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RELATIVE SENSITIVITY OF LIFE-CYCLE AND BIOMARKER RESPONSES IN FOUR EARTHWORM SPECIES EXPOSED TO ZINC

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Abstract—Life-cycle (survival, weight change, and cocoon production rate) and biomarker (neutral-red retention by coelomocytes lysosomes) responses to zinc in four earthworm species were measured in laboratory tests. In all species, dose-dependent effects on survival, cocoon production, and neutral-red retention times were found (one-way ANOVA $p < 0.05$). However, for weight change, only *Aporrectodea caliginosa* showed a clear response. Comparisons of the effects of zinc on these parameters in the different earthworm species indicated similar order of sensitivity. Thus, *Lumbricus rubellus* and *Aporrectodea caliginosa* were more sensitive to zinc than *Lumbricus terrestris* and *Eisenia fetida* in all cases. To compare the relative sensitivities of life-cycle and biomarker responses, a sublethal sensitivity index (SSI) was applied. For cocoon production, SSIs indicated that the EC10s were below LC50s by a factor of between 8.3 and 16.5. These values are toward the high end of the range found previously for soil invertebrates, indicating an emphasis on maximizing survival. For neutral red, SSIs ranged from 4.5 to 41.2. Thus, the biomarker was predictive of life-cycle effects in some (*Lumbricus rubellus* and *Aporrectodea caliginosa*), although not the other two species tested.

Keywords—Species sensitivity Life-cycle traits Biomarker Neutral red Sublethal sensitivity index

INTRODUCTION

The fact that soil invertebrates vary in their sensitivity to pollutants [1,2] has been recognized within the regulatory framework of some European countries. Thus, soil quality criteria that were initially set on expert opinion are now derived using a more scientifically robust approach. To derive justifiable soil protection values, a number of techniques have been suggested based on the use of available soil invertebrate toxicity data [3,4]. These methods yield a frequency distribution of sensitivity for species exposed in a standard soil from which soil protection values, expressed as the fraction of species potentially affected, can be calculated.

The use of species toxicity data to predict the effects of pollutants on communities is an important development in ecotoxicology. However, despite this increased emphasis, the number of studies that have measured relative responses for a number of parameters remains low. One such study, which determined the sensitivity of reproduction and survival parameters to cadmium for a range of soil arthropods, was conducted by Crommentuijn et al. [5]. Results indicated that for some species, such as the oribatid mite *Platynothrus peltifer,* reproduction was inhibited at concentrations far below those affecting survival. However, for others, such as the isopod *Porcellio scaber,* sublethal and lethal effects occurred at similar concentrations. Thus, the species tested varied considerably in their relative sensitivities to the selected parameters.

Variation in the relative sensitivity of life-cycle traits between species would be expected to reflect differences in subindividual level responses. Such lower organizational level effects, which have been termed biomarkers, can be used as an early warning system for measuring effects caused by environmental pollutants [6]. A number of earthworm-based low organizational level biomarkers have been developed in recent years [7–10]. One of the most widely used techniques is the neutral-red retention (NRR) assay [11]. This procedure assesses lysosomal membrane stability by measuring the retention of cationic neutral-red dye in coelomocyte lysosomes. The technique has been used to establish dose–response relationships for a range of metals and organic chemicals in a variety of earthworm species [8,12–14]. However, to date, these tests have been conducted using a range of laboratory, semifield, and real field methods, making it difficult to compare sensitivities of responses between species or to compare the relative sensitivities of biomarker and life-cycle effects.

To compare the relative sensitivities of life-cycle and biomarker responses in different species, measurements should be conducted simultaneously using a standardized test system. Thus, in the current study, survival, weight change, cocoon production rate, and NRR were all measured in standardized laboratory toxicity tests using four earthworm species. Relative sensitivities of life-cycle and biomarker responses were compared after exposure to zinc since this metal has a key role in determining effects on soil invertebrates in many soils polluted with mixtures of metals [15,16]. The relative sensitivities of the life-cycle and biomarker responses to zinc were then compared for each of the four species by use of the SSI as proposed by Crommentuijn et al. [5].

MATERIALS AND METHODS

Collection and zinc exposure of selected earthworms

The four earthworm species used in the studies were *Eisenia fetida, Lumbricus terrestris, Lumbricus rubellus,* and *Aporrectodea caliginosa.* These species were selected because they represent a range of earthworm ecotypes. *Eisenia fetida* inhabits only organic matter-rich locations, such as animal

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Table 1. Characteristics of the soil used for the preparation of the earthworm exposure media

Texture	Sandy loam
Organic matter content (% loi)	2.35
pH	6.35
% Clay	9.7
% Silt	16.3
% Sand	74

manure or compost heaps. *Lumbricus rubellus* is epigeic, living in the upper soil and litter layers. *Aporrectodea caliginosa* is endogeic and lives in the mineral soil. *Lumbricus terrestris* is anecic and lives in deep vertical burrows. In addition to variations in ecotype, the four species vary in the activity of the calcium-secreting glands located in the pharyngeal cavity. Thus, *E. fetida, L. rubellus,* and *L. terrestris* have high calcium gland activity, while activity in *A. caliginosa* is low. It has been suggested that the activity of the calcium gland may be important for determining the relative sensitivities of earthworms to metals [2].

All zinc exposures were conducted in a natural soil-based test system. The soil used was a mixture of a commercially available sandy loam soil (Rockalls, Wokingham, Berkshire, UK) (Table 1) and commercially available *Sphagnum* peat (Bullrush Ltd, County Tyrone, UK). Initially, it was intended to use unamended soil during the study. However, results from initial trials indicated low earthworm survival and reproduction, particularly for *L. terrestris* and *L. rubellus.* Thus, to ensure adequate survival and cocoon production, 10% by weight of finely ground *Sphagnum* peat was added. The introduction of additional organic matter to the soil would be expected to alter zinc availability [17]. However, the use of peat from the same commercial batches ensured that differences in zinc availability at the same nominal concentrations were minimized among the four tests.

To prepare the test medium, soil was sieved through a 2-mm mesh to remove larger particles and mixed thoroughly with the required quantities of finely ground *Sphagnum* peat. One kilogram of the soil mix was then added to each experimental container (plastic boxes 220 × 160 × 80 mm), with four replicate containers used for each test concentration. The zinc concentrations used in all tests were 0, 190, 350, 620, 1,200, 2,000, and 3,600 μg/g. To spike the soils with zinc, aqueous solutions of the nitrate salt (Zn $[NO_3]_2.6H_20$) were added to the test soils to give the required water content (60% water-holding capacity) and metal concentrations in the test soil. The same volume of distilled water was added to controls. After addition of the relevant metal solutions, soils were left to stabilize for 1 week prior to the introduction of the earthworms.

The earthworms for each test were obtained from a number of diverse sources. *Eisenia fetida* were reared in a synchronous laboratory culture maintained on uncontaminated horse manure. Fully clitellate individuals with an average wet weight of 422 mg were selected for the study. Adult *L. terrestris* were obtained from a commercial supplier. These worms were picked from the soil surface at a parkland site during nightly surface activity. All worms used were adult, with an average wet weight of 6,063 mg. *Lumbricus rubellus* and *A. caliginosa* were collected from an unpolluted pasture on the University of Reading campus, Reading, United Kingdom, by digging and hand sorting. Collected worms were adult, with mean wet weights of 732 mg for *L. rubellus* and 724 mg for *A. caliginosa.*

Prior to exposure, all worms were acclimatized in the uncontaminated test medium for 7 d. After this time, worms were sorted from the soil, washed, weighed individually, and added to the relevant test soil in a random order. In the test with *E. fetida,* which has a small mean size and can exist at high population densities, 10 worms were added to each treatment replicate. For the remaining three species, which have a larger mean size and live at lower natural population densities, only six worms per container were used. To maintain the worms during the exposure period, the test containers were covered to limit water loss and kept in constant light at 15°C for 42 d. This temperature was selected since it was known to be optimal for the growth, reproduction, and survival of *L. terrestris, L. rubellus,* and *A. caliginosa* [18,19]. For *E. fetida,* higher temperatures (20–25°C) are considered optimal [20]. However 15°C was used in this study to maintain consistency with the other species.

During all tests, a number of life-cycle measurements were made. Mortality was measured every 7 d by sorting soils and counting the number of surviving worms. Weight change was assessed by comparing mean final weight with mean initial values for each container, while cocoon production was assessed by wet sieving the soil and collecting all cocoons. The number of cocoons produced during the test was compared to survival data to allow cocoon production rates (cocoons/worm/week) to be calculated. To increase rates of survival, growth, and cocoon production, finely ground fresh horse manure (dried and rewetted to 75% water content) was added as a source of food in all tests [21,22]. To ensure sufficient food, 4 g dry weight of food was added weekly to each container. In the test with *E. fetida,* manure was added as two small pellets into holes in the soil surface, while for the remaining species, food was mixed with surface soil.

Neutral-red retention assay

In addition to measuring the effects of zinc on selected life-cycle traits, the neutral-red retention assay was used to assess relative sensitivity of this biomarker response. The NRR assay, which measures contaminant-induced lysosomal membrane damage, is conducted on cells present in earthworm coelomic fluid. For this assay, coelomocytes were collected using an invasive technique. This involved inserting a fine-needled syringe containing earthworm physiological ringer [23] into the coelomic cavity of the earthworm at a site posterior to the clitellum. The syringe was filled with a small volume (20–50 μl) of coelomic fluid using a gentle drawing action. This technique has been found to be suitable for collecting live (viability >95%), intact earthworm coelomocytes [24].

To determine the neutral-red retention time, a stock solution of 20 mg neutral red (Sigma Chemical, St. Louis, MO, USA) dissolved in 1 ml of dimethyl sulfoxide was prepared. Ten microliters of the stock solution were then diluted with 2.5 ml of earthworm ringer to give a working neutral-red concentration of 80 μg/ml. The working solution was renewed every hour because of the crystallization of nonpolar neutral red in the aqueous ringer. Next, collected earthworm coelomocytes were placed on a slide and allowed to adhere to the surface for 30 s. Twenty microliters of neutral-red working solution were applied to the slide onto which a coverslip was placed. The preparation is scanned under a light microscope for 2 min, during which time several fields of view were chosen at ran-

dom and the number of unstained cells and cells with a stained cytosol (exhibiting dye loss from the lysosomes to the cytosol) counted. Following each observation period, the slide was returned to a humidity chamber for a further 2 min prior to the next observation. Observation was stopped when the number of stained cells exceeded 50% of the total. This time was taken as the neutral-red retention time. Neutral-red measurements were conducted for two earthworms from each replicated container (total of eight worms per treatment).

Metal analysis of soils and earthworm tissues

Analysis of zinc concentrations in the exposure soils was used to verify nominal concentrations. For the analysis, approx. 1 g of test soil was placed into a conical flask with 10 ml of concentrated Analar® grade nitric acid (Merck, Darmstadt, Germany). Flasks were heated to the boiling point until all organic matter had been broken down, and the digests were diluted to 100 ml with double-distilled water. Solutions were analyzed by flame atomic absorption spectrometry (Varian Spectra 30 AAS, Varian, Walnut Creek, CA, USA).

On completion of the neutral-red retention measurements, individual worms were prepared for analysis of body zinc concentration. Prior to analysis, all animals were maintained on filter paper for 48 h to allow them to void any soil present in the gut. This period should have been sufficient to ensure removal of almost all ingested soil since gut transit time of 8, 12 to 24, and 2.5 h have been found for *L. terrestris, L. rubellus,* and *E. fetida,* respectively [25–27].

For analysis of *E. fetida,* whole worms were placed into acid-washed test tubes and dried to constant weight. Two milliliters of Analar grade nitric acid were added, and the solution was heated until all tissue had been digested. Once cool, the digest was diluted to 10 ml with double-distilled water and analyzed for zinc by flame atomic absorption spectrophotometry. For the remaining three species, worms were dried in conical flasks. Ten milliliters of acid were added and the flasks heated to allow digestion. Resulting solutions were then diluted to 100 ml with deionized water and analyzed using flame atomic absorption spectrophotometry. During all analyses, standard reference materials (tomato leaf and bovine liver from the National Bureau of Standards, Washington, DC; lobster hepatopancreas from the National Research Council, Ottawa, ON, Canada; and calcareous loam soil from the Community Bureau of Reference, Brussels, Belgium) were measured. Values obtained were within 10% of certified values in all cases.

Statistics

Significant differences in mortality, growth, cocoon production rate, neutral-red retention, and zinc content were calculated using analysis of variance (ANOVA). When differences were found, Tukey's multiple comparison test was used to determine differences between specific treatments. Collected lethal and sublethal data were also used to calculate LC50 and EC50 values. LC50 values with 95% confidence intervals were determined by the log-probit method using the MicroProbit 3.0 statistical software package (MicroProbit, U.S. Environmental Protection Agency, Cincinnati, OH). The EC50 values were calculated using the linear interpolation technique within the IC_p 2.0 software system (U.S. Environmental Protection Agency, Cincinnati, OH). In both cases, the effect concentrations were calculated using measured soil zinc concentrations. For calculation of weight-change EC50s, weight-loss data would be required for all concentrations. However, for the containers in which no worms survived after 42 d, such data were not available. Thus, to allow weight-change EC50s to be calculated, a weight loss of 50% was assumed in the containers with no worms alive at 42 d. To compare the relative sensitivity of the measured life-cycle and biomarker responses, SSIs were determined from calculated effect concentration (EC10) values. Sublethal sensitivity indices were calculated for each trait as follows: SSI = LC50/EC10 [5].

RESULTS

Total soil zinc concentrations

Analysis of nitric acid–extractable zinc levels in control soils indicated that total zinc concentrations (102–133 μg/g) were within the range typical for an uncontaminated soil. Because of the presence of zinc in the unamended medium, measured zinc concentrations generally exceeded nominal values, particularly at the lowest nominal soil zinc concentration (Table 2). Thus, measured soil zinc concentrations have been used in all statistical interpretation.

Effects of zinc on life-cycle traits

Survival of all four species was significantly reduced at 2,000 and 3,600 μg Zn/g, while no significant reductions in survival were found at any of the remaining (lower) soil zinc concentrations. Dose-mortality data was used to calculate 14- and 48-d LC50s. Values calculated after 14 d indicated that the lowest LC50 of 1695 (1,621–1,775) μg Zn/g was for *A. caliginosa. Lumbricus rubellus* was the next most sensitive, with an LC50 of 1,734 (1,651–1,791) μg Zn/g, followed by *L. terrestris* with a 14-d LC50 of 2,378 (2,132–2,636) μg Zn/g and finally *E. fetida,* which was least sensitive, with an LC50 of 3,172 (3,150–3,215) μg Zn/g. Calculated 48-d LC50s were similar to 14-d values for all four species and were 1,619 (1,537–1,636) μg Zn/g for *A. caliginosa,* 1,709 (1,630–1,759) μg Zn/g for *L. rubellus,* 2,217 (1,940–2,646) μg Zn/g for *L. terrestris,* and 3,350 (3,130–3,171) μg Zn/g for *E. fetida.* Thus, the order of sensitivity was the same as after 14 d of exposure.

Analysis of mean weight change over the exposure period by one-way ANOVA indicated a significant effect of zinc concentration only for *A. caliginosa* ($p < 0.001$) and *L. terrestris* ($p < 0.05$). For *L. terrestris,* a post hoc multiple comparison test indicated no significant differences in weight change between any of the tested concentrations. However, for *A. caliginosa,* significant differences in weight change between treatments were found. Significantly higher weight loss was found for the worms exposed to 1,200 and 2,000 μg Zn/g when compared to control soil values. Because of the absence of clear dose–response relationships, it was not possible to calculate an EC50 for weight change in *E. fetida, L. terrestris,* or *L. rubellus.* For *A. caliginosa,* an EC50 of 417 (26–681) μg Zn/g was calculated.

The soil medium and test protocols used gave high rates of reproduction in the control for three of the four species tested. For *L. terrestris, L. rubellus,* and *A. caliginosa,* control cocoon production rates were 0.42, 2.1, and 1.84 cocoons/worm/week, respectively. These values compared favorably with the rates of 0.5 cocoons/worm/week for *L. terrestris,* 1.8 cocoons/worm/week for *L. rubellus,* and 2.6 cocoons/worm/week for *A. caliginosa* found in culture by other authors [18,28,29]. For *E. fetida,* the control cocoon production rate

Table 2. Nominal and actual measured concentrations of zinc in soils to which *Eisenia fetida*, *Lumbricus terrestris*, *Lumbricus rubellus*, and *Aporrectodea calignosa* were exposed for 42 d. The effects of exposure on survival, percentage weight change relative to initial weight, cocoon production rate, and mean worm zinc concentration are shown also. All values marked with asterisks are significantly different from controls at $p < 0.05$

Nominal zinc concn. (μg/g)	Actual zinc concn. (μg/g)	Percentage survival		Percentage weight change relative to initial weight	Cocoon production, cocoons/worm/week	Mean worm zinc concn. (μg/g dry wt)
		14 d	42 d			
Eisenia fetida						
0	124	100	100	3.4 ± 5.6	0.48 ± 0.06	120 ± 5
190	440	100	93 ± 4	−0.2 ± 7.2	0.6 ± 0.04	117 ± 6
350	643	100	100	5.3 ± 9.8	0.38 ± 0.03	118 ± 5
620	838	98 ± 2	98 ± 2	11.7 ± 6.9	0.38 ± 0.04	126 ± 14
1,200	1,650	100	98 ± 2	−9.2 ± 5.2	0.33 ± 0.07*	170 ± 22
2,000	2,470	93 ± 2	88 ± 2*	−6.4	0.26 ± 0.02*	282 ± 77
3,600	4,000	18 ± 8*	0*	—	0*	—
Lumbricus terrestris						
0	133	96 ± 4	75 ± 25	−23.4 ± 3.6	0.42 ± 0.05	667 ± 19
190	222	91 ± 4	50 ± 25	−24.9 ± 2.5	0.34 ± 0.05	738 ± 16
350	361	91 ± 4	67 ± 24	−19.9 ± 1.9	0.33 ± 0.13	884 ± 31
620	718	100	100	−17.2 ± 1.5	0.34 ± 0.09	1,109 ± 40
1,200	1,767	100	83 ± 12	−24 ± 1.5	0.16 ± 0.06	1,628 ± 38
2,000	2,217	54 ± 11*	38 ± 25	−20.5 ± 3.1	0.07 ± 0.05*	1,805 ± 42
3,600	3,615	0*	0*	—	0*	—
Lumbricus rubellus						
0	118	100	100	3.4 ± 3.6	2.1 ± 0.21	614 ± 52
190	388	100	100	−0.2 ± 7.2	1.63 ± 0.25	746 ± 123
350	638	100	96 ± 4	5.3 ± 9.8	1.67 ± 0.19	956 ± 86
620	724	100	100	11.7 ± 6.9	1 ± 0.13*	1,029 ± 107
1,200	1,213	92 ± 5	92 ± 5	−9.2 ± 5.9	0.2 ± 0.05*	1,186 ± 74
2,000	2,255	8 ± 5*	4 ± 5*	−14.1	0.02 ± 0.02*	1,002[a]
3,600	5,818	0*	0*	—	0*	—
Aporrectodea caliginosa						
0	102	100	100	24.9 ± 6	1.84 ± 0.05	352 ± 37
190	297	100	100	32.3 ± 2	1.71 ± 0.11	484 ± 50
350	400	100	100	30.6 ± 2.6	1.22 ± 0.19*	414 ± 32
620	638	100	96 ± 4	6.2 ± 8.7	0.35 ± 0.13*	618 ± 110
1,200	1,377	100	100	−37.4 ± 7.8*	0.01 ± 0.01*	785 ± 143
2,000	1,811	33 ± 10*	29 ± 14*	−38 ± 10.5*	0*	572 ± 93
3,600	3,975	0*	0*	—	0*	—

[a] Only one worm available for analysis.

of 0.48 cocoons/worm/week was somewhat below optimum. For example, Venter and Reinecke [30] found reproduction rates for this species of 2.5 cocoons/worm/week. However, the fact that the latter study was conducted at 25°C as compared to 15°C used in the current study probably accounts for any differences found.

Analysis of cocoon production rates using ANOVA indicated a significant effect of zinc in each of the four species tested. Furthermore, post hoc comparisons also indicated significant dose-dependent effects. For *E. fetida*, cocoon production rates were significantly reduced at 1,200 μg Zn/g and at all higher zinc concentrations. For *L. terrestris*, cocoon production rates were significantly reduced at 2,000 and 3,600 μg Zn/g. For *L. rubellus*, cocoon production was significantly reduced at 620 μg Zn/g and all higher soil zinc concentrations, while for *A. caliginosa*, cocoon production was significantly reduced at all concentrations in excess of 350 μg Zn/g. Cocoon production rates were used to estimate EC50 (and EC10) values. Calculations indicated that sensitivity, when expressed by cocoon production EC50s, increased in the following order: *A. caliginosa* (442 [370–522] μg Zn/g) > *L. rubellus* (599 [483–757] μg Zn/g) > *L. terrestris* (1,029 [567–1,430] μg Zn/g) > *E. fetida* (1,898 [953–2,170] μg Zn/g).

Neutral-red measurements

Neutral-red retention times in all *E. fetida* and *A. caliginosa* maintained in the control soil were longer than the 60-min observation period. This was not the case for *L. terrestris* and *L. rubellus.* For these species, the retention time for some of the control soil worms was below the maximum value, indicating higher variability in the stress responses when kept in a control situation. Calculation of the mean control NRR times in these latter species gave values of 32 and 43.5 min for *L. terrestris* and *L. rubellus*, respectively.

One-way ANOVA indicated a significant impact of zinc on NRR in all four species, and these were accompanied by clear dose-dependent effects (Fig. 1a to d). For *E. fetida*, NRR time was significantly reduced in surviving worms incubated at 2,000 μg Zn/g (Fig. 1a). For *L. terrestris*, NRR times were significantly reduced at 620, 1,200, and 2,000 μg Zn/g (Fig. 1b), while for both *L. rubellus* and *A. caliginosa*, NRR times were significantly lower in all zinc-contaminated soils (Fig. 1c and d). Neutral-red retention times were used to estimate EC50 (concentration required to reduce neutral-red retention times to half the control value) and EC10 values. Calculations indicated that the sensitivity of the biomarker response ex-

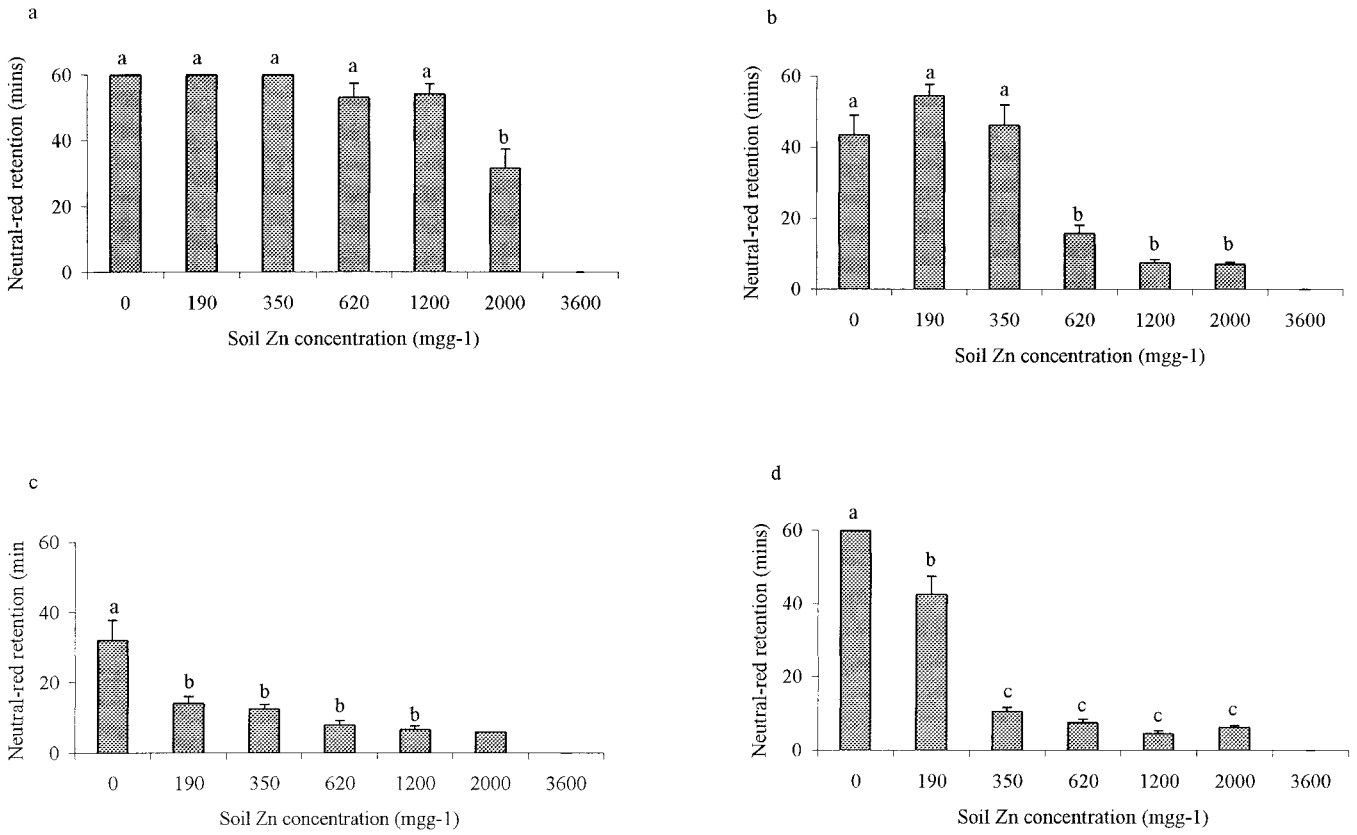

Fig. 1. Mean (± SEM) neutral-red retention times (min) for (**a**) *Eisenia fetida,* (**b**) *Lumbricus terrestris,* (**c**) *Lumbricus rubellus,* and (**d**) *Aporrectodea caliginosa* exposed to an increasing range of soil zinc concentrations in separate laboratory tests.

pressed as an EC50 increased in the following order: *L. rubellus* (168 [140–447] μg Zn/g) > *A. caliginosa* (252 [249–271] μg Zn/g) > *L. terrestris* (542 [491–572] μg Zn/g) > *E. fetida* (>2,000 μg Zn/g).

Earthworm zinc concentration

Analysis of the relationship between (measured) soil and mean earthworm zinc concentrations using linear regression of log-transformed values indicated a significant relationship in all species (Fig. 2). Comparisons of regressions for the four tested species using a general linear model indicated no significant differences between slope parameters. However, a comparison of regression intercepts indicated significant differences between tested species. Thus, the concentration of zinc found in animals maintained in the control soil, which are representative of minimum physiological requirements, differed between species. Initial concentrations were lowest in *E. fetida,* intermediate for *A. caliginosa,* and highest in *L. rubellus* and *L. terrestris.*

Comparative sensitivity of biomarker and measured life-cycle traits using SSI

For three of the four species (*E. fetida, L. terrestris,* and *L. rubellus*), EC10 values for the effects of zinc on weight change could not be calculated because of the absence of a clear dose–response relationship. Thus, sublethal sensitivity indices could not be calculated for this parameter in these species. Of the remaining sublethal parameters, calculated SSIs indicated that the threshold concentration for sublethal life-cycle and biomarker responses (EC50) occurred at concentrations below the median lethal concentration (LC50) by at least a factor of four (Table 3). For *E. fetida* and *L. terrestris,* the highest SSIs of 13 and 19.3 were found for cocoon production. However, for the remaining two species, *L. rubellus* and *A. caliginosa,* the highest SSIs of 50.3 and 24.9 were found for NRR time. The SSI calculated for the effects of zinc on weight change in *A. caliginosa* was 3.9. Thus, the threshold concentration for this parameter was less sensitive to zinc than either cocoon production rate or NRR time.

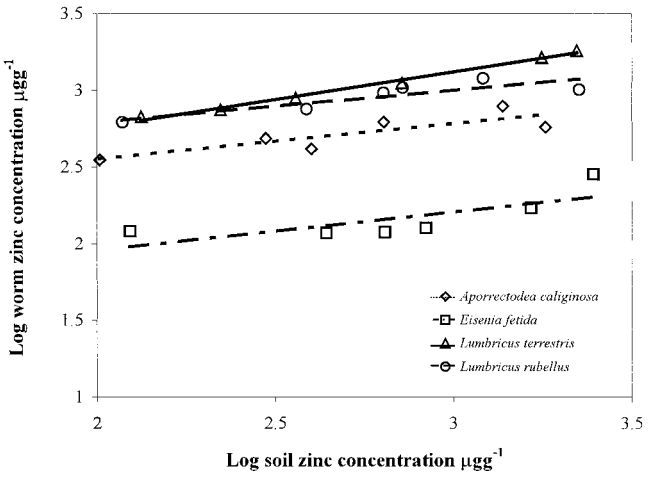

Fig. 2. Relationships between $\log_{10}$ worm zinc concentration and $\log_{10}$ measured soil zinc concentration in four species of earthworms exposed in a laboratory test system. All values are expressed on a dry-weight basis and are means of eight individuals.

Table 3. Toxicity values for the effects of zinc on four earthworm species exposed in a soil-based laboratory toxicity test system for 42 d. Values calculated for effects on survival are expressed as LC50 and LC10 values (with 95% confidence interval where available). Values for effects on cocoon production, weight change, and neutral-red retention (NRR) time are as EC50s and EC10s. All values are calculated from measured zinc concentrations. Sublethal sensitivity indexes (SSIs) are calculated as LC50/EC10 for each measurement parameter

Species	Parameter	EC(LC)50 (μg Z/ng)	EC(LC)10 (μg Z/ng)	SSI (LC50/EC10)
Eisenia fetida	14-d survival	3,172 (3,150–3,215)	2,511 (2,470–2,588)	
	48-d survival	3,150 (3,130–3,171)	2,740 (2,470–2,470)	
	Cocoon production	1,898 (953–2,174)	246 (212–311)	13
	Weight change	N/A —	N/A —	N/A
	NRR time	>2,000 —	599 (501–1,356)	5
Lumbricus terrestris	14-d survival	2,378 (2,132–2,636)	1,870 (1,811–1,962)	
	48-d survival	2,217 (1,940–2,646)	1,857 (1,588–2,037)	
	Cocoon production	1,029 (567–1,426)	115 (62–706)	19.3
	Weight change	N/A —	N/A —	N/A
	NRR time	542 (491–572)	368 (241–396)	6.0
Lumbricus rubellus	14-d survival	1,734 (1,651–1,791)	1,234 (1,115–1,321)	
	48-d survival	1,709 (1,630–1,759)	1,232 (1,017–1,303)	
	Cocoon production	599 (483–757)	88 (52–404)	19.4
	Weight change	N/A —	N/A —	N/A
	NRR time	168 (140–447)	34 (28–67)	50.3
Aporrectodea caliginosa	14-d survival	1,695 (1,621–1,775)	1,417 (1,229–1,457)	
	48-d survival	1,619 (1,537–1,636)	1,402 (1,242–1,427)	
	Cocoon production	442 (370–522)	206 (133–268)	7.9
	Weight change	868 (748–968)	417 (26–681)	3.9
	NRR time	252 (249–271)	65 (39–123)	24.9

DISCUSSION

Effects of zinc on life-cycle traits

A number of studies have examined the toxicity of zinc for selected life-cycle traits in earthworms [31,32]. However, only in the study of Spurgeon and Hopkin [2] have the responses of different species been compared. Results indicated that LC50s ranged from 1,106 μg Zn/g (for *E. fetida*) to 561 μg Zn/g (for *Aporrectodea rosea*), while cocoon production EC50s ranged from 623 μg Zn/g for *E. fetida* to 348 μg Zn/g for *L. rubellus.* Comparisons of current results with those of Spurgeon and Hopkin [2] indicate lower zinc toxicity in the current tests. These differences can be attributed primarily to variations in the soil type used for each study. For the current tests, exposures were in a natural loam soil amended with 10% peat. This soil almost certainly complexes metals more effectively than the artificial soil used by Spurgeon and Hopkin [2]. As a result, the bioavailability and thus toxicity of zinc is likely to be lower in the loam/peat mix than in the artificial soil. [17,33,34].

Comparisons of responses to zinc in the tested species indicated relatively small differences in sensitivity when related to those found for other chemicals. For example, in a study of the toxicity of chlorpyrifos to six earthworm species, Ma and Bodt [35] found that the LC50 for the most sensitive species (*L. rubellus*) was higher than for the least sensitive (*Eisenia veneta*) by a factor greater than nine. Similarly, Heimbach [36] compared the toxicity of a range of pesticides to *E. fetida* and *L. terrestris.* Results indicated that relative sensitivity was dependent on the chemical to which the worms were exposed. Thus, for copper-oxichloride, the LC50 for *E. fetida* was higher than that for *L. terrestris* by a factor greater than nine, while for aldicarb, *L. terrestris* was less sensitive than *E. fetida* by a factor of 7.8. The relatively small differences in the toxicity of zinc found in this study suggests that for this pollutant, which has a number of diverse effects, differences

in species sensitivity may be lower than for chemicals such as pesticides with a single mode of action.

Similar orders of sensitivity were found for effects on survival and cocoon production. Thus, *A. caliginosa* was the most sensitive, followed by *L. rubellus, L. terrestris,* and *E. fetida.* Variations in the sensitivity of species to pollutants can result from variations in behavior and ecology or from physiological differences in detoxification and elimination strategies. For earthworms, Spurgeon and Hopkin [2] have suggested that differences in zinc sensitivity may be partially explained by calcium gland activity, and this theory is, to an extent, supported by the current results. Thus, *E. fetida* and *L. terrestris,* both of which have active calcium metabolism, were least sensitive for both traits, while *A. caliginosa,* which has low calcium gland activity, was most sensitive for cocoon production. Differences in calcium metabolism do not, however, explain all species sensitivity differences found in this study. For example, *L. rubellus* was more sensitive than *L. terrestris* for effects on both survival and cocoon production despite the fact that these species have similar-size calcium glands. Instead, it is the smaller size of *L. rubellus,* with a larger surface-area-to-mass ratio (and thus higher potential uptake rate), that probably accounts for the observed sensitivity differences between these two species.

To assess the relationship between life-cycle effects, the relative sensitivity of acute and chronic life-cycle responses was studied using the sublethal sensitivity index of Crommentuijn et al. [5]. Comparisons of cocoon production SSIs calculated by comparing EC10s for this parameter to their respective LC50s indicated that values ranged from 19.4 for *L. rubellus* to 7.9 for *A. caliginosa.* Variations in reproduction SSIs have been found in a number of previous studies. Thus, Crommentuijn et al. [5] found SSIs for four soil invertebrate species ranging from 14.6 in *Folsomia candida* to <2.07 in *Orchesella cincta.* Similarly, Van Straalen et al. [37] also found large differences in reproductive SSIs between Collembola and mites. For the springtail *O. cincta,* the reproductive SSI was 3.2, while for the oribatid *Platynothrus peltifer,* a value of 280 could be calculated.

Differences in reproductive SSI are representative of variations in life-cycle responses to pollutant stress. For species with high SSIs, reproduction is affected at pollutant concentrations far below those affecting survival. Such large differences result because resources normally available for reproduction are diverted toward the detoxification and repair function required to increase survival probabilities. Low SSI values indicate that effects on reproduction occur at levels only marginally below those causing an acute response. In species exhibiting such low values, emphasis is given to maximizing reproduction rates rather than the maintenance and repair required to prevent death. Comparisons of the reproductive SSIs for earthworms determined in the current study with those for other soil invertebrates calculated in previous work indicate that the earthworm values are relatively high [5,37]. Thus, it appears that greater significance is given to maximizing survival than to maintaining reproductive rates when this group is subjected to pollutant stress as a result of zinc exposure. This emphasis on ensuring survival is probably related to the fact all species tested in this study are long-lived (maximum life span greater than five years) and exhibit iteroparous reproduction.

Earthworm zinc concentrations

Zinc was accumulated by all tested species, with highest concentrations found for worms in the most contaminated soil (excluding treatments in which substantial partial mortality occurred). Comparison of the regressions of log soil and log earthworm zinc concentrations indicated significant differences in intercepts that reflect variations in normal physiological zinc levels. Thus, *L. terrestris* and *L. rubellus* from uncontaminated sites contained approx. 600 μg Zn/g, *A. caliginosa* approx. 350 μg Zn/g, and *E. fetida* approx. 100 μg Zn/g (all on a microgram-per-gram dry-weight basis). Comparisons of the regression slopes indicated no significant differences between species, indicating similar net zinc assimilation at each soil concentration. In all cases, the slope parameter for the regression relationship was below one, indicating that zinc is regulated in the four earthworm species tested.

Zinc assimilation is determined by the relationship between accumulation and elimination, and separate studies have indicated that rates for these parameters may differ between earthworm species. For example, Spurgeon and Hopkin [38] found rapid zinc uptake and elimination in *E. fetida,* while Marino and Morgan [39] found slow uptake in *L. rubellus.* Differences in zinc kinetics can be expected to influence rates of assimilation. However, for the two earthworm species studied, the fact that high accumulation is matched by high excretion in *E. fetida* while low accumulation is probably matched by low excretion in *L. rubellus* means that, when exposed to a given soil zinc concentration, net assimilation rates are similar.

Neutral-red responses

Neutral-red measurements indicated significantly reduced retention times at selected zinc doses in all species (Fig. 1a to d). A comparison of the EC50 values for NRR response indicated that *L. rubellus* was the most sensitive, followed by *A. caliginosa, L. terrestris,* and finally *E. fetida.* The reasons underlying species-specific differences in the NRR responses, and in particular the low sensitivity of *E. fetida,* are not at present fully clear; however, two possible explanations can be given. First, the fact that *E. fetida* is able to regulate internal zinc levels by rapid elimination means that this species may be able to maintain free zinc concentrations in coelomic fluid at levels below those required to cause cytological damage [38]. Second, variations in the composition of the coelomic fluid affect assay results. Flow cytometric studies of coelomic fluids from selected earthworm species have indicated differences in the cell assemblages present. For example, Diogene et al. [40] found that the coelomic fluid of *E. fetida* contained mainly (57%) small chloragocyte cells of between 2 and 10 μm, while in *L. terrestris,* the largest fraction (52%) was of larger coelomocytes of greater than 20 μm. Chloragocytes, which are common in *E. fetida,* are known to strongly accumulate neutral red [41]. Thus, in *E. fetida,* the abundance of chloragocytes may reduce the concentration of neutral-red dye available for uptake by the coelomocytes monitored during the NRR assay, making detection of dye leaching from the lysosomes to the cytosol more difficult.

Despite the problems of using the NRR assay in *E. fetida,* results from the current study suggest that the method can be used to detect zinc exposure in a range of earthworm species. Previous studies have also indicated that the technique is broadly responsive for a range of organic pollutants and metals and in laboratory, mesocosm, and field exposures [7,12]. This

widespread applicability for the NRR assay in terms of species, chemical, and exposure protocols suggests that the method may be applicable as a general biomarker for quantifying pollutant exposure in earthworms. However, prior to adoption for widespread use, it is important to establish clear links between the response and ecologically important life-cycle endpoints such as survival, length of juvenile period, and fecundity. Additionally, confirmation that the biomarker is truly predictive, in the sense that responses are induced at concentrations below those resulting in effects on key life-cycle parameters, should also be sought.

A number of studies have found clear links between NRR times and effects on key life-cycle parameters. For example, Svendsen et al. [8] found that reduced NRR time for *E. andrei* exposed to copper in a laboratory test system was accompanied by effects on cocoon production, although effect concentrations for NRR were lower than the cocoon production values. The link between the biomarker and life-cycle responses is also apparent from the results of the current study. Thus, NRR times were significantly reduced in all treatments in which lower rates of cocoon production were found (except 1,200 $\mu g g^{-1}$ in *E. fetida*). The presence of clear links between neutral-red retention and measured life-cycle parameters supports the use of this biomarker for quantification of pollutant exposure in earthworms.

Calculation of SSIs from NRR EC10s and respective LC5050s gave values ranging from 4.5 for *E. fetida* to 41.2 for *L. rubellus.* For two species, *L. rubellus* and *A. caliginosa*, the SSI for NRR was higher than the SSIs for cocoon production, indicating that the assay is predictive of life-cycle effects. However, for the two remaining species, NRR SSIs were below cocoon production values, indicating that in these species, effects on neutral-red retention occur at concentrations above those affecting life-cycle traits.

The absence of a predictive element for the NRR assay in *E. fetida* and *L. terrestris* could present some problems when using this technique in practical risk-monitoring studies (for zinc). For example, in some cases a biomarker response may be absent in soils at which substantive effects on life-cycle traits occur. The species-dependent differences in life-cycle and biomarker sensitivities found in this study emphasize the importance of having a suite of organisms and methods available for monitoring purposes. In the United Kingdom (and northern Europe), both *L. rubellus* and *A. caliginosa* are among the most common earthworm species, and *L. rubellus* in particular is frequently present in a wide range of contaminated soils [2,42–44]. Thus, these species (for which the NRR assay is truly predictive) should, where possible, be favored for biomarker assessment and subsequent risk assessment using the neutral-red retention technique.

Currently, guidelines for the use of risk assessment tools based on soil invertebrate biomarkers, and in particular lysosomal membrane stability, have yet to be developed. The success of these biomarkers as tools for risk assessment purposes depends largely on the choice of biomarker assay (both for effect and for exposure measurement), which should both be sensitive and reflect the relevant ecological effects of toxicity at a site. The field of biomarker research has developed rapidly in the last decade and has triggered an ongoing debate on the pros and cons of various biomarkers to be included in risk assessment procedures. Of great consideration is the validity of a predictive role for biomarkers in the assessment of risks to soil invertebrate communities and the potential for the circumvention of lasting damage to populations [45]. Weeks et al. [46] set out a stepwise approach for the implementation of a biomarker-monitoring survey using soil invertebrate species to generate data for risk assessment purposes. There are, however, no rigid guidelines currently established to implement such a scheme.

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REFERENCES

1. Kammenga JE, Riksen JAG. 1996. Comparing differences in species sensitivity to toxicants: Phenotypic plasticity versus concentration-response relationships. *Environ Toxicol Chem* 15:1649–1653.
2. Spurgeon DJ, Hopkin SP. 1996. The effects of metal contamination on earthworm populations around a smelting works—Quantifying species effects. *Appl Soil Ecol* 4:147–160.
3. Aldenberg T, Slob W. 1993. Confidence limits for hazardous concentrations based on logistically distributed NOEC toxicity data. *Ecotoxicol Environ Saf* 25:48–63.
4. Van Straalen NM, Denneman CAJ. 1989. Ecotoxicological evaluation of soil quality criteria. *Ecotoxicol Environ Saf* 18:241–251.
5. Crommentuijn T, Doodeman C, Van Der Pol JJC, Doornekamp A, Rademaker MCJ, Van Gestel CAM. 1995. Sublethal sensitivity index as an ecotoxicity parameter measuring energy allocation under toxicant stress—Application to cadmium in soil arthropods. *Ecotoxicol Environ Saf* 31:192–200.
6. Weeks JM. 1995. The value of biomarkers for ecological risk assessment: Academic toys or legislative tools. *Appl Soil Ecol* 2: 215–216.
7. Scott-Fordsmann JJ, Weeks JM. 1998. A review of selected biomarkers in earthworms. In Sheppard SC, Bembridge JD, Holmstrup M, Posthuma L, eds, *Advances in Earthworm Ecotoxicology.* Society of Environmental Toxicology and Chemistry, Pensacola, FL, USA.
8. Svendsen C, Weeks JM. 1997. Relevance and applicability of a simple biomarker of copper exposure. I. Links to ecological effects in a laboratory study with *Eisenia andrei. Ecotoxicol Environ Saf* 36:72–79.
9. Stürzenbaum SR, Kille P, Morgan AJ. 1998. Identification of heavy metal induced changes in the expression patterns of the translationally controlled tumour protein (TCTP) in the earthworm *Lumbricus rubellus. Biochim Biophys Acta* 1398:294–304.
10. Stürzenbaum SR, Kille P, Morgan AJ. 1998. Identification of new heavy-metal-responsive biomarker in the earthworm. In Sheppard SC, Bembridge JD, Holmstrup M, Posthuma L, eds, *Advances in Earthworm Ecotoxicology.* Society of Environmental Toxicology and Chemistry, Pensacola, FL, USA.
11. Weeks JM, Svendsen C. 1996. Neutral-red retention by lysosomes from earthworm (*Lumbricus rubellus*) coelomocytes: A simple biomarker for exposure to soil copper. *Environ Toxicol Chem* 15: 1801–1814.
12. Olesen TME. 1998. The use of an earthworm biomarker to assess the effects of sewage sludge applications to agricultural land. MS thesis. Odense University, Odense, Denmark.
13. Svendsen C, Weeks JM. 1997. Relevance and applicability of a simple biomarker of copper exposure. II. Validation and applicability under field conditions in a mesocosm experiment with *Lumbricus rubellus. Ecotoxicol Environ Saf* 36:80–88.
14. Svendsen C, Meharg AA, Freestone P, Weeks JM. 1996. Use of an earthworm lysosomal biomarker for the ecological assessment of pollution from an industrial plastics fire. *Appl Soil Ecol* 3:99–107.
15. Spurgeon DJ, Hopkin SP. 1995. Extrapolation of the laboratory-based OECD earthworm test to metal-contaminated field sites. *Ecotoxicology* 4:190–205.
16. 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.
17. Spurgeon DJ, Hopkin SP. 1996. Effects of variations of the or-

ganic-matter content and pH of soils on the availability and toxicity of zinc to the earthworm *Eisenia fetida*. *Pedobiologia* 40: 80–96.
18. Butt KR. 1992. The intensive production of *Lumbricus terrestris* L. for soil amelioration. *Soil Biol Biochem* 24:1321–1325.
19. Daniel O, Kohli L, Bieri M. 1996. Weight-gain and weight-loss of the earthworm *Lumbricus terrestris* L at different temperatures and body weights. *Soil Biol Biochem* 28:1235–1240.
20. Reinecke AJ, Kriel JR. 1981. Influence of temperature on the reproduction of the earthworm *Eisenia foetida* (Oligochaeta). *S Afr J Zool* 16:96–100.
21. Spurgeon DJ, Hopkin SP, Jones DT. 1994. Effects of cadmium, copper, lead and zinc on growth, reproduction and survival of the earthworm *Eisenia fetida* (Savigny): Assessing the environmental impact of point-source metal contamination in terrestrial ecosystems. *Environ Pollut* 84:123–130.
22. Van Gestel CAM, Dirven-Van Breemen EM, Baerselman R. 1992. Influence of environmental conditions on the growth and reproduction of the earthworm *Eisenia andrei* in an artificial soil substrate. *Pedobiologia* 36:109–120.
23. Lockwood APM. 1963. *Animal Body Fluids and Their Regulation.* Heinemann Educational Books, London, UK.
24. Svendsen C. 1995. Development of an in vitro neutral-red retention assay for testing toxicant-induced cell damage in soil invertebrates: Field extrapolation and validation. MS thesis. Odense University, Odense, Denmark.
25. Hartenstein F, Hartenstein E, Hartenstein R. 1981. Gut load and transit time in the earthworm *Eisenia foetida*. *Pedobiologia* 22: 5–20.
26. Piearce TG. 1972. The calcium relations of selected Lumbricidae. *J Anim Ecol* 41:167–188.
27. Hartenstein R, Amico L. 1983. Production and carrying capacity for the earthworm *Lumbricus terrestris* in culture. *Soil Biol Biochem* 15:51–54.
28. Lofs-Holmin A. 1983. Reproduction and growth of common arable land and pasture species of earthworms (Lumbricidae) in laboratory cultures. *Swed J Agric Res* 13:31–37.
29. Boström U. 1988. Growth and cocoon production by the earthworm *Aporrectodea caliginosa* in soil mixed with various plant materials. *Pedobiologia* 32:77–80.
30. Venter JM, Reinecke AJ. 1988. The life-cycle of the compost worm *Eisenia-fetida* (Oligochaeta). *S Afr J Zool* 23:161–165.
31. Neuhauser EF, Loehr RC, Milligan DL, Malecki MR. 1985. Toxicity of metals to the earthworm *Eisenia foetida*. *Biol Fertil Soils* 1:149–152.
32. Van Gestel CAM, Dirven-Van Breemen EM, Baerselman R. 1993. Accumulation and elimination of cadmium, copper, chromium and zinc and effects on growth and reproduction in *Eisenia andrei* (Oligochaeta, Annelida). *Sci Total Environ* (Suppl. 1):585–597.
33. Smit CE, Van Gestel CAM. 1996. Comparison of the toxicity of zinc for the springtail Folsomia candida in artificially contaminated and polluted field soils. *Appl Soil Ecol* 3:127–136.
34. Weltje L. 1998. Mixture toxicity and tissue interactions of Cd, Cu, Pb and Zn in earthworms (Oligochaeta) in laboratory and field soils: A critical evaluation of data. *Chemosphere* 36:2643–2660.
35. Ma WC, Bodt J. 1993. Differences in toxicity of the insecticide chlorpyrifos to six species of earthworms (Oligochaeta, Lumbricidae) in standardized soil tests. *Bull Environ Contam Toxicol* 50:864–870.
36. Heimbach F. 1985. Comparison of laboratory methods, using *Eisenia fetida* and *Lumbricus terrestris*, for the assessment of the hazard of chemicals to earthworms. *Z Pflanzenkr Pflanzenschutz* 92:186–193.
37. Van Straalen NM, Schobben JHM, De Goede RGM. 1989. Population consequences of cadmium toxicity in soil micro-arthropods. *Ecotoxicol Environ Saf* 17:190–204.
38. Spurgeon DJ, Hopkin SP. 1999. Comparisons of metal accumulation and excretion kinetics in earthworms (*Eisenia fetida*) exposed to contaminated field and laboratory soils. *Appl Soil Ecol* 11:227–243.
39. Marino F, Morgan AJ. 1998. The time-course of metal (Ca, Cd, Cu, Pb, Zn) accumulation from a contaminated soil by three populations of the earthworm *Lumbricus rubellus*. *Appl Soil Ecol* 9: 169–177.
40. Diogene J, Dufour M, Poirier GG, Nadeau D. 1997. Extrusion of earthworm coelomocytes: Comparison of the cell populations recovered from the species *Lumbricus terrestris*, *Eisenia fetida* and *Octolasion tyrtaeum*. *Lab Anim* 31:326–336.
41. Aziz NA, Morgan AJ, Kille P. 1998. Metal resistance on earthworms: Genetic adaption or physiological accumulation. *Pedobiologia* 43:594–601.
42. Van Brummelen TC, Verweij SA, Wedzinga SA, Van Gestel CAM. 1996. Polycyclic aromatic hydrocarbons in earthworms and isopods from contaminated forest soils. *Chemosphere* 32: 293–314.
43. Morgan JE, Morgan AJ. 1988. Earthworms as biological monitors of cadmium, copper, lead and zinc in metalliferous soils. *Environ Pollut* 54:123–138.
44. Dworschak UR. 1997. Earthworm populations in a reclaimed lignite open-cast mine of the Rhineland. *Eur J Soil Biol* 33:75–81.
45. Weeks JM. 1998. Effects of pollutants on soil invertebrates: Links between levels. In Schuurmann G, Markert B, eds, *Ecotoxicology*, John Wiley & Sons, Chichester, UK, pp 645–646.
46. Weeks JM, Ekdokimova G, Ernst WHO, Scott-Fordsmand J, Sousa JP, Sergeev VE, Nakonieczny M. 1999. Report of the working group on the strategy for the implementation of a biomarker biomonitoring programme for the Kol Peninsula, Russia. In Peakall DB, Walker CH, Migula P, eds, *Biomarkers: A Pragmatic Basis for the Remediation of Severe Pollution in Eastern Europe*, Kluwer, Dordrecht, The Netherlands, pp 279–298.