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The effect of environmental parameters on growth, cholinesterase activity and glutathione S-transferase activity in the earthworm (*Apporectodea caliginosa*)

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The effect of age and environmental parameters on growth, cholinesterase (ChE), and glutathione S-transferase (GST) activities were assessed in juvenile *Apporectodea caliginosa* earthworms. Earthworms were maintained in three types of soil – loam, sandy, and clay – at a range of moisture contents (15–30 %), and temperatures (5–20 °C). Earthworm age (1–3 months) had no significant affect on ChE activity. Growth rates were influenced by all environmental parameters tested and these effects were interrelated. Optimum conditions for growth appeared to be in loam or sandy soil with 25–30 % moisture at 10–15 °C. The GST activity was influenced by soil temperature and activity was significantly higher at 15 °C than at 5 °C and 10 °C. Soil type also influenced GST activity and this influence was dependent on moisture content. In sandy soil GST activity was significantly lower at 30 % moisture than at lower moisture contents, in loam soil GST activity was significantly higher at 15 % moisture than at higher moisture contents, while in clay soil GST activity was not affected by moisture content. These results indicate that in field experiments when evaluating GST activity soil temperature and soil type need to be consistent between control and 'contaminated sites'. ChE activity was only affected by temperature, so this should be considered when comparing control and treated areas.

Keywords: earthworm, temperature, moisture content, biomarkers, cholinesterase, glutathione S-transferase.

Abbreviations: GST, glutathione S-transferase; ChE, cholinesterase; GSH, glutathione; DTNB, 5,5′,-dithio-bis-2-nitrobenzoic acid; CDNB,1-chloro-2,4-dinitrobenzene.

Introduction

A biomarker is 'a biological response that can be related to an exposure to, or toxic effect of, an environmental chemical or chemicals' (Peakall 1994). The development of biomarker research has had close links with medicine and vertebrate biology (NRC 1987), and over the last 5 years there has been increasing interest in invertebrates as bioindicator species for the development of biomarkers (Edwards and Fisher 1991, Depledge and Fossi 1994, Lagadic *et al.* 1994).

Earthworms have been selected as one of the five key indicator organisms for ecotoxicological testing of industrial chemicals by the European Community (EC), the Organization for Economic Co-operation and Development (OECD), and the Food and Agriculture Organization of the United Nations (FAO) (Edwards and Bater 1992). Earthworms are key components of most soil biota. They contribute to the overall productivity of agricultural soils through their feeding, casting, and burrowing activities. These contributions include breakdown of organic matter,

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mixing of soil layers, formation of soil aggregates, and increased soil porosity, water drainage, and aeration. Healthy earthworm populations positively influence many physical, chemical, and biological soil attributes (Culy and Berry 1995). *Apporectodea caliginosa* (Savigny) was selected for the following experiments as an indicator species in New Zealand because of its widespread occurrence in arable and pasture lands (Martin 1977, Springett 1992), and, because it lives in the topsoil, it is vulnerable to surface-applied pesticides.

Two enzyme systems in earthworms have been identified as potential biomarkers of pesticide exposure. Cholinesterase (ChE) is the target enzyme for organophosphate and carbamate pesticides, which act by inhibiting its activity. Edwards and Fisher (1991) have shown that acetylcholinesterase activity in several terrestrial and aquatic invertebrates can be significantly decreased in cases of sublethal exposure to a number of pesticides. Changes in the activity of this enzyme have long been used to diagnose organophosphorus poisoning in birds and fish, along with other measurements such as chemical residues or behavioural changes (Greig-Smith 1991).

Glutathione S-transferase (GST) is involved in detoxification of various xenobiotic chemicals. In insects, GST plays an important role in biotransformation of various insecticides (Motoyama 1980, Lamoureux and Rusness 1987), including the degradation of some organophosphorus compounds (Yang 1976). In addition, GST activity in insects and other animals has also been shown to be induced by a number of chemicals, including pesticides, e.g. lindane, dimethoate, carbaryl, and permethrin (Lagadic *et al.* 1993, Parkes *et al.* 1993, El-Sharkawy *et al.* 1994).

In order to utilize these enzyme systems in earthworms as biomarkers of exposure to pesticides, it is critical to establish their stability under the variable environmental conditions likely to be encountered during field exposure. Juvenile earthworms were chosen for these experiments as juveniles are generally more sensitive to pesticides than adults, and they are more commonly found in the upper topsoil and are therefore more vulnerable to surface-applied pesticide. In the following experiments, the effect of earthworm age on the ChE and GST activities was first determined to assess whether activity would vary greatly between age classes (1–3 months). The effect of soil type, temperature, and moisture content on earthworm growth (as defined by change in weight), ChE, and GST activity was then investigated.

Materials and methods

Invertebrates

A laboratory colony of *A. caliginosa* was derived from adult earthworms collected in Canterbury, New Zealand, in 1997. Templeton silt loam (3.8% organic matter) was prepared by drying the soil at 30 °C for 24 h to kill any extant earthworms and other macro invertebrates. The soil was then rehydrated to produce a moisture content of 20–25 % by mass. Dry grass-meal was added at the rate of 7.5 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.

Experimental outline

Preliminary experiment to determine the effect of age on ChE and GST activity. It is well known that age affects enzyme activities. A preliminary experiment was conducted to enable selection of juvenile

earthworms in an age range in which enzyme activities are similar. Earthworms were reared to 1, 2, and 3 months of age in Templeton silt loam as above and a moisture content of 20–25%. For each age group, 50 randomly chosen earthworms were frozen at –20 °C until animals for all age groups had been collected. Of the 50 earthworms collected, 25 were randomly chosen and analysed for ChE activity and the remaining 25 for GST activity.

Effect of environmental parameters on biomarkers. Juvenile earthworms of mixed age (1–3 months) were used to determine the effects of environmental parameters on biomarkers. Ten earthworms were placed in 500 g of either loamy, sandy, or clay soils at four moisture levels and incubated at four different temperatures (as described below). This resulted in 48 treatments and for each treatment there were four replicates of ten earthworms. In an additional set of soil samples moisture content was monitored and there was no significant change in soil moisture over the 3-week experiment ($F_{1,60} = 2.317$, $p = 0.133$).

Soil type. Soil was collected from sites in Canterbury, New Zealand where there had been no pesticide use in the previous 5 years. Particle size analysis of the soils was conducted by the Physics Laboratory, Landcare Research, Hamilton by the pipette method following hydrogen peroxide pretreatment, suspension in sodium hexametaphosphate and ultrasonic dispersion (Claydon 1989). For description of soil properties see table 1. All soils were dried at 30 °C for 24 h and pH was adjusted to 6.5–7.0 by adding calcium carbonate.

Moisture content. The moisture content of each soil type was adjusted to 15, 20, 25, and 30 % by mass. These moisture contents cover the range in which earthworms would normally exist in Canterbury.

Temperature. Earthworms were incubated in temperature-controlled chambers with a 16/8 h light/dark cycle with light intensity of approximately 40 lux at 5, 10, 15, and 20 °C which are in the range of temperatures to which earthworms would be exposed throughout the year.

Analysis

To assess the effects of environmental parameters on growth (defined as change in wet weight) earthworms were weighed at the start of the experiment, and sorted from the soil and reweighed after 3 weeks, at which point the experiment was terminated. Earthworms were removed from the soil and five earthworms from each replicate were frozen for subsequent ChE activity analysis. The remaining five earthworms were frozen for subsequent GST activity analysis.

Enzyme biomarker assays

Enzyme activity and protein concentrations were determined using a Reader 340 AT TC (SLT Instruments) with 96-well microtitre plates and the 'Software 2000' package (SLT). Frozen samples of earthworms were defrosted on ice. Earthworms for each replicate were pooled and homogenized in ice-cold phosphate buffer containing 0.02 M potassium dihydrogen phosphate. The samples for determination of ChE activity were homogenized in phosphate buffer adjusted to pH 7.5. Samples were maintained on ice for 60 min before analysis and the crude homogenate was used for analysis. For GST activity, earthworm samples were homogenized in buffer adjusted to pH 7.0. The buffer contained 1 mM of reduced glutathione (GSH) to prevent enzyme oxidation (Motoyama *et al.* 1978). Analysis was conducted using the supernatant following centrifugation of homogenate at 15 000 rpm for 5 min.

The ChE activity was determined using a modified method based on Ellman *et al.* (1961). This method has been adapted for use in earthworms and scaled down for use in microtitre plates. In summary, the reaction mixture containing buffer, enzyme homogenate, and 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB) was incubated at 37 °C for 5 min and the reaction was started by adding the substrate acetylthiocholine iodide. Acetylthiocholine iodide is hydrolysed by the cholinesterase and releases acetylthiocholine which reacts with DTNB to produce a yellow anion which can be detected spectrophotometrically at 405 nm. Activity is expressed as nmol acetylthiocholine hydrolysed min^{-1} mg^{-1} protein.

The GST activity was determined by the method of Habig *et al.* (1974) which has been modified to suit earthworms and scaled down for use in microtitre plates. The reaction mixture containing buffer and enzyme homogenate was incubated at 35 °C for 5 min and the reaction was started by adding 1 mM 1-chloro-2,4-dinitrobenzene (CDNB), the enzyme substrate. The enzyme catalyses the conjugation of

Table 1. Particle size analysis of soils.

Soil type	Clay content (%)	Silt (%)	Sand (%)	Organic content (%)
Loam soil	18	71	11	5.27
Sandy soil	14	43	43	3.87
Clay soil	25	61	14	6.23

CDNB to glutathione producing *S*-(2,4-dinitrophenyl)glutathione, and enzyme activity is measured spectrophotometrically at 340 nm. Activity is expressed as nmol GSH conjugated min^{-1} mg^{-1} 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.

Statistical analysis

Results were analysed by ANOVA to determine the effect of age and temperature and moisture content on ChE and GST activities. Significant treatment effects ($p < 0.05$) were further investigated using Tukey's HSD test.

Results

Effect of age on ChE and GST activity

There was no significant difference in the activity of either GST ($F_{2,72} = 0.0629$, $p = 0.939$) or ChE ($F_{2,72} = 0.935$, $p = 0.398$) for any age group (table 2). Based on these results, 1–3 month old worms were used to further investigate the effects of environmental parameters on enzyme activities.

Effect of environmental parameters on growth

There was a significant three-way interaction effect of soil, moisture content, and temperature on earthworm growth ($F_{18,144} = 2.03$, $p = 0.012$) (figure 1 and table 3). This interaction means that we cannot consider the effect of one parameter in isolation from the others, and consequently makes reporting and interpretation of the results more complex.

At 5 °C and 20 °C there was no difference in earthworm growth between any soil type at any moisture content ($p > 0.05$). However, at 10 °C and 15 °C growth in sandy soil was significantly higher than in clay and loam soil at any temperature ($p < 0.05$).

In clay soil there were no differences in growth rates between any treatment except between earthworms maintained at 30% moisture and 10–15 °C, which exhibited higher growth than earthworms at 15 °C moisture for all temperatures studied.

At 15 and 20 % moisture there was no significant difference in growth between any soil type at any temperature, except for in sandy soil at 10 and 15 °C (20 % moisture) which was significantly higher ($p < 0.05$) than both clay and loam at any temperature.

At 25 % moisture in sandy soil there was significantly higher growth at 10 °C and 15 °C than at 5 °C ($p < 0.05$). A similar pattern was observed in loam soil,

Table 2. The effect of age of juvenile earthworms on ChE and GST activities. Values are mean activity ± standard error ($n = 25$).

Earthworm age	ChE activity[a]	GST activity[b]
1 month	134.5 ± 7.0	156.1 ± 8.6
2 months	128.7 ± 6.1	154.4 ± 9.2
3 months	143.2 ± 8.5	158.9 ± 8.9

[a] Che (cholinesterase) activity is expressed as nmol acetylthiocholine hydrolysed min^{-1} mg^{-1} protein.
[b] GST (glutathione S-transferase) activity is expressed as nmol glutathione conjugated min^{-1} mg^{-1} protein.

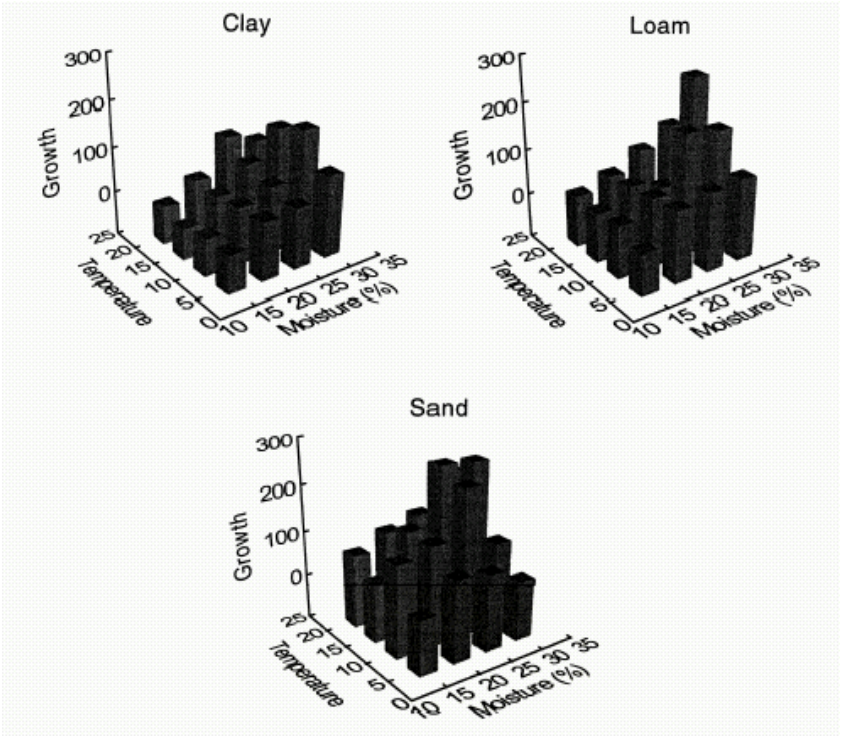

Figure 1. Mean growth (expressed as % change in body weight) in earthworms maintained at different combinations of soil moisture and temperature for 3 weeks. For actual data refer to table 3.

Table 3. The effect of environmental parameters on earthworm growth. Values are the % increase in body weight after 3 weeks and represent the mean value from four replicate jars of worms (SE).

Soil type	Moisture content (%)	5 °C	10 °C	15 °C	20 °C
Loam	15	–14.5 (10.8)	11.1 (9.9)	4.7 (20.6)	11.9 (18.6)
Loam	20	53.2 (24.6)	47.1 (34.15)	43.3 (34.2)	37.2 (17.5)
Loam	25	69.3 (7.1)	165.9 (12.6)	199.4 (50.5)	75.7 (7.9)
Loam	30	76.9 (7.7)	154.8 (24.2)	244 (24.4)	116.9 (25.2)
Sand	15	16.2 (12.6)	100 (38.3)	25.8 (8.3)	60.6 (50.4)
Sand	20	76.1 (9.9)	118.8 (10.9)	122.3 (20.9)	93.6 (24.3)
Sand	25	65.2 (11.9)	218.7 (48.8)	244 (31.7)	117.2 (28.4)
Sand	30	28.2 (7.1)	85.1 (11.9)	236.8 (63.8)	195.2 (37.4)
Clay	15	–21.2 (5.4)	–22.2 (5.2)	–33.6 (5.5)	–17.4 (18.6)
Clay	20	24 (12.5)	23.4 (4.7)	14.6 (11.3)	21.1 (7.8)
Clay	25	29.7 (17.6)	45.8 (10.8)	67.6 (18.9)	101.9 (39.9)
Clay	30	78.6 (18.6)	151.1 (6.3)	128.9 (19.9)	74.8 (44.3)

except that growth at 5 °C was not significantly lower than at 10 °C. At 10–15 °C, growth in sandy and loam soils was not significantly different, but growth in sand was significantly higher than in clay soil, while growth in loam soil was not significantly different from clay except for at 15 °C which was significantly higher than growth in clay soil at 10 °C.

At 30 % moisture, growth in sandy soil at 5 °C and 10 °C was significantly lower than at 15 °C, but growth at 20 °C was not significantly higher than at 10 °C.

In loam soil, growth is not significantly different between moisture contents and temperatures except for at 10 °C and 15 °C (25–30 % moisture), which is significantly higher than at 15 % moisture at any temperature. At 15 °C (25–30 % moisture), growth is also significantly higher than at 20 % moisture across all temperatures.

In sandy soil, growth at 15 % and 20 % moisture was not effected by temperature. In sandy soil at 25 % and 30 % moisture there was a significant increase in growth from 5 °C through to 15 °C. Moisture content had no effect on growth at 5 °C and 20 °C.

Effect of environmental parameters on enzyme activities

GST activity. Temperature had a significant effect on GST activity ($F_{3,135} = 2.993$, $p = 0.0014$), but this was not influenced by soil type or moisture content (figure 2). The GST activity at 5 °C and 10 °C was lower than activity at 15 °C ($p = 0.007$, $p = 0.002$ respectively). Activity at 15 °C was not significantly different to activity at 20 °C.

There was a significant two-way interaction effect of soil type and moisture content on GST activity ($F_{6,135} = 3.707$, $p = 0.0019$) (figure 3).

Earthworm GST activity in clay soil was not affected by moisture content and activity was not significantly different between loam and clay soil at any moisture content. However, GST activity in loam soil was affected by moisture content, at 15 % moisture activity was significantly higher than at all other moisture contents ($p = 0.0362$ [20%]; $p = 0.0015$ [25 %]; $p = 0.0294$ [30 %]).

In sandy soil, GST activity was significantly affected by moisture, at 30 % moisture GST activity was significantly lower than at other moisture contents ($p < 0.0001$ [15 %]; $p = 0.0006$ [20 %]; $p = 0.004$ [25 %]).

GST activity differed significantly between sand and clay soil only at extremes of moisture (sand 15 % cf. clay 30 %, $p = 0.0214$, sand 30 % cf. clay 15 %, $p = 0.0207$). Similarly activity in loam soil at 30 % moisture was significantly different from sandy soil at 15 % ($p = 0.0082$) and loam at 15 % moisture was significantly different from sand at 30 % ($p = 0.0001$). Activity in loam at 25 % moisture was significantly different from sand of equal or lesser moisture content ($p < 0.01$), but not from sand at 30 %.

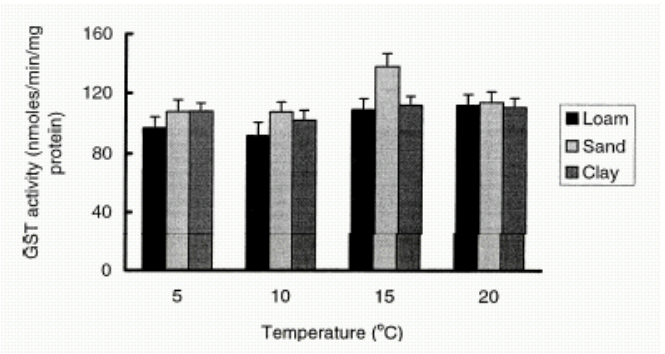

Figure 2. The effect of temperature on GST activity.

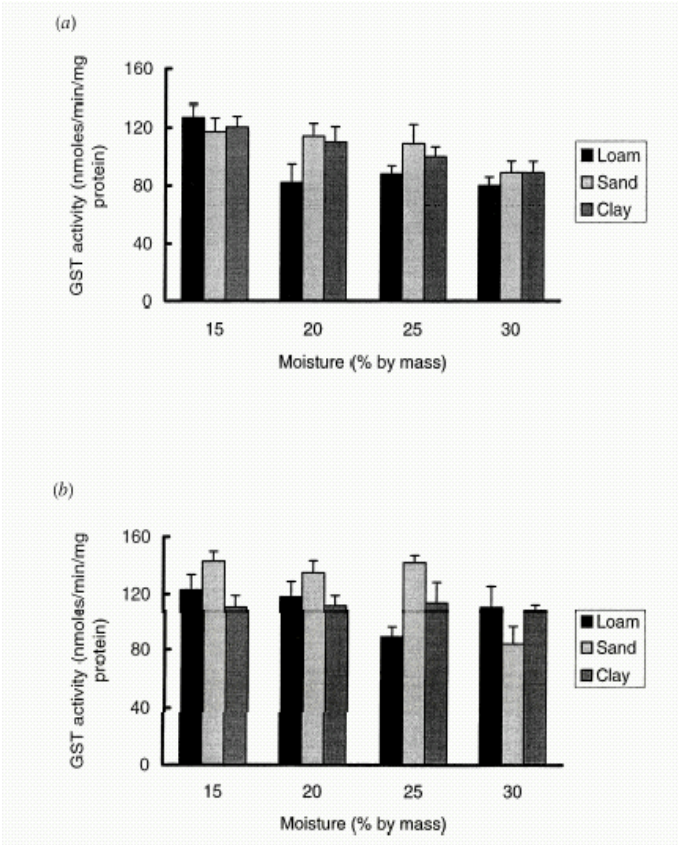

Figure 3. The effect of moisture content on GST activity in different soil types. (a) 5 °C and 10 °C and (b) 15 °C and 20 °C.

Effect on ChE activity. Temperature had a significant effect on ChE activity ($F_{3,133} = 4.477$, $p = 0.005$). The ChE activity was significantly higher at 5 °C ($p = 0.0318$) and 20 °C ($p = 0.0091$) than at 10 °C, while at 15 °C ChE activity was not significantly different from activity at any other temperature. Soil type and moisture content did not affect ChE activity ($F_{2,133} = 1.9374$, $p = 0.1481$ [soil type]; $F_{3,133} = 1.8003$, $p = 0.1502$ [moisture content]) (table 4).

Discussion

The effect of age and environmental parameters on cholinesterase and glutathione S-transferase activity in the common earthworm *Apporectodea caliginosa* was investigated. Ultimately, these biomarkers will be developed for use in a field situation, i.e. for comparison of enzyme activities in earthworms from a contaminated area with earthworms from an organic control area. The effect on 'growth' was also evaluated to provide a guide to the general health of the earthworms in these different conditions.

The activity of GST and ChE was not significantly affected by age of the juvenile earthworms (1–3 months old). This may indicate that juvenile earthworms collected from the field can be analysed together rather than trying to separate them

Table 4. The effect of environmental parameters on earthworm cholinesterase activity. Values represent the mean value from four replicate jars of worms (SE).

Soil type	Moisture content (%)	5 °C	10 °C	15 °C	20 °C
Loam	15	87.8 (3.6)	60.3 (12.3)	87.2 (6.5)	83.7 (12.1)
Loam	20	65.2 (13.7)	57.1 (7.7)	50.5 (5.1)	68.9 (13.5)
Loam	25	91.3 (15.7)	57.7 (12.3)	59.6 (8.4)	89.6 (22.9)
Loam	30	70.2 (16.6)	52.2 (7.0)	78.8 (6.3)	86.2 (8.9)
Sand	15	75.4 (10.1)	74.5 (13.0)	53.3 (11.3)	75.5 (13.0)
Sand	20	62.6 (2.2)	58.0 (20.0)	49.1 (7.5)	66.2 (11.1)
Sand	25	52.2 (3.9)	52.0 (12.1)	69.8 (8.0)	66.8 (10.6)
Sand	30	73.6 (1.0)	64.0 (16.9)	62.3 (4.3)	78.6 (9.9)
Clay	15	87.8 (12.3)	60.3 (8.7)	87.2 (6.5)	83.7 (12.1)
Clay	20	65.2 (13.7)	57.1 (7.7)	50.5 (5.1)	68.9 (13.5)
Clay	25	91.3 (15.7)	57.7 (12.3)	59.6 (8.4)	89.6 (22.9)
Clay	30	70.2 (16.6)	52.2 (7.0)	78.8 (6.3)	86.2 (8.9)

into age classes. If so, then the amount of time spent ageing field collected worms could be greatly reduced. However to confirm that these laboratory results can be extrapolated to the field requires testing of juvenile worms collected from the field and from a variety of locations.

Growth was shown to be affected by all the environmental parameters tested, and these effects were interrelated. The key temperature range for this earthworm species appeared to be 10–15 °C. This is consistent with Lee (1985) who showed that the optimal temperature for growth in *Apporectodea caliginosa* was 10–15 °C, but Reinecke (1974) showed that the range of temperatures from 10 to 23 °C was suitable for growth.

Previous studies have found that earthworms prefer light and medium loams to heavier clays, sandy, and alluvial soils. El Duweini and Ghabbour (1965) showed that soil moisture influenced the suitability of soil for earthworms, and that at higher moisture contents sand was a suitable medium.

We observed good growth in loam as well as in sandy soil. Further to this we found that earthworm growth was influenced by soil moisture content in conjunction with soil type and temperature. Different combinations of soil type, moisture and temperature were found to have significantly different effects on worm growth. Clay soil appeared to be a poor medium for earthworm growth, except at high moisture (30 %) and at temperatures of 10–15 °C where growth was not significantly different from loam or sand of a similar moisture content and temperature.

Field soil in New Zealand where earthworms are typically found is around 20 % moisture content (unpublished data), and in dry conditions worms will retreat to deeper soil. Adult *A. caliginosa* have previously been shown to prefer a soil moisture content of 18–22 % (Daughberger 1988) and moisture content is also a dominant factor in cocoon production. Pesticides can be more toxic at low moisture contents because the concentration of pesticide in pore water can be higher if less moisture is present (Bauer and Rombke 1997). At moisture contents below 16 % earthworms show lower physical activity, but there is little data for higher moisture contents. Our results suggest that optimal conditions for growth of *A. caliginosa* are around 25–30 % soil moisture at 10–15 °C in loam or sandy soil.

Temperature significantly affected GST activity. Activity at 5 °C and 10 °C was significantly lower than at 15 °C.

Soil type was found to influence GST activity and this was dependent on the moisture content. At higher moisture contents, GST activity decreased for all soil types except clay, in which GST activity was not affected by moisture content. The GST activity in loam soil with moisture content of 15 % was significantly higher than in loam soil at higher moisture contents. Earthworms maintained in sandy soil had significantly lower GST activity at 30 % moisture than at other moisture levels.

In our laboratory experiments ChE activity was suprisingly robust and was not affected by soil type or moisture content, but was significantly affected by temperature. Therefore temperature is a parameter that should be taken into account when considering the effect of pesticides on ChE activity and when comparing ChE activity in earthworms taken from different sites.

These results show that the biomarker response for both GST and ChE activity are influenced by temperature and that GST activity is further influenced by soil type/moisture content interaction. These results indicate that soil type, moisture content, and temperature may be important sources of variation in the parameters measured and warrant consideration when assessing the effect of pesticide contamination in the field.

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References

Bradford, M. M. 1976, A rapid and sensitive method for the quantification of microgram quantities of protein utilising the principle of protein-dye binding. *Analytical Biochemistry,* **72**, 248–254.

Claydon, J. J. 1989, Determination of particle size distribution in fine grained soils – pipette method. DSIR Div. *Land & Soil Sciences Technical Record LH5.*

Culy, M. D. and Berry, E. C. 1995, Toxicity of soil-applied granular insecticides to earthworm populations in cornfields. *Down to Earth,* **50**, 20–25.

Daughberger, P. 1988, Temperature and moisture preference of three earthworm species (Oligochaeta: Lumbricidae). *Pedobiologia*, **32**, 57–64.

Depledge, M. D. and Berry, E. C. 1995, Toxicity of soil-applied granular insecticides to earthworm populations in cornfields. *Down to Earth,* **50**, 20–25.

Depledge, M. H. and Fossi, M. C. 1994, The role of biomarkers in environmental assessment (2). Invertebrates. *Ecotoxicology,* **3**, 161–172.

Edwards, C. A. and Bater, J. 1992, The use of earthworms in environmental management. *Soil Biology and Biochemistry,* **24**,1683–1689.

Edwards, C. A. and Fisher, S. W. 1991, The use of cholinesterase measurements in assessing the impacts of pesticides on terrestrial and aquatic invertebrates. In *Cholinesterase-inhibiting Insecticides: their Impact on Wildlife and the Environment,* P. Mineau, ed. (Amsterdam: Elsevier), pp. 256–275.

Ellman, G. L., Courtenay, K. D., Valentino, A. J. and Featherstone, E. M. 1961, A new rapid colorimetric determination of acetylcholinesterase activity. *Biochemical Pharmacology,* **7**, 88–95.

El-Duweini, A. K. and Ghabbour, S. I. 1965, Population density and biomass of earthworms in different types of Egyptian soils. *Journal of Applied Ecology,* **2**, 271–287.

El-Sharkawy, A. M., Abdel-Rahman, S. Z., Hassan, A. A., Gabr, M. H., El-Zoghby, S. M. and El-Sewedy, S.M. 1994, Biochemical effects of some insecticides on the metabolic enzymes regulating glutathione metabolism. *Bulletin of Environmental Contamination and Toxicology,* **52**, 505–510.

Greig-Smith, P. W. 1991, Use of cholinesterase measurements in surveillance of wildlife poisoning in farmland. In *Cholinesterase-inhibiting Insecticides: their Impact on Wildlife and the Environment,* P. Mineau, ed. (Elsevier, Amsterdam), pp. 127–150.

FAIRBROTHER, A., MARDEN, B. T., BENNETT, J. K. and HOOPERDS, M. J. 1991, Methods used in determination of cholinesterase activity. In *Cholinesterase-inhibiting Insecticides: their Impact on Wildlife and the Environment,* P. Mineau, ed. (Elsevier, Amsterdam), pp. 35–72.

HABIG, W. H., PABST, M. J. and JACOBY, W. B. 1974, Glutathione S-transferases, the first enzymatic step in mercapturic acid formation. *Journal of Biological Chemistry,* **249**, 321–336.

LAGADIC, L., CUVANY, A., BERGE, J. and ECHAUBARD, M. 1993, Purification and partial characterisation of glutathione S-transferases from insecticide-resistant and Lindane-induced susceptible *Spodoptera littoralis* (Boisd) larvae. *Insect Biochemistry and Molecular Biology,* **23**, 467–474.

LAGADIC, L., CAQUET, T. and RAMADE, F. 1994, The role of biomarkers in environmental assessment (5). Invertebrate populations and communities. *Ecotoxicology,* **3**, 193–208.

LAMOUREUX, G. L. and RUSNESS, D. G. 1987, Synergism of diazinon toxicity and inhibition of diazinon metabolism in the house fly by Tridiphane: inhibition of glutathione-S-transferase activity. *Pesticide Biochemistry and Physiology* **27**, 318–329.

LEE, K. E. 1985, *Earthworms: Their Ecology and Relationships with Soil and Land Use* (Sydney: Academic Press).

MARTIN, N. A. 1977, Guide to the lumbricid earthworms of New Zealand pastures. *New Zealand Journal of Experimental Agriculture,* **5**, 301–319.

MOTOYAMA, N. 1980, Glutathione S-transferases: Their role in the metabolism of organophosphorus insecticides. *Reviews of Biochemical Toxicolology,* **2**, 49–69.

MOTOYAMA, N., KULKARNI, A. P., HODGSON, E. and DAUTERMANN, W. C. 1978, Endogenous inhibitors of glutathione-S-transferase in house flies. *Pesticide Biochemistry and Physiology,* **9**, 155.

NRC (National Research Council) 1987, Committee on biological markers. *Environmental Health Perspectives.* **74**, 3–9.

PARKES, T. L., HILLIKER, A. J. and PHILLIPS, J. P. 1993, Genetic and biochemical analysis of glutathione-S-transferase in the oxygen defence system of *Drosophila melanogaster*. *Genome,* **36**, 1007–1014.

PEAKALL, D. B. 1994, Biomarkers, the way forward in environmental assessment. *Toxicology and Ecotoxicology News,* **1**, 55–60.

REINECKE, A. J. 1974, The upper lethal temperature of *Eisenia rosea* (Oligochaeta). *Natuurwetenskappe,* **62**, 1–14.

SPRINGETT, J. A. 1992, Distribution of lumbricid earthworms in New Zealand. *Soil Biology and Biochemistry,* **24**, 1377–1381

YANG, R. S. H. 1976, Enzyme conjugation and insecticide metabolism. In *Biochemistry and Physiology of Insecticides,* C.F. Wilkinson, ed. (New York: Plenum Press), pp. 177–187.