Ecotoxicology **7**, 291–295 (1998)

Toxicity of nickel to the earthworm and the applicability of the neutral red retention assay

JANECK J. SCOTT-FORDSMAND[1]*, JASON M. WEEKS[2] and STEPHEN P. HOPKIN[3]

[1]*Dept. Terrestrial Ecology, National Environmental Research Institute, Vejlsøvej 25, PO Box 314, DK-8600 Silkeborg, Denmark*

[2]*Institute of Terrestrial Ecology, Natural Environment Research Council, Monks Wood, Abbots Ripton, Huntingdon, Cambridgeshire, PE17 2LS, UK*

[3]*Division of Zoology, School of Animal and Microbial Sciences, University of Reading, PO Box 228, Reading RG6 6AJ, UK*

Received 13 March 1998; accepted 14 May 1998

The toxic effects of nickel on survival, growth, and reproduction of *Eisenia veneta* were investigated following 4 weeks of exposure to a nickel-chloride spiked loamy sand soil. The ability of a simple earthworm biomarker, the lysosomal membrane stability of coelomocytes, to reflect nickel exposure was also studied. Nickel caused a significant toxic effect on *E.veneta* at soil concentrations above 85 mg Ni/kg. Reproduction (cocoon production) was the most sensitive parameter being reduced at soil concentrations above 85 mg Ni/kg (EC10 = 85 mg Ni/kg). Survival of adults was only reduced at concentrations above 245 mg Ni/kg, while adult and cocoon wet weight were not affected by soil nickel concentrations up to 700 mg Ni/kg. The lysosomal membrane stability, measured as neutral-red retention time, was reduced at soil nickel concentrations similar to those that reduced reproduction, and demonstrated a dose-response relationship. The neutral-red retention time showed large individual variation for the earthworms within each exposure concentration. It was concluded that the lysosomal membrane stability, measured as neutral red retention time, has a potential role in risk assessment, but care should be taken conducting this test.

Keywords: earthworm; nickel; soil; toxicology; eisenia veneta.

Introduction

Nickel (Ni) is a naturally occurring element that is present in soil, water, air, and biological material. Elevated concentrations in the environment may also be caused by anthropogenic input such as deposition from the burning of fossil fuels, wear of nickel coated surfaces, and spreading of sewage sludge and manure, especially pig manure (Scott-Fordsmand, 1997). Such anthropogenic nickel discharges, and in certain places naturally occurring concentrations, may lead to elevated levels in terrestrial and other ecosystems. Although many studies have been performed on the toxic effects of nickel on terrestrial plants and microorganisms, little is known on the toxicity of nickel toward soil dwelling invertebrates (Scott-Fordsmand, 1997).

One important group of terrestrial invertebrates are earthworms. They are abundant and widespread in soils and play a key role in the functioning of such ecosystems. Although information on the toxicity of a range of metals toward earthworms is available (*e.g.*, Bengtsson and Tranvik, 1989; Morgan *et al.*, 1993; Spurgeon *et al.*, 1994; Spurgeon and Hopkin, 1995; Spurgeon and Hopkin, 1996a,b; van Gestel *et al.*, 1989; -1991; -1993), very little information is available on the toxicity of nickel (Scott-Fordsmand, 1997). Previous studies have mostly dealt with test designs that are difficult to extrapolate to soil concentrations and field conditions, for example using a two layer design (soil-sludge) (Hartenstein *et al.*, 1981; Malecki *et al.*, 1982; Neuhauser *et al.*, 1983, 1984) or direct injection of nickel into coelomic fluid (Furst *et al.*, 1993). The few studies that have been reported on soil exposure give highly different effect values for nickel on earthworms (Ma, 1983; Neuhauser *et al.*, 1985, 1986).

When performing laboratory studies with metals on soil organisms, it is always difficult to extrapolate these results to field conditions. One way to reduce this step is by applying a biomarker which may also be used in the field. For the effects of metals on earthworms a biomarker has recently been suggested by Weeks and Svendsen (1996). This biomarker, the Neutral-Red Retention assay (NRR), measures the membrane stability of lysosomes within the coelomocytes of earthworms. It has so far almost entirely been applied to studies dealing with copper exposure and it is not known whether this technique would work for nickel exposed earthworms (Svendsen *et al.*, 1996; Svendsen and Weeks, 1997a,b; Harreus *et al.*, 1997).

The aims of the present study are to evaluate the lethal and sublethal toxicity of nickel to the earthworm, *Eisenia*

*To whom correspondence should be addressed.

veneta, and to test the suitability of lysosomal stability (measured as neutral-red retention time) as a biomarker reflecting sublethal effects relevant for population dynamics.

Methods and materials

Earthworms

Adult *Eisenia veneta* were incubated under conditions identical to the experimental conditions (in the absence of added nickel) for one week prior to running the experiment. The worms (all adults) ranged in live weight from 1.310 to 1.811 g, and were maintained on moist filter paper for 24 hours to allow them to evacuate the gut.

Preparation and contamination of soil

The soil used was a loamy sand soil (LUFA-Speyer 2.2, Sp 2121, LUFA Speyer, Speyer, Germany) with a pH of 5.5, total organic carbon 2.3%, clay 5%, silt 13%, and sand 82%. Prior to the experiment the soil was oven-dried (Memmert, Type UL40) at 80°C overnight, to eliminate undesired soil fauna and to obtain nickel concentrations on a dry weight basis. Nickel was added as a chloride salt ($NiCl_2$-$6H_2O$, Merck Pro Analysis, UK) from a stock solution (4.545 g Ni/l).

Experimental design

The experiment was conducted in plastic containers containing 610 g moist loamy sand soil, *i.e.*, 500 g dry soil and 110 ml de-mineralized water giving a 22% water content (50% of the water holding capacity). The water, in the relevant cases also containing the final nickel content, was added one day prior to the start of the experiment. The earthworms (10 per replicate) were exposed to seven nickel concentrations, including controls, ranging between 0 and 1000 mg Ni/kg, specifically 0, 50, 100, 300, 500, 700 and 1000 mg Ni/kg dry weight, with four replicates per concentration. For all soils the pH was adjusted to 5.5–6.0, by the addition of $CaCO_3$. The experiment was continued for four weeks, at constant temperature 20°C and with a 12/12 hr 295 Lux/25 Lux regime. Food in the form of 3 g horse manure was added to the soil surface every 7 d. Prior to re-wetting with 10 ml glass-distilled water, the manure had been dried at 100 EC, ground, and sieved through a 2 mm sieve. Water lost from the containers by evaporation was replenished every 7 d.

Experimental measurements

At the end of the experiment, adult survival, growth, reproduction, and lysosomal membrane stability were measured for each exposure concentration. The surviving adult earthworms were hand collected, counted, and weighed after 24 hours on moist filter paper. Cocoons, used as a measure of reproductive output, were obtained by wet-sieving the soil and were counted and weighed.

To measure the lysosomal membrane stability of any surviving worms, coelomic fluid was extracted and stained as described by Weeks and Svendsen (1996). The viability of the extracted cells was checked with Eosin Y. For each earthworm, the lysosomal membrane stability was determined as the number of cells with fully stained (red) cytosols (*i.e.*, exhibiting dye leakage from the lysosomes to the cytosol) compared to the number of cells with unstained cytosols (no leakage); the ratio (leaked to non-leaked) was determined. For each worm this ratio was assessed during 2 minutes of counting every 4 minutes, until >50% of the cytosols were stained red or 60 m had elapsed. The time required to obtain a ratio of 1 between the cells with leaked and non-leaked lysosomes was termed the neutral-red retention time (NRR-time).

Statistics

The NOEC (No Observable Effect Concentration) and LOEC (Lowest Observable Effect Concentration) were estimated by multiple comparison using Dunnett's procedure, on a 5% significance level (Nordberg-King, 1993). Effect concentrations, EC10 and EC50, and bootstrapping intervals (equivalent to 95% confidence intervals) were estimated by fitting a logistic model to the data (Nordberg-King, 1993).

Results

Adult survival and growth

The mortality of the earthworms increased with increasing soil nickel concentration. All worms exposed to 1000 mg Ni/kg died. The EC10 for mortality (corrected for control mortality) was 247 mg Ni/kg and the EC50 684 mg Ni/kg. The NOEC (no observed effect concentration) was 700 mg Ni/kg and the LOEC (lowest observed effect concentration) was 1000 mg Ni/kg (Table 1).The mean earthworm wet-weight showed no significant change with exposure concentration (Table 2).

Cocoon production and cocoon wet weight

Cocoon production was more severely affected by nickel than adult survival, with no cocoons produced at concentrations of 700 and 1000 mg Ni/kg, and only in one replicate at 500 mg Ni/kg. For reproduction, the NOEC was 100 mg Ni/kg and the LOEC 300 mg Ni/kg, with an EC10 of 85 mg Ni/kg and an EC50 of 300 mg Ni/kg (Table 1). The mean cocoon wet weight showed no change with soil nickel concentrations, the mean wet-weight being 0.0227–0.0246 grams (Table 2).

Lysosomal membrane stability

The neutral-red retention time for earthworms exposed to a range of soil nickel concentrations showed a general decrease with increasing nickel concentrations, although large individual variation was observed. Earthworms ex-

Table 1. No Observable Effect Concentrations (NOEC), Lowest Observable Effect Concentrations (LOEC) at a 5% significance level, and Effect Concentrations with a 10% effect (EC10) and a 50% effect (EC50) mg Ni/kg dry weight for various endpoints in *Eisenia veneta* following exposure to nickel-chloride-spiked LUFA soil. Bracketed values are bootstrapping values on the EC10/EC50 estimates

Endpoint	NOEC	LOEC	EC10	EC50
Mortality	700	1000	247[45–825]	684[588–812]
Reproduction	100	300	85[−25–445]	300[104–458]
Neutral-Red retention time	300	500	85[58–300]	366[14–616]

Table 2. Mean (±SD) cocoon and earthworm (*Eisenia veneta*) wet weight following 28 d exposure to nickel chloride in a loamy sand soil (LUFA-Speyer 2.2)

Soil nickel concentration (mg Ni/kg dry weight soil)	Cocoon wet weight (±SD) (grams)	Final worm weight (±SD) (grams)
Control	0.0246 (0.0024)	1.247 (0.138)
50	0.0233 (0.0069)	1.350 (0.106)
100	0.0236 (0.0018)	1.255 (0.162)
300	0.0246 (0.0052)	1.434 (0.051)
500	0.0227	1.341 (0.129)
700	–[a]	1.151 (0.192)
1000	–[a]	–[b]

[a]No cocoons present. [b]No survivors.

posed to 700 mg Ni/kg all had neutral-red retention times (NRR-times) of less than 2 minutes, while this was the case for two replicates at 500 mg Ni/kg and one at 300 mg Ni/kg. The control and 50 mg Ni/kg in general exhibited NRR-times around 50 minutes or more. For the NRR-time an EC10 of 85 mg Ni/kg and an EC50 of 366 mg Ni/kg, with a NOEC at 300 mg Ni/kg and a LOEC at 500 mg Ni/kg (Table 1) were observed.

The pH of the soil remained constant during the experimental period.

Discussion

This study has shown that reproduction of the earthworm *Eisenia veneta* was affected in the laboratory by nickel soil concentrations above 85 mg Ni/kg. The most sensitive toxicological parameters were reproduction (cocoon production) and lysosomal membrane stability, with survival being reduced only at higher nickel concentrations. Adult and cocoon wet-weights were not affected by nickel at the concentrations tested, *i.e.*, the concentrations at which the cocoons were produced.

As discussed previously (see Forbes and Forbes, 1993; Hoekstra and Ewijk, 1993, Hopkin, 1993) the present results highlight the problems with using NOEC or LOEC values, for example in risk assessment. In the present experiment the NOEC value for mortality may be equivalent to 50% mortality of the worms. The results discussed in this paper are based on the trends observed using the EC10 and EC50 data obtained.

The soil nickel concentrations that caused mortality of *E. veneta* in the present study were at the same level or lower than previous records for other earthworm species (Ma, 1983; Neuhauser *et al.*, 1985; Neuhauser *et al.*, 1986). In a sandy loam, Ma (1983) measured an LC50 of between 2000 and 2500 mg Ni/kg for *Lumbricus rubellus*. Using an artificial OECD soil contaminated with $Ni(NO_3)_2$, Neuhauser *et al.* (1985, 1986) recorded an LC50 of 757 mg Ni/kg for *E. fetida*. In the various experiments performed so far, different species, soil types, and nickel salts have been used, which all contribute to the differences in effect levels. For example, Malecki *et al.* (1982) and Neuhauser *et al.* (1985) found that chloride salts of Ni were more toxic than other salts of nickel. In relation to this it may be speculated that the chloride ions have an additive toxic effect toward earthworms. The magnitude of this problem is as yet not well studied and more attention should be devoted to it. In the present experiment the worms were stressed by factors other than nickel as the control earthworms lost weight during the four weeks of exposure. Also under field conditions many stressors interact, probably influencing the toxicity of xenobiotics.

The relative sensitivity of measured endpoints (*e.g.*, reproduction, growth, and mortality) may vary with species and experimental conditions. It is important to establish such differences when establishing soil quality criteria, as discussed by Scott-Fordsmand *et al.* (1997). In our study, reproduction was three times more sensitive than adult mortality, which was equivalent to, or less than, that found in other studies on earthworms exposed to other metals (*e.g.*, Spurgeon *et al.*, 1994; van Gestel *et al.*, 1991, 1993).

Exposing *E. fetida* to a soil/sludge (equal size layers) system, Hartenstein *et al.* (1981), Malecki *et al.* (1982) and Neuhauser *et al.* (1983, 1984) observed no differences in sensitivity between mortality and growth. In our study, growth was not significantly affected at any concentration, although mortality occurred. It was, however, noted that there was a general reduction in the weight of the worms during the experiment, indicating that *E. veneta* did not have optimal conditions in the loamy sand soil.

It may be speculated that the survivability of hatchlings in nickel contaminated soil depends on the initial size of the hatchling. In this case a measure of the cocoon size may provide a prediction of the survivability of the offspring, as hatchling size has been shown to depend on the cocoon size in four other earthworm species, *i.e.*, *Allolobophora chlorotica*, *Aporrectodea calignosa*, *A. longa*, and *Lumbricus terrestris* (Pedersen and Bjerre, 1991). In the present experiment, however, the cocoon size was independent of the exposure concentration, only differing in the number produced. Nevertheless, it is not known whether the quality of the cocoons is the same at all concentrations. Previously it has been reported that the cocoon mass may depend on metal exposure, *i.e.*, collecting *Dendrobaena octaedra* from various heavy metal contaminated forests, Rozen (1996) observed that worms from the most heavily contaminated areas produced cocoons with a smaller mass.

There is an increasing interest in the development of biological markers to estimate the toxicity of different compounds in the environment. The lysosomal membrane stability (neutral-red retention time) has mainly been applied to earthworms following exposure to copper, although one published study has been conducted at a plastic contaminated site and another was concerned with the effects of sewage-sludge application to land (Weeks and Svendsen, 1996; Svendsen *et al.*, 1996; Svendsen and Weeks, 1997a, b). In agreement with these previous studies the lysosomal membrane stability was reduced following nickel exposure at soil concentrations as low as those that affect reproduction (Fig. 1). The sensitivity of the lysosomal membrane response, and the finding of a dose-response relationship between soil nickel concentrations and the lysosomal response, agreed with observations on copper exposed earthworms (Svendsen and Weeks, 1997a). In our study, however, the variation between individuals from the same exposure concentration was much larger than previously observed. Given this large variation, the present experimental design does not indicate whether the depressed reproduction correlates with the lysosomal membrane stability at the individual level, *i.e.*, it may or may not be the case that the individuals with the most reduced NRR-time had the most severely depressed reproduction. There may be several reasons for the observed variation in the NRR-time between individuals. For example, this biomarker is known to be affected by

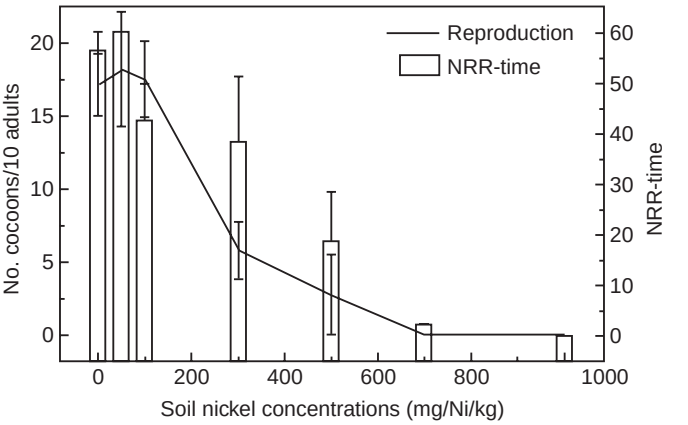

Fig. 1. Mean (±SEM) neutral-red retention time of coelomocyte lysosomes (NRR-time) and mean (±SEM) number of cocoons per 10 adult worms (reproduction), following 28 days of exposure to soil contaminated with nickel chloride.

conditions other than the presence of toxic metals (Moore, 1990). In our experiment we used a loamy sand soil which proved not to be optimal for earthworms, *i.e.*, a generally low reproduction rate and weight-loss was observed for earthworms in this soil. The lysosomal membrane stability (measured as the neutral-red retention time) seems very sensitive to nickel. However, in order to use it as a biomarker in the field, factors other than the toxicological ones which may affect this marker need to be identified. Furthermore, given the high variability between individuals, it is important to sample more animals in field studies in order to get a representative biomarker response.

Acknowledgements

This study was carried out under the European BIOPRINT project (supported by EU Environmental Research Programme contract no. EV5V-CT94-0406) during a PhD programme at University of Reading, UK. We are grateful for technical assistance from Miss Nicola Maguire. We also wish to thank two anonymous reviewers for useful comments on the manuscript.

References

Bengtsson, G. and Tranvik, L. (1989) Critical metal concentrations for forest soil invertebrates. *Water Air Soil Pollut.* **47**, 381–417.

Forbes, T.L. and Forbes, V.L. (1993) A critique of the use of distribution-based extrapolation models in ecotoxicology. *Funct. Ecol.* **7**, 249–254.

Furst, A., Chien Y. and Chien P.K. (1993) Worms as a substitute for rodents in toxicology: acute toxicity of three nickel compounds. *Toxicol. Meth.* **3**, 19–23.

Harreus, D., Köhler, H.-R. and Weeks, J.M. (1997) Combined non-invasive cell isolation and neutral-red retention assay for measuring the effect of copper on the lumbricid *Aporrectodea rosea* (Savigny). *Bull. Environ. Contam. Toxicol.* **59**, 44–49.

Hartenstein, R., Neuhauser, E.F. and Narahara, A. (1981) Effects of heavy metal and other elemental additives to activated sludge on growth of *Eisenia foetida. J. Environ. Qual.* **10**, 372–376.

Hoekstra, J. A. and van Ewijk, P.H. (1993) Alternatives for the No-Observed-Effect level. *Environ. Toxicol. Chem.* **12**, 187–194.

Hopkin, S.P. (1993) Ecological implication of '95% protection levels' for metals in soil. *Oikos* **66**, 137–141.

Ma W.C. (1983) Biomonitoring of soil pollution: ecotoxicological studies of the effect of soil-borne heavy metals on lumbricid earthworms. In *Annual Report 1982*, pp. 83–97. Research Institute for Nature Management, The Netherlands.

Malecki, M.R., Neuhauser, E.F. and Loehr, R.C. (1982) The effect of metals on the growth and reproduction of *Eisenia foetida* (Oligochaeta, Lumbricidae). *Pedobiologia* **24**, 129–137.

Moore, M.N. (1990) Lysosomal reaction to environmental stress and pollution. In *Estarine Ecotoxicology*, P.L. Chambers and C.M. Chambers, eds, pp. 81–87, *JAPAGA*, UK.

Morgan, A.J., Morgan, J.E., Turner, M., Winters, C. and Yarwood, A. (1993) Metal relationships of earthworms. In *Ecotoxicology of Metals in Invertebrates*. R. Dallinger and P.S. Rainbow, eds, pp. 333–358, Chelsea, UK: Lewis Publishers.

Neuhauser, E.F., Malecki, M.R. and Loehr, R.C. (1983) Methods using earthworms for the evaluation of potentially toxic materials in soils. In *Proceedings of the Second Symposium on Hazardous and Industrial Solid Waste Testing. ASTM STP*, R.A. Conway, ed. pp. 313–203, Gulledge WP, USA.

Neuhauser, E.F., Malecki, M.R. and Loehr, R.C. (1984) Growth and reproduction of the earthworm *Eisenia fetida* after exposure to sublethal concentrations of metals. *Pedobiologia* **27**, 89–97.

Neuhauser, E.F., Loehr, R.C., Milligan, D.L. and Malecki, M.R. (1985) Toxicity of metals to the earthworm *Eisenia fetida. Biol. Fert. Soil.* **1**, 149–152.

Neuhauser, E.F., Loehr, R.C. and Malecki, M.R. (1986) Contact and artificial soil tests using earthworms to evaluate the impact of waste in soil. In *Proceedings of the Fourth Symposium on Hazardous and Industrial Solid Waste Testing, ASTM STP. 886*, J.K. Petros, W.J., Lacy, and R.A. Conway, eds. pp. 192–203, Gulledge WP, USA.

Nordberg-King, T.J. (1993) A Linear Interpolation Method for Sublethal Toxicity: The inhibition concentration (Icp) Approach (Version 2.0). and Dunnett Program (Version 1.5), *NETAC Technical Report* 03-93, Duluth, MN: U.S. Environmental Protection Agency.

Pedersen, M.B. and Bjerre, A. (1991) The relationship between mass of newly hatched individuals and cocoon mass in lumbricid earthworms. *Pedobiologia* **35**, 35–39.

Rozen, A. (1996) Do earthworms (*Dendrobaena octaedra*) originating from polluted forests vary in body mass and cocoon production? In *Proceedings of the International Colloquium on Soil Zoology*, J.P. Curry, T. Bolger, B. Kaye and Purvis G., eds. p. 262.

Scott-Fordsmand, J.J. (1997) Toxicity of nickel to soil organisms in Denmark. *Rev. Environ. Contam. Toxicol.* **148**, 1–34.

Scott-Fordsmand, J.J., Krogh, P.H., and Weeks, J.M. (1997) Sublethal toxicity of copper to a soil-dwelling springtail, *Folsomia fimetaria* (Collembola: Isotomidae). *Environ. Toxicol. Chem.* **16**, 2538–2542.

Spurgeon, D.J., Hopkin, S.P. and Jones, D.T. (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.

Spurgeon, D.J. and Hopkin, S.P. (1995) Extrapolation of the laboratory based OECD earthworm test to metal-contaminated field sites. *Ecotoxicology* **4**, 190–205.

Spurgeon, D.J. and Hopkin, S.P. (1996a) Effect of variation in organic matter content and pH of soil on the availability and toxicity of zinc to the earthworm *Eisenia fetida. Pedobiologia* **40**, 80–96.

Spurgeon, D.J. and Hopkin, S.P. (1996b) 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.

Svendsen, C. and Weeks, J.M. (1997a) Relevance and applicability of a simple earthworm biomarker of copper exposure. I: Links to ecological effects in a laboratory study with *Eisenia andrei. Ecotoxicol. Environ. Saf.* **36**, 72–79.

Svendsen, C. and Weeks, J.M. (1997b) Relevance and applicability of a simple earthworm biomarker of copper exposure. II: Validation and applicability under field conditions in a mesocosm experiment with *Lumbricus rubellus. Ecotoxicol. Environ. Saf.* **36**, 72–79.

Svendsen, C., Meharg, A.A., Freestone, P. and Weeks, J.M. (1996) Use of an earthworm lysosomal biomarker for the ecological assessment of pollution from an industrial plastics fire. *Appl. Soil Ecol.* **3**, 99–107.

Van Gestel, C.A.M., van Dis, W.A., Van Breemen, E.M. and Sparenburg, P.M. (1989) Development of a standardized reproduction toxicity test with the earthworm species *Eisenia fetida* using copper, pentachlorophenol and 2,4-dichloroaniline. *Ecotoxicol. Environ. Saf.* **18**, 305–312.

Van Gestel, C.A.M., van Dis, W.A., Dirren-Van Breemen, E.M., Sparenburg, P.M. and Baerselman, R. (1991) Influence of cadmium, copper and pentachlorophenol on growth and sexual development of *Eisenia andrei* (Oligochaeta: Annelida). *Biol. Fert. Soils* **12**, 117–121.

Van Gestel, C.A.M., van Dis, W.A., Dirren-Van Breemen, E.M. and Baerselman, R. (1993) Accumulation and elimination of cadmium, copper, chromium and zinc and effects on growth and reproduction in *Eisenia andrei* (Oligochaeta: Annelida). *Sci. Tot. Environ. Suppl.*, (**Part 1**), 585–597.

Weeks, J.M. and Svendsen, C. (1996) Neutral-red retention by lysosomes from earthworm (*Lumbricus rubellus*) coelomocytes: A simple biomarker for exposure of soil invertebrates. *Environ. Toxicol. Chem.* **15**, 1801–1805.