$ [28 mg/kg chlorpyrifos]) (Table 5). Like cocoon production, cocoon viability had recovered by week 8 of the recovery period.

*Comparison of cocoon production*

To compare the sensitivity of cocoon production in earthworms exposed to pesticide as juveniles or as adults, the number of cocoons produced per adult was expressed as a percent of the control. Cocoon production in adults exposed as juveniles to pesticide for four weeks was significantly lower than in earthworms exposed as adults, but this was dependent on treatment ($F_{3,20} = 6.03$, $p < 0.005$). Juveniles exposed to the simulated field rates of each pesticide subsequently produced fewer cocoons than did earthworms exposed as adults ($p < 0.01$ [diazinon], $p < 0.005$ [chlorpyrifos]). Diminished cocoon production in earthworms exposed to the high concentration of each pesticide was similar for both age groups.

## DISCUSSION

Three biomarkers and two life-stages of *A. caliginosa* were assessed for their relative sensitivities when exposed to two organophosphorus insecticides in common use in New Zealand, diazinon and chlorpyrifos.

Cholinesterase activity was strongly inhibited by both pesticides and was sufficiently sensitive to detect pesticide levels comparable with those applied to fields at recommended rates. Activity was inhibited rapidly (within one week) and did not show any return to preexposure levels even after four weeks, indicating that the biomarker response is continuously depressed in the presence of the contaminant and is therefore a mechanistic marker. At the equivalent of the recommended field application rate, ChE activity was inhibited by up to 70% by diazinon and 24% by chlorpyrifos compared with controls. This apparent difference in potency could be due to the diazinon field rate having a threefold higher concentration of active ingredient than that for chlorpyrifos. Pesticide-induced effects on growth in juvenile earthworms were observed at both concentrations of diazinon but only at the higher concentration of chlorpyrifos. To compare the relative sensitivity of growth and biomarker response as indicators of pesticide exposure, the lowest level of pesticide applied that produced an effect was compared (Table 6). For ChE activity, the concentration of chlorpyrifos that caused a significant effect was lower than that for growth (4 mg/kg for ChE and 28 mg/kg for growth), while for diazinon, concentrations affecting ChE activity and growth were the same (12 mg/kg). However, as Figure 1 illustrates, the impacts of diazinon on ChE activity in juveniles are far greater than those on growth (70% inhibition of ChE activity compared with 30% reduction in growth). Therefore, ChE activity is a more responsive indicator than is growth for detecting exposure to low concentrations of diazinon and chlorpyrifos. The mechanism of biocidal action of the organophosphorus insecticide compounds is via inhibition of ChE enzymes, resulting in abnormalities in neurotransmission and subsequent paralysis and death of the target organism. It is likely that the same sequence of events is occurring in the earthworm, where, once ChE is inhibited below a certain threshold, interference with normal neural function may be translated into an inhibition of growth.

Induction of GST activity has been reported in the earthworm *Pheretima posthuma* exposed to pesticides (lindane, aldrin, and endosulfan) [21], but Stokke and Stenersen [22] have reported that GST activity is not inducible in earthworms by known inducers of GST activity. The current research confirmed that GST activity was not induced by the two organ-

Table 4. Growth of adult *Aporrectodea caliginosa* exposed to diazinon and chlorpyrifos for four weeks and recovery after removal to clean soil (mean ± standard error)

| | Mean weight per earthworm (mg) | | | |
|---|---|---|---|---|
| | Exposure period | | Recovery period | |
| Treatment | Week 0 | Week 4[a] | Week 8 | Week 12 |
| Control | 708 ± 21 | 1,007 ± 26 | 1,347 ± 44 | 1,163 ± 78 |
| 12 mg/kg diazinon | 701 ± 15 | 889 ± 20 | 1,071 ± 34 | 1,136 ± 18 |
| 60 mg/kg diazinon | 772 ± 48 | 870 ± 63A | 1,078 ± 27 | 1,036 ± 51 |
| 4 mg/kg chlorpyrifos | 715 ± 30 | 943 ± 42 | 1,089 ± 74 | 1,117 ± 62 |
| 28 mg/kg chlorpyrifos | 677 ± 8 | 837 ± 12A | 948 ± 75 | 1,096 ± 46 |

[a] Significant differences ($p < 0.05$) between treatment and control exist only in week 4. Values with the same letter were significantly different from the control but not significantly different from each other.

Table 5. Cocoon production and cocoon viability in *Aporrectodea caliginosa* exposed as adults to diazinon and chlorpyrifos for four weeks and recovery after removal to clean soil (mean ± standard error)[a]

| | Exposure period | | Recovery period | | | |
|---|---|---|---|---|---|---|
| | Week 4 | | Week 8 | | Week 12 | |
| Treatment | Cocoons per adult | Cocoon viability | Cocoons per adult | Cocoon viability | Cocoons per adult | Cocoon viability |
| Control | 8.7 ± 1.0 | 19 ± 7 | 12.8 ± 1.6 | 70 ± 8 | 9.8 ± 0.4 | 67 ± 6 |
| 12 mg/kg diazinon | 4.3 ± 0.6A | 2 ± 1 | 10 ± 1.6 | 35 ± 3A | 9.5 ± 0.5 | 57 ± 9 |
| 60 mg/kg diazinon | 0.9 ± 0.2A | 11 ± 11 | 6.1 ± 1.2A | 31 ± 5A | 8.6 ± 1.3 | 69 ± 5 |
| 4 mg/kg chlorpyrifos | 4.9 ± 0.3 | 15 ± 5 | 8.3 ± 2.1A | 59 ± 2 | 7.4 ± 0.2 | 69 ± 6 |
| 28 mg/kg chlorpyrifos | 2.7 ± 0.5A | 12 ± 6 | 7.1 ± 1.1A | 29 ± 4A | 7.3 ± 0.4 | 78 ± 3 |

[a] Significant differences ($p < 0.05$) between treatment and control are indicated for each week. Values with the same letter were significantly different from the control but not significantly different from each other.

ophosphorus insecticides used in this study, and in fact, diazinon inhibited GST activity. Atrazine has been shown to inhibit GST activity at low concentrations, while at high concentrations, induction has been observed [23]. Therefore, diazinon and chlorpyrifos may cause induction at higher concentrations, but these concentrations would not be realistic for field exposure.

The NRRA in *A. caliginosa* was very sensitive to low concentrations of pesticide and responded similarly to both pesticides in a dose-dependent manner. Similarly, Eason et al. [11] reported a significant reduction in NRRT in adult *Eisenia andrei* after exposure to chlorpyrifos. The NRRA in *A. caliginosa* has been further evaluated in a controlled field mesocosm experiment and was reduced in response to exposure to these pesticides at the recommended application rates for vegetable crops [24]. Therefore, this biomarker is sufficiently sensitive to detect exposure to field-relevant concentrations of these pesticides. An analysis of growth in adult earthworms used in the NRRT determinations showed that significant reductions occurred only at the higher concentration for each pesticide. This indicates that, as for ChE activity, changes in NRRT may occur prior to any observed growth inhibition.

Growth in adult and juvenile earthworms was compared to determine the relative sensitivity of each age group to pesticide exposure. Given the differences in growth rates (a 20% increase in adult weight after four weeks compared with 100% weight increase in juveniles for the same time period), it is likely that growth in juveniles will be more sensitive to exposure to pesticides than growth in adults. However, although results for diazinon supported this supposition, as evidenced by growth effects at lower concentrations for juveniles than adults, chlorpyrifos did not.

Table 6. Summary of concentrations of diazinon and chlorpyrifos causing adverse impacts on biomarkers, growth, and fecundity in *Aporrectodea caliginosa*

| | Lowest-observed-response concentration | |
|---|---|---|
| Parameter being assessed | Diazinon (mg/kg) | Chlorpyrifos (mg/kg) |
| Juvenile parameters | | |
| Cholinesterase activity | 12 | 4 |
| Growth | 12 | 28 |
| Maturation | 60 | 28 |
| Cocoon production | 12 | 28 |
| Adult parameters | | |
| Neutral red retention time | 12 | 4 |
| Growth | 60 | 28 |
| Cocoon production | 12 | 28 |
| Cocoon viability | 12 | 28 |

The effects of diazinon and chlorpyrifos on life-table parameters in juvenile earthworms has been assessed previously [25], and effects were only observed at the high concentration of chlorpyrifos, and no effects were seen at the laboratory-simulated field rates for either pesticide. However, earthworms were only exposed to the pesticide-treated soil for four weeks. In the field situation, these pesticides could persist in the soil for longer time periods [16], e.g., chlorpyrifos typically persists for 60 to 120 d in soil, but this is largely dependent on environmental conditions, and chlorpyrifos has been found to persist for up to two years in soil [26]. Newer products such as sustained-release pellets, which release low levels of pesticide into the soil over a much longer time frame, can persist for up to 18 months [27]. Therefore, further experiments investigating the effects of long-term pesticide exposure on earthworm growth and fecundity were conducted with juveniles. Growth in juveniles exposed to pesticide for 12 weeks was significantly reduced by exposure to both pesticides at both concentrations, although these effects varied with time. Interestingly, diazinon appeared to have only a transitory effect at the laboratory-simulated field rate, but at the sublethal concentration, the effect was continued. The laboratory-simulated field rate of chlorpyrifos had the opposite effect, with no significant effect on growth initially, but with long-term exposure, as would occur with sustained-release pellets, growth was eventually compromised. Growth in controls appeared to level off once earthworms reached a certain weight (around 650 mg mean body wt). This was likely due to overcrowding of the resulting adult earthworms in the jars. For future experiments, earthworms will be maintained in 1 kg of soil rather than in 500 g.

Exposure to a pollutant can impact on growth of an earthworm either by direct toxic effects on the organism or indirectly, by impacting on the energy budget of the organism as it attempts to detoxify the contaminant [13]. This is especially important for juvenile earthworms, as this will reduce the amount of energy available for maturation and will result in slower maturation and a delay in cocoon production.

Cocoon production typically increases markedly in the first weeks of adulthood and has been shown to be sensitive to

exposure to contaminants. For example, Spurgeon and Hopkin [13] found cocoon production in newly mature adults (exposed as juveniles) to be very sensitive to metal exposure. The sensitivity of the earthworms in the Spurgeon and Hopkin study was attributed to a delayed maturation in the exposed earthworms because, by 20 weeks, cocoon production by these earthworms was similar to earthworms that were exposed as adults. In our study, juvenile-exposed earthworms produced fewer cocoons than the control earthworms for all treatments except at the field application rate of chlorpyrifos. The effect of the sublethal concentrations of each pesticide on cocoon production could, in part, be due to the slower maturation rates. However, the effects of pesticide exposure on cocoon production relative to the control appeared to increase with time, indicating an additional effect of pesticide exposure on cocoon production. The similarity in responses between adult- and juvenile-exposed earthworms is further evidence that the pesticides are affecting fecundity directly. Also, cocoon production was reduced by the low concentration of diazinon in the absence of any obvious effect on maturation or growth (growth had returned to normal by eight weeks). Therefore, we cannot attribute the fecundity effect of these pesticides to growth, maturation, or the age of the earthworms.

When comparing the toxicity potency of diazinon and chlorpyrifos with respect to fecundity, these results indicate that, at recommended field rates, diazinon is more inherently toxic to earthworms than chlorpyrifos. This is in agreement with Potter et al. [28], who found some short-term reductions in earthworm abundance caused by diazinon during field trials, while chlorpyrifos has been reported to have no effect on earthworm abundance [29,30]. The higher toxicity of diazinon compared with chlorpyrifos is also evidenced by a greater impact on both cholinesterase activity and NRRT.

In adult earthworms, the effects of both pesticides were apparent during the four-week exposure period. However, growth, cocoon production, and cocoon viability showed rapid recovery once removed from pesticide-contaminated soil. Neuhauser [15] showed a similar recovery in *E. fetida* eight weeks after termination of exposure to the carbamate pesticide carbaryl. Cocoon production by adult-exposed earthworms was affected in the same manner as that in the juvenile experiment, which suggests similar sensitivity in the two age groups to pesticide exposure, and adverse effects concentrations were the same for the four-week exposure period in each age group (Table 6). However, if cocoon production (after four weeks of exposure) by juvenile-exposed earthworms was compared directly to adult-exposed earthworms, then juveniles are clearly more sensitive to the simulated field rates (low concentrations) of both pesticides.

When comparing fecundity and growth effects, it is apparent that these pesticides have a much greater impact on cocoon production and cocoon viability than growth, which is consistent with previous reports that cocoon production can be more predictive of long-term effects of exposure to a contaminant than growth [31].

As shown previously, the suborganismal biomarker responses (ChE activity and the NRRA) were more sensitive to pesticide exposure than growth. In order to ascertain the relationship between biomarker responses and effects on fecundity and to determine their ability to predict long-term impacts, the concentrations causing adverse effects on the biomarkers and fecundity were compared. For chlorpyrifos exposure, the biomarkers ChE activity in juveniles and NRRA in adults were more sensitive than cocoon viability in adults (juveniles not tested) and cocoon production in each age group. The increased sensitivity of biomarkers compared with growth and fecundity impacts was not apparent for diazinon exposure due to its potency on all of the parameters measured at the simulated field rate.

Despite the lack of differentiation between biomarker responses and fecundity impacts for diazinon at the low concentration, the onset of biomarker changes for both pesticides is likely to be more rapid than the onset of fecundity impacts, which generally require a minimum of four weeks of exposure. For example, ChE activity is inhibited within 24 h by exposure to pesticides [6] and the NRRA responds far more rapidly than other physiological responses [32]. In conclusion, impacts of diazinon and chlorpyrifos exposure on ChE activity and the NRRA occur at lower concentrations and/or prior to adverse impacts on growth and fecundity in earthworms, thus indicating that these two biomarkers can provide some early warning of subsequent toxicity of these organophosphorus insecticides. Growth and cocoon production in juvenile earthworms appeared to be more sensitive to pesticide exposure than in adults, confirming that juveniles represent a more sensitive indicator for predicting long-term impacts on populations compared with adult earthworms.

In conclusion, this research has shown that these two biomarker assays have potential as biomarkers of organophosphorus insecticides and can be linked to ecologically relevant endpoints such as growth and fecundity. Therefore, they could be used as early warning indicators of an adverse impact of pesticides on earthworm populations, i.e., by measuring enzyme activities in earthworms from a contaminated area and comparing these with earthworms from a matched organic control area. This approach avoids the influence of environmental parameters on the basal enzyme levels, although the NRRA appears to be insensitive to the effects of the environmental parameters (L. Booth, unpublished data) and ChE activity was only affected by temperature [33].

*Acknowledgement*—The authors thank V. Heppelthwaite for assistance, R. Webster for statistical analysis, S. Wratten, J. Clemons, C. Eason, and C. Bezar for advice and editorial comment. This project was funded by the Foundation for Research, Science and Technology, New Zealand.

## REFERENCES

1. Neuhauser EF, Malecki MR, Loehr RC. 1984. Growth and reproduction of the earthworm after exposure to sublethal concentrations of metals. *Pedobiologia* 27:89–97.
2. McCarthy JF, Shugart LR. 1990. *Biomarkers of Environmental Contamination.* Lewis, Boca Raton, FL, USA.
3. Organization for Economic Cooperation and Development. 1984. Earthworm acute toxicity tests. OECD Guideline 207. Paris, France.
4. Springett JA. 1992. Distribution of lumbricid earthworms in New Zealand. *Soil Biol Biochem* 24:1377–1381.
5. Edwards CA, Bohlen PJ. 1996. Effects of agricultural practices and chemicals on earthworms. In Edwards CA, Bohlen PJ, eds, *Biology and Ecology of Earthworms.* Chapman & Hall, London, UK, pp 268–299.
6. Booth LH, Hodge S, O'Halloran K. 2000. The use of cholinesterase as a biomarker in *Aporrectodea caliginosa* (Oligochaeta; Lumbricidae) to detect organophosphate contamination: A comparison of laboratory tests, mesocosms and field studies. *Environ Toxicol Chem* 19:417–422.
7. Edwards CA, Fisher SW. 1991. The use of cholinesterase measurements in assessing the impacts of pesticides on terrestrial and aquatic invertebrates. In Mineau P, ed, *Cholinesterase-Inhibiting*

*Insecticides: Their Impact on Wildlife and the Environment.* Elsevier, Amsterdam, The Netherlands, pp 256–275.
8. Fairbrother A, Marden BT, Bennett JK, Hooperds MJ. 1991. Methods used in determination of cholinesterase activity. In Mineau P, ed, *Cholinesterase-Inhibiting Insecticides: Their Impact on Wildlife and the Environment.* Elsevier, Amsterdam, The Netherlands, pp 35–72.
9. Mannervik B, Danielson UH. 1988. Glutathione transferases—Structure and catalytic activity. *Crit Rev Biochem* 23:283–337.
10. Weeks JM, Svendsen C. 1996. Neutral red retention by lysosomes from earthworm (*Lumbricus rubellus*) coelomocytes: A simple biomarker of exposure to soil copper. *Environ Toxicol Chem* 15: 1801–1805.
11. Eason CT, Svendsen C, O'Halloran K, Weeks JM. 1999. An assessment of the lysosomal neutral red retention test and immune function assay in earthworms (*Eisenia andrei*) following exposure to chlorpyrifos, benzo-*a*-pyrene (B*a*P) and contaminated soil. *Pedobiologia* 43:641–645.
12. Van Gestel CAM, Dirven-Van Breemen EM, Baerselman R, Emans HJB, Janssen JA, Posthuma R, Van Vliet PJM. 1992. Comparison of sublethal and lethal criteria for nine different chemicals in standardised toxicity tests using the earthworm *Eisenia andrei. Ecotoxicol Environ Saf* 23:206–220.
13. Spurgeon DJ, Hopkin SP. 1996. Effects of metal-contaminated soils on the growth, sexual development, and early cocoon production of the earthworm *Eisenia fetida,* with particular reference to zinc. *Ecotoxicol Environ Saf* 35:86–95.
14. Van Gestel CAM, Van Dis WA, Dirven-Van Breemen EM, Sparenburg PM, Baerselman R. 1991. Influence of cadmium, copper and pentachlorophenol on growth and sexual development of *Eisenia andrei* (Oligochaeta: Annelida). *Biol Fertile Soils* 12:117–121.
15. Neuhauser EF. 1990. Growth and reproduction of the earthworm *Eisenia fetida* exposed to sub-lethal concentrations of organic chemicals. *Soil Biol Biochem* 22:175–179.
16. Kuhr RJ, Tashiro H. 1978. Distribution and persistence of chlorpyrifos and diazinon applied to turf. *Bull Environ Contam Toxicol* 20:652–656.
17. Ellman GL, Courtenay KD, Valentino AJ, Featherstone EM. 1961. A new rapid colorimetric determination of acetylcholinesterase activity. *Biochem Pharmacol* 7:88–95.
18. Habig WH, Pabst MJ, Jacoby WB. 1974. Glutathione-*S*-transferases, the first enzymatic step in mercapturic acid formation. *J Biol Chem* 249:7130–7139.
19. Bradford M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilising the principle of protein-dye binding. *Anal Biochem* 72:248–254.
20. Lockwood APM. 1963. *Animal Body Fluids and Their Regulation.* Heinemann, London, UK.
21. Hans RK, Khan MA, Farooq M, Beg MU. 1993. Glutathione *S*-transferase activity in an earthworm (*Pheretima posthuma*) exposed to three insecticides. *Soil Biol Biochem* 25:509–511.
22. Stokke K, Stenersen J. 1993. Non-inducibility of the glutathione transferases of the earthworm *Eisenia andrei. Comp Biochem Physiol C* 106:753–756.
23. Baturo W, Lagadic L, Caquet T. 1996. Growth, fecundity and glycogen utilization in *Lymnaea palustris* exposed to atrazine and hexachlorobenzene in freshwater mesocosms. *Environ Toxicol Chem* 14:503–511.
24. Booth LH, Heppelthwaite VJ, Webster R, O'Halloran K. 2001. Lysosomal neutral red retention time as a biomarker of organophosphate exposure in the earthworm *Aporrectodea caliginosa.* Laboratory and semi-field experiments. *Biomarkers* 6:77–82.
25. Booth LH, Heppelthwaite VJ, O'Halloran K. 2000. Growth, development, and fecundity of the earthworm *Aporrectodea caliginosa* after exposure to two organophosphates. *Proceedings,* 53rd New Zealand Plant Protection Society Conference, Christchurch, New Zealand, August 7–9, 2001, pp 221–225.
26. Cox C. 1995. Insecticide fact sheet. Chlorpyrifos, part 2: Human exposure. *J Pestic Reform* 15:14–20.
27. Racke KD. 1993. Environmental fate of chlorpyrifos. *Rev Environ Contam Toxicol* 131:1–151.
28. Potter DA, Buxton MC, Redmond CT, Patterson CG, Powell AJ. 1990. Toxicity of pesticides to earthworms (Oligochaeta: Lumbricidae) and effect on thatch degradation in Kentucky bluegrass turf. *J Econ Entomol* 83:2362–2369.
29. Culy MD, Berry EC. 1995. Toxicity of soil-applied granular insecticides to earthworm populations in cornfields. *Down Earth* 50:20–25.
30. Thompson AR, Sans WW. 1974. Effects of soil insecticides in southwestern Ontario on non-target invertebrates: Earthworms in pasture. *Environ Entomol* 3:305–308.
31. Choo LPD, Baker GH. 1998. Influence of four commonly used pesticides on the survival, growth, and reproduction of the earthworm *Aporrectodea trapezoides* (Lumbricidae). *Aust J Agric Res* 49:1297–1303.
32. Svendsen C, Weeks JM. 1995. The use of a lysosome assay for the rapid assessment of cellular stress from copper to the freshwater snail *Viviparus contectus* (Millet). *Mar Pollut Bull* 31:139–142.
33. Booth LH, Heppelthwaite V, McGlinchy A. 2000. The effect of environmental parameters on growth, cholinesterase activity and glutathione *S*-transferase activity in the earthworm (*Apporectodea caliginosa*). *Biomarkers* 5:46–55.