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Comparison of Sublethal and Lethal Criteria for Nine Different Chemicals in Standardized Toxicity Tests Using the Earthworm *Eisenia andrei*

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In this study, the effects of nine different chemicals on the survival, growth, and reproduction of the earthworm species *Eisenia andrei* were determined using a recently developed method. Earthworms were exposed for 3 weeks to the test chemicals in an artificial soil substrate. Additional data on the acute toxicity of these chemicals were derived from the literature. For some chemicals, cocoon production was the most sensitive parameter (cadmium, chromium, paraquat, fentin, benomyl, phenmedipham), while for others cocoon hatchability was most sensitive (pentachlorophenol, parathion, carbendazim). In the case of parathion, growth of the worms seemed to be even more sensitive than reproduction. As an overall parameter for the effect on earthworm reproduction, the total number of juveniles produced per worm appeared to be a useful parameter. Differences between (acute) LC₅₀ values and the lowest NOEC value for effects on growth and reproduction were different for each chemical. Difference was greatest for cadmium (a factor of >100) and smallest for fentin, benomyl, and pentachlorophenol (a factor of 5-6). © 1992 Academic Press, Inc.

1. INTRODUCTION

At present, two guidelines for determining the earthworm toxicity of chemicals are available (OECD, 1984; EEC, 1985). Both guidelines use mortality as the only parameter. It is known, however, that survival is less sensitive and from an ecological point of view also less relevant compared to growth and reproduction (Moriarty, 1983). For that reason, recently a test method has been described for determining the effects of chemical substances on the reproduction of earthworms (Van Gestel *et al.*, 1989). This method is an extension of the existing guidelines for acute toxicity testing with earthworms (OECD, 1984; EEC, 1985), and the same earthworm species and artificial soil substrate are used.

From aquatic ecotoxicology, it is well known that there is no constant difference between acute LC₅₀ and no-observed-effect concentrations (NOEC) for effects on growth and reproduction (e.g., Slooff and Canton, 1983). To investigate whether this is also the case for earthworm toxicity, we tested nine chemicals with different modes of action. For these chemicals effects on survival, growth, and reproduction of earthworms were determined in the newly developed reproduction toxicity test according to Van Gestel *et al.* (1989). And results were compared with those on acute toxicity. The chemicals chosen are heavy metals (cadmium, chromium) and pesticides belonging

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Earthworms

All tests were of the species *Eis. andrei* and were ages ranged betw and 582 mg.

Chemicals

Cadmium chloride, fentin (triphenyltin chloride), Riedel de Haën a Merck and was 9 flüssig, and phen

Artificial Soil

The artificial soil consisted of ca. 69% fine sand, 27% of 6.0 ± 0.5. In the higher: 6.7-6.9 and sieved peat was used. demineralized water was used, and the pH (<0.5 mm) cow dung substrate was som pH 6.5-6.9.

Cocoon Production

Earthworm reproduction (Van Gestel *et al.* (1989). Fentin was subsequently added in an aqueous solution and the moisture content of the water. Amounts equivalent to the middle of the ground cow dung. Ten days before use. Ten adults with a glass petri dish at 20°C in an illuminated incubator. Before exposure, the artificial soil with 4 g



to different chemical classes: fungicides (benomyl, carbendazim, pentachlorophenol, fentin), insecticides (parathion), and herbicides (phenmedipham, paraquat). This paper presents the results.

2. MATERIALS AND METHODS

Earthworms

All tests were performed with adult earthworms, with a well-developed clitellum, of the species *Eisenia andrei* (Bouché, 1972). The worms were obtained from our own culture and were grown on horse dung at an ambient temperature of $20 \pm 5^\circ\text{C}$. Worm ages ranged between 8.5 and 15.5 weeks, and individual weights were between 170 and 582 mg.

Chemicals

Cadmium chloride, chromium(III) nitrate, paraquat dichloride, parathion-ethyl, fentin (triphenyltin chloride), and benomyl were obtained from Baker, Merck, and Riedel de Haën and were $\geq 99\%$ pure. Pentachlorophenol (PCP) was obtained from Merck and was 95% pure. Carbendazim was tested as the 60% formulation Derosal flüssig, and phenmedipham as the 16.2% formulation Betanal.

Artificial Soil

The artificial soil comprised (by dry weight) 10% sphagnum peat, 20% kaolin clay, ca. 69% fine sand, and some calcium carbonate to adjust soil pH (1 N KCl) to a level of 6.0 ± 0.5 . In the tests with chromium and carbendazim soil pH was somewhat higher: 6.7–6.9 and 7.3, respectively. In the case of the cocoon production tests 1 mm sieved peat was used, and the soil was moistened up to a level of 55% (w/w) using demineralized water. For the incubation of cocoons finely ground (<0.5 mm) peat was used, and the final substrate had a moisture content of 35% (w/w). Finely ground (<0.5 mm) cow dung was used as a food source. The pH of the cocoon incubation substrate was somewhat higher in the tests with chromium, fentin, and cadmium: pH 6.5–6.9.

Cocoon Production Toxicity Tests

Earthworm reproduction toxicity tests were performed as described by Van Gestel *et al.* (1989). Fentin, benomyl, and PCP were mixed with a small amount of sand and subsequently added to the artificial soil. All other chemicals were added to the soil as an aqueous solution. The substrate was mixed intensively with a household mixer and the moisture content was raised to the desired level by the addition of demineralized water. Amounts equivalent to 0.4 kg dry artificial soil were placed in 1-liter glass jars. In the middle of the soil, a hole was made and filled with 4 or 8 g (dry weight) finely ground cow dung. The cow dung was moistened up to 55% with demineralized water before use. Ten adult earthworms were added to each jar. Jars were loosely covered with a glass petri dish to prevent moisture loss by evaporation and incubated at $20 \pm 5^\circ\text{C}$ in an illuminated climatic chamber (ca. 400 lx).

Before exposure, earthworms were preincubated for 1 week (phase A) in untreated artificial soil with 4 g cow dung (8 g for carbendazim and phenmedipham). As described

by Van Gestel *et al.* (1989), this preincubation period was necessary to let the worms get adapted to the artificial soil substrate and to stimulate cocoon production during the exposure period. After this, earthworms were exposed for 3 weeks to the chemical substances (phase B). In the case of cadmium and chromium, the test was extended with a recovery period of 3 weeks in untreated soil to study the influence of the duration of exposure. For benomyl the test was prolonged with a second 3-week exposure period (phase C). In phases B and C, 4 g cow dung was added to the soil, and after 8-9 days (benomyl, carbendazim, phenmedipham) or after 7 and 14 days another 4 g cow dung was added. At the end of each period the number of cocoons produced, soil pH, soil moisture content, and worm weights were determined as described by Van Gestel *et al.* (1989).

Each test was performed at five or six test concentrations and a control. The tests had four jars per concentration, each jar containing 10 earthworms. In the case of benomyl and chromium three replicates were used. In the test with PCP 12 controls were used. Because earthworms are capable of avoiding chemical substances (Edwards and Lofty, 1977), they might escape from the treated soil by moving into the hole with untreated cow dung. For this reason, the test with cadmium was carried out with 8 jars per concentration, and in 4 of these jars cow dung was treated at the same cadmium concentration as the soil.

In the case of paraquat and fentin, a range finding was carried out to assess proper test concentrations. In this range finding, only two replicate jars were used per concentration. Paraquat was added dry to the soil, whereas fentin was added as a suspension in water. In the case of paraquat, the range-finding test lasted for 3 weeks and had no preincubation period. For the range-finding test with fentin, a 1-week preincubation period was included. In both range-finding tests cocoon production was the only parameter tested.

Cocoon Incubation

To determine the influence of the chemical substances on cocoon hatchability, cocoons produced in phases B and C were incubated for 5 weeks in untreated artificial soil as described by Van Gestel *et al.* (1988). The artificial soil was mixed with 1% finely ground cow dung as a food source for the juvenile worms.

To study the influence of PCP on cocoon hatching, cocoons produced in the control were incubated in soil treated with PCP concentrations corresponding with those in the cocoon production test. And cocoons produced in the treated soil were incubated in either untreated soil or soil treated at the same concentration. In this way, cocoon incubation tests with PCP could be performed in duplicate, and three different combinations of cocoon production and cocoon incubation (i.e., untreated-untreated, treated-untreated, treated-treated) could be tested.

Statistics

LC₅₀ values were calculated using the trimmed Spearman-Kärber method (Hamilton *et al.*, 1977/1978). EC₅₀ values for the effect of the chemicals on cocoon production were determined according to a logit model. No-observed-effect concentrations (NOEC) were calculated using the Williams test (Williams, 1971, 1972) and Student's *t* test or by analysis of variance (ANOVA) using the software package Genstat 5.

3. RESULTS AND DISCUSSION

Cadmium

The cadmium concentrations tested were 0, 10, 18, 32, 56, and 100 mg Cd · kg⁻¹ dry soil. Upon analysis corresponding actual concentrations appeared to be 0.1, 11, 17, 29, 50, and 94 mg Cd · kg⁻¹ dry soil, respectively.

Analysis of variance comparing the results of the tests with treated and untreated food did not reveal significant differences ($F_{1,5}^2 = 3.00$; n.s.). Treatment of the cow dung did have a significant effect on earthworm growth ($F_{1,5}^2 = 19.3$; $P < 0.001$); growth was higher when worms were fed untreated cow dung. And this difference was significant at 10 and 100 mg Cd · kg⁻¹ in soil and dung. Van Gestel *et al.* (1989) performed a similar study with copper and did not find any difference in cocoon production between worms fed treated and those fed untreated cow dung. Despite the difference observed for growth, results of both tests are combined and summarized in Table 1.

Cadmium did not influence the growth of earthworms, although growth tended to increase with increasing concentration. Cocoon production was significantly reduced at all concentrations tested. In the control, 0.9% of the cocoons were abnormally shaped and passed a 2-mm sieve; at 10 and 18 mg Cd · kg⁻¹ this was 2.4–2.6% and at the higher concentrations 5.7–6.6%. The number of fertile cocoons was not cadmium dose related, although it was significantly reduced at 18 mg Cd · kg⁻¹. Cadmium did not influence the number of juveniles hatching per fertile cocoon, but as a consequence of the effect on cocoon production the number of offspring (juveniles/worm/week) was significantly reduced at 18 mg Cd · kg⁻¹ dry soil and higher. During the 3 weeks recovery period cocoon production and growth of all groups completely recovered to the level of the control.

Van Gestel and Van Dis (1983) determined an LC₅₀ of >1000 mg Cd · kg⁻¹ for *E. andrei* in an artificial soil at pH 7.0. Ma (1983) reported a 6-week LC₅₀ and a 12-week NOEC of 500 and 10 mg Cd · kg⁻¹ dry soil, respectively, for the effect of cadmium on the survival and cocoon production of *Lumbricus rubellus* in a loamy sand. Bengtsson *et al.* (1986) determined the effect of soil pH and cadmium on the reproduction of *Dendrobaena rubida*. At pH 5.5 and 6.5 cocoon production was stimulated by 10 mg

TABLE I

EFFECT OF CADMIUM ON THE GROWTH AND REPRODUCTION OF *Eisenia andrei* (IN ARTIFICIAL SOIL (AVERAGE OF EIGHT REPLICATES))

Concentration (mg Cd · kg ⁻¹)	% growth	Cocoons/worm/week	% fertile cocoons	Juveniles/fertile cocoon	Juveniles/worm/week
0	3.2 ± 2.3	1.37 ± 0.11	94 ± 3	2.58 ± 0.29	3.33 ± 0.49
10	3.0 ± 3.6	1.11 ± 0.23*	90 ± 8	2.66 ± 0.37	2.66 ± 0.72
18	3.3 ± 2.4	1.06 ± 0.23**	81 ± 10**	2.41 ± 0.28	2.11 ± 0.61**
32	3.7 ± 2.2	1.07 ± 0.16**	94 ± 5	2.60 ± 0.15	2.61 ± 0.49*
56	4.6 ± 1.5	0.90 ± 0.18**	91 ± 5	2.39 ± 0.21	1.94 ± 0.36**
100	5.5 ± 2.4	0.85 ± 0.15**	93 ± 8	2.39 ± 0.36	1.86 ± 0.37**

* $P < 0.05$.

** $P < 0.01$.

Cd · kg⁻¹ and at pH 4.5 and 5.5 it was reduced by 100 mg Cd · kg⁻¹. At the highest concentration, cocoons were abnormally shaped and aberrantly colored. Hatching of the cocoons and embryonic development time were significantly increased by 10 mg Cd · kg⁻¹ at pH 5.5 and by 100 mg Cd · kg⁻¹ at pH 6.5.

In a substrate of untreated soil covered with a layer of treated horse dung, Malcom *et al.* (1982) found NOEC values for the effect on cocoon production of *E. fetida* <25 and 50 mg Cd · kg⁻¹ dung for acetate and chloride salts of cadmium, respectively. As earthworms may avoid toxic substances (Edwards and Lofty, 1977), these results cannot be compared with those of this study.

Chromium

Chromium concentrations tested were 0, 10, 32, 100, 320, and 1000 mg Cr · kg⁻¹ dry soil. Upon analysis corresponding actual soil concentrations appeared to be 6.3, 16, 40, 101, 287, and 972 mg Cr · kg⁻¹, respectively.

Table 2 shows the results of this test. No mortality occurred at any of the concentrations tested.

From Table 2, it can be concluded that growth of the worms was significantly ($P < 0.05$) reduced at 1000 mg Cr · kg⁻¹. During the 3-week recovery period growth was dose related increased; only for the worms exposed to 1000 mg Cr · kg⁻¹ was this increase significantly different from control at $P < 0.05$.

Cocoon production was increased (not significantly) at 10 and 32 mg Cr · kg⁻¹ and reduced (not significantly) at 100 and 320 mg Cr · kg⁻¹, and significantly ($P < 0.05$) reduced at 1000 mg Cr · kg⁻¹. The EC₅₀ for the effect of chromium on cocoon production was 155 mg Cr · kg⁻¹ dry soil. During the 3-week recovery period cocoon production was (not significantly) higher at 10 and 32 mg · kg⁻¹ and lower at 320 and 1000 mg Cr · kg⁻¹.

The fertility of the cocoons was significantly ($P < 0.05$) lower at 1000 mg Cr · kg⁻¹. During the 3-week recovery period, the number of fertile cocoons was no longer affected. The number of juvenile worms per fertile cocoon was not affected by chromium. The number of juveniles produced per worm per week was significantly reduced at ≥ 100 mg Cr · kg⁻¹ dry soil.

TABLE 2
EFFECT OF CHROMIUM (III) NITRATE ON THE GROWTH AND REPRODUCTION OF *Eisenia andrei* IN ARTIFICIAL SOIL (AVERAGE OF THREE REPLICATES)

Concentration (mg Cr · kg ⁻¹)	% growth	Cocoons/worm/week	% fertile cocoons	Juveniles/fertile cocoon	Juveniles/worm/week
0	33.2 ± 11.6	0.53 ± 0.09	89 ± 5	2.50 ± 0.27	1.19 ± 0.26
10	29.8 ± 3.7	0.67 ± 0.15	81 ± 8	2.30 ± 0.27	1.23 ± 0.18
32	23.4 ± 6.4 ^a	0.69 ± 0.15	79 ± 10	2.43 ± 0.12	1.30 ± 0.20
100	26.7 ± 3.4	0.38 ± 0.10 ^a	69 ± 14 ^a	2.30 ± 0.62	0.58 ± 0.18 ^{**}
320	26.3 ± 3.3	0.38 ± 0.16 ^a	68 ± 20 ^a	1.93 ± 0.42 ^a	0.49 ± 0.22 ^{**}
1000	22.0 ± 4.3 ^{**a}	0.21 ± 0.05 ^{**b}	52 ± 28 ^b	2.27 ± 0.64	0.23 ± 0.13 ^{**}

^a $P < 0.05$.

^{**} $P < 0.01$.

In this study, the growth of the worms was much higher than in the previous test with cadmium. It should be noted that as a consequence of this, cocoon production was much lower than that in the test on cadmium. The high soil pH in this test may be responsible for the low cocoon production. Van Gestel *et al.* (in press) found an inverse relationship between growth and cocoon production for *E. andrei* in artificial soil and observed a decreased cocoon production at soil pH ≥ 7.0 .

In the literature, not many data are available on the reproduction toxicity of chromium(III). Molnar *et al.* (1989) found a 50% reduction in the reproduction of *E. fetida* at 250 mg \cdot kg⁻¹ chromium(III) in a substrate of peat and horse manure. Chromium(VI) appeared to be more toxic, but due to its rapid conversion into chromium(III) its toxicity decreased with time. Abbasi and Soni (1983) found an LC₅₀ of 15 mg \cdot kg⁻¹ for the effect of chromium(VI) on *Octochaetis pattoni* in a substrate of soil with animal dung; reproduction was not affected till the worms died.

Paraquat

Table 3 lists the results of the paraquat test. Paraquat did not affect the growth of the earthworms at concentrations up to 1000 mg \cdot kg⁻¹ dry soil. Cocoon production was lower at 450 mg \cdot kg⁻¹ and significantly reduced at 1000 mg \cdot kg⁻¹. At these two highest concentrations, more cocoons appeared to have an abnormal shape and passed a 2-mm sieve (4.5 and 10.3%, respectively, compared to 0.7% in the control); the number of fertile cocoons was reduced (not significantly). Apparently, the abnormal shape of the cocoons was induced by paraquat and influenced cocoon fertility as most abnormally shaped cocoons did not hatch. The number of juveniles per fertile cocoon was not affected by paraquat, but the total number of offspring (juveniles/worm/week) was significantly reduced at 1000 mg \cdot kg⁻¹ dry soil.

Haque and Ebing (1983) determined LC₅₀ values of >200 mg \cdot kg⁻¹ for paraquat (in the formulation Gramoxone) for *E. fetida* and *L. terrestris*. Fischer (1989) studied the effect of paraquat (as Gramoxone) on *E. fetida* in a substrate of peaty soil with horse dung. At the lowest concentration tested (165 mg \cdot kg⁻¹ dry substrate) cocoon production was not affected. Effects on growth became apparent after 6 weeks at this

TABLE 3
THE EFFECT OF PARAQUAT ON THE GROWTH AND REPRODUCTION OF *Eisenia andrei*
IN ARTIFICIAL SOIL (AVERAGE OF FOUR REPLICATES)

Concentration of paraquat (mg \cdot kg ⁻¹)	% growth	Cocoons/worm/week	% fertile cocoons	Juveniles/fertile cocoon	Juveniles/worm/week
0	2.8 \pm 1.6	1.21 \pm 0.22	92 \pm 7	2.60 \pm 0.14	2.88 \pm 0.62
20	3.9 \pm 1.2	1.21 \pm 0.29	96 \pm 5	2.72 \pm 0.12	3.12 \pm 0.68
45	5.2 \pm 1.4	1.20 \pm 0.32	87 \pm 6	2.33 \pm 0.36	3.00 \pm 0.81
100	2.6 \pm 0.8	1.33 \pm 0.16	89 \pm 9	2.69 \pm 0.27	3.15 \pm 0.37
200	3.9 \pm 1.8	1.28 \pm 0.20	91 \pm 3	2.44 \pm 0.17	2.83 \pm 0.44
450	5.4 \pm 1.4	0.93 \pm 0.20 ^a	78 \pm 16	2.52 \pm 0.36	2.03 \pm 0.30 ^a
1000	2.0 \pm 1.0	0.72 \pm 0.11 ^b	77 \pm 12	2.12 \pm 0.40	1.19 \pm 0.28 ^b

^a P < 0.05.

^b P < 0.01.

concentration and growth was slightly reduced after 8 weeks. At 230 mg · kg⁻¹ dry substrate after 6 weeks worms started to die. When surviving worms were placed on untreated substrate, they did not recover. Apparently, paraquat may have a long lasting irreversible action on earthworms.

Parathion

Table 4 lists the results of the study on parathion. At the highest concentration tested 47.5% of the worms died; the LC₅₀ therefore is >180 mg · kg⁻¹ dry soil. From Table 4 it can be concluded that parathion significantly affected earthworm growth at all concentrations tested. This effect was reproduced in a later study (Van Gestel, unpublished data). Cocoon production was lower at 56 mg · kg⁻¹ and significantly reduced at 100 mg · kg⁻¹. The EC₅₀ for the effect of parathion on cocoon production was 68 (56–84) mg · kg⁻¹ dry soil. A high number of cocoons appeared to have an abnormal shape and passed a 2-mm sieve: 0.5, 3.5, 3.8, 5.9, 14.9, and 14.0% at 0, 10, 18, 32, 56, and 100 mg · kg⁻¹, respectively. The number of fertile cocoons that was dose related reduced from 18 mg · kg⁻¹ onward. From this it can be concluded that as with paraquat, parathion induced the formation of abnormally shaped cocoons leading to a reduced number of fertile cocoons. The number of juveniles per fertile cocoon was significantly reduced at 32 mg · kg⁻¹ and the number of juveniles/worm/week at 56 mg · kg⁻¹ dry soil.

Fayoile (1979) determined a 7-day LC₅₀ of 80 mg · kg⁻¹ for *Allolobophora chlorotica* in a sandy soil. Cathey (1982) reported a 6-week LC₅₀ of 44 mg · kg⁻¹ for *L. terrestris* in a substrate of shredded paper based buss-bedding. No literature data were found on the effects of parathion on earthworm reproduction.

Fentin

In the range-finding study, all worms died at fentin concentrations of 100 and 1000 mg · kg⁻¹ dry soil. Cocoon production was 50% reduced at 10 mg · kg⁻¹. From the

TABLE 4
THE EFFECT OF PARATHION ON THE GROWTH AND REPRODUCTION OF *Eisenia andrei*
IN ARTIFICIAL SOIL (AVERAGE OF FOUR REPLICATES)

Concentration of parathion (mg · kg ⁻¹)	% growth	Cocoons/worm/week	% fertile cocoons	Juveniles/fertile cocoon	Juveniles/worm/week
0	7.5 ± 1.2	1.36 ± 0.29	96 ± 4	2.88 ± 0.34	3.83 ± 1.26
10	0.8 ± 1.1**	1.40 ± 0.15	88 ± 6	2.67 ± 0.29	3.36 ± 0.82
18	2.4 ± 2.1**	1.26 ± 0.09	86 ± 5*	2.63 ± 0.26	2.86 ± 0.40
32	3.1 ± 2.4*	1.36 ± 0.13	82 ± 6*	2.17 ± 0.17* [△]	2.43 [△] ± 0.43
56	1.1 ± 2.6**	0.86 ± 0.36 [△]	78 ± 7**	2.03 ± 0.22* [△]	1.37 [△] ± 0.53*
100	-9.4 ± 6.6**	0.33 ± 0.28**	53 ± 3** [△]	1.82 ± 0.84** [△]	0.47 [△] ± 0.4***
180	-20.9 ± 1.5**	0.00**	—	—	—

* P < 0.05.

** P < 0.01.

TABLE 5
EFFECT OF FENTIN ON THE GROWTH AND REPRODUCTION OF *Eisenia andrei*
IN ARTIFICIAL SOIL (AVERAGE OF FOUR REPLICATES)

Concentration of fentin (mg · kg ⁻¹)	% growth	Cocoons/worm/week	% fertile cocoons	Juveniles/fertile cocoon	Juveniles/worm/week
0	3.3 ± 1.3	1.40 ± 0.10	37 ± 5	2.71 ± 0.29	3.31 ± 0.64
0.32	2.8 ± 0.9	1.38 ± 0.27	93 ± 9	2.64 ± 0.27	3.39 ± 0.96
1	0.9 ± 1.4	1.51 ± 0.08	93 ± 3	2.75 ± 0.24	3.85 ± 0.43
3.2	1.9 ± 0.9	1.55 ± 0.09	89 ± 4	2.48 ± 0.28	3.43 ± 0.30
10	1.9 ± 1.8	1.43 ± 0.14	95 ± 3	2.70 ± 0.16	3.71 ± 0.59
32	-3.8 ± 4.1*	0.55 ± 0.19*	94 ± 7	2.47 ± 0.43	1.26 ± 0.44*

* P < 0.05.

results of both the range finding and the definitive test with fentin, an LC₅₀ of 57 mg · kg⁻¹ dry soil can be calculated.

Table 5 shows the results of the study with fentin. From this table it can be concluded that fentin significantly reduced growth, cocoon production, and the number of juveniles/worm/week at 32 mg · kg⁻¹ dry soil. The results indicate that the dose-response curve for fentin is rather steep. The EC₅₀ for the effect of fentin on cocoon production was 28 (22-35) mg · kg⁻¹ dry soil. The fertility of the cocoons and the number of juveniles per fertile cocoon were not affected by fentin. In the control all cocoons had a normal shape; in the treated soil some small and abnormally shaped cocoons were produced: 1.1-4.2% (not dose related).

Analysis of the soil treated at a nominal fentin concentration of 0.32 mg · kg⁻¹ gave actual concentrations of 0.56 and 0.34 mg · kg⁻¹ dry soil at the start and at the end of the test, respectively. No literature data were found on the effect of fentin on the reproduction of earthworms.

Benomyl

Table 6 shows the results of the reproduction test with benomyl. To study the influence of the duration of the exposure period on the effect of benomyl, worms were

TABLE 6
EFFECT OF BENOMYL ON THE GROWTH AND REPRODUCTION OF *Eisenia andrei*
IN ARTIFICIAL SOIL (AVERAGE OF THREE REPLICATES; 6 WEEKS EXPOSURE)

Concentration of benomyl (mg · kg ⁻¹)	% growth	Cocoons/worm/week	% fertile cocoons	Juveniles/fertile cocoons	Juveniles/worm/week
0	61.1 ± 12.1	0.50 ± 0.05	80 ± 15	2.1 ± 0.2	0.86 ± 0.13
0.1	46.6 ± 3.3*	0.56 ± 0.09	80 ± 8	2.1 ± 0.3	1.09 ± 0.23
0.32	58.8 ± 6.7	0.59 ± 0.15	84 ± 7	1.9 ± 0.2	0.91 ± 0.03
1.0	63.9 ± 12.6	0.55 ± 0.23	71 ± 1	2.0 ± 0.1	0.76 ± 0.28
3.2	-16.1 ± 6.6**	0.05 ± 0.05**	0**	—	0**

** P < 0.01.

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ighest concentration · kg⁻¹ dry soil. From d earthworm growth r study (Van Gestel, g⁻¹ and significantly n cocoon production

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lobophora chlorotica · kg⁻¹ for *L. terrestris* ure data were found

tions of 100 and 100 · mg · kg⁻¹. From d

NOF *Eisenia andrei* ES)

Concentration of benomyl (mg · kg ⁻¹)	Juveniles/cocoon	Juveniles/worm/week
0.34	3.83 ± 1.26	
0.29	3.36 ± 0.82	
0.26	2.86 ± 0.40	
0.17*	2.43 ± 0.43	
0.22*	1.37 ± 0.63*	
0.84**	0.47 ± 0.47**	

incubated for 6 weeks in treated soil. Differences between both 3-week incubation periods were small, however, and therefore in Table 6 results are averaged for the incubation periods. At 10 mg · kg⁻¹ high mortality occurred, resulting in LC₅₀ values of 6.0 and 5.7 mg · kg⁻¹ dry soil after 3 and 6 weeks of incubation, respectively.

From Table 6, it is obvious that benomyl significantly affected growth and reproduction of *E. andrei* at 3.2 mg · kg⁻¹, and no effects were noted at 1.0 mg · kg⁻¹. EC₅₀ values for the effect of benomyl on cocoon production did not differ significantly for both test periods, and the EC₅₀ was 1.6 (1.2–2.3) mg · kg⁻¹ dry soil for the entire 6-week test period. From these results, it can be concluded that also in the case of benomyl, dose-response curves are rather steep.

LC₅₀ values for the effect of benomyl on *E. andrei* or *E. fetida* reported in the literature range between 11 and 27 mg · kg⁻¹, and for *L. terrestris* between 0.4 and 3.5 mg · kg⁻¹ (Heimbach, 1984, 1985; Haque and Ebing, 1983; Roark and Dale, 1979; Karnak and Hamelink, 1982). In a substrate of clay with cattle dung (1:1), an NOEC of 0.25 mg · kg⁻¹ was found for the effect of benomyl on the cocoon production of *Aporectodea caliginosa* (Lofs-Holmin, 1982). This value corresponds with that found in this study, although a completely different substrate was used.

Carbendazim

Carbendazim was tested at concentrations of 0, 1, 3.2, 10, 32, and 100 mg · kg⁻¹ of the formulation Derosal, corresponding to 0, 0.6, 1.92, 6.0, 19.2, and 60 mg a.i. · kg⁻¹ dry soil, respectively. Table 7 shows the results of this test.

At the two highest concentrations all worms died: the LC₅₀ was 5.7 (4.7–6.9) mg a.i. · kg⁻¹ dry soil. Carbendazim significantly affected growth at 6.0 mg a.i. · kg⁻¹ dry soil, and reproduction (cocoon production, number of fertile cocoons, number of juveniles) was significantly reduced at ≥1.92 mg a.i. · kg⁻¹ dry soil. The EC₅₀ for the effect of carbendazim on cocoon production was 2.9 (2.2–3.8) mg a.i. · kg⁻¹ dry soil.

As was indicated by Van Gestel *et al.* (in press), the low number of cocoons produced in this test can be attributed to the high soil pH (7.3). Vonk *et al.* (1986) reported an LC₅₀ of 9.3 mg · kg⁻¹ for the effect of carbendazim on *E. fetida* in an artificial soil, and the NOEC for cocoon production in the same study was 2.0 mg · kg⁻¹ dry soil.

TABLE 7
EFFECT OF CARBENDAZIM ON THE GROWTH AND REPRODUCTION OF *Eisenia andrei*
IN ARTIFICIAL SOIL (AVERAGE OF FOUR REPLICATES)

Concentration of carbendazim (mg · kg ⁻¹)	% growth	Cocoons/ worm/week	% fertile cocoons	Juveniles/ fertile cocoon	Juveniles/ worm/week
0	10.0 ± 6.0	0.77 ± 0.16	84 ± 15	1.90 ± 0.16	1.27 ± 0.48
0.6	9.5 ± 6.5	0.69 ± 0.07	74 ± 8	1.88 ± 0.29	0.94 ^a ± 0.18
1.92	5.5 ± 5.3 ^c	0.55 ± 0.11 ^{*a}	24 ± 14 ^{*c}	1.44 ± 0.13 ^{*a}	0.18 ± 0.12 [*]
6.0 ^a	-52.8 ± 6.3 ^{**}	0.05 ± 0.06 ^{**}	—	—	—

^a Only 1⁷ worms survived.

^{*} P < 0.05.

^{**} P < 0.01.

both 3-week incubation are averaged for the two resulting in LC₅₀ values of 5.7 and 6.0 mg a.i. · kg⁻¹ dry soil, respectively. The EC₅₀ values did not differ significantly for dry soil for the entire 6-week incubation, that also in the case of *E. fetida* reported in the literature between 0.4 and 3; Roark and Dale, 1979; the dung (1:1), an NOEC for the cocoon production of *E. fetida* responds with that found in the literature.

TABLE 8
EFFECT OF PHENMEDIPHAM ON THE GROWTH AND REPRODUCTION OF *Eisenia andrei* IN ARTIFICIAL SOIL (AVERAGE OF FOUR REPLICATES)

Concentration of phenmedipham (mg · kg ⁻¹)	% growth	Cocoons/worm/week	% fertile cocoons	Juveniles/fertile cocoon	Juveniles/worm/week
0	-4.4 ± 4.7	1.13 ± 0.07	90 = 5	2.53 ± 0.22	2.57 ± 0.27
1.62	-3.0 ± 6.6	0.74 ± 0.09 ^a	90 = 9	2.22 ± 0.23	1.46 ± 0.25*
5.18	-0 ± 6.0	0.66 ± 0.13 ^a	93 = 6	2.42 ± 0.20	1.48 ± 0.33*
16.2	-5.0 ± 2.0	0.53 ± 0.17 ^b	33 = 3	2.42 ± 0.42	1.23 ± 0.23*
51.8	-16.5 ± 6.9*	0.35 ± 0.15*	76 = 9*	2.31 ± 0.50	0.64 ± 0.34*
162 ^d	-57.8 ± 10.6*	0*	—	—	—

^a Only 13 worms survived.
* P < 0.05.

Phenmedipham

Phenmedipham was tested at concentrations of 0, 10, 32, 100, 320, and 1000 mg · kg⁻¹ of the formulation Betanal, corresponding to 0, 1.62, 5.18, 16.2, 51.8, and 162 mg a.i. · kg⁻¹ dry soil, respectively. Table 8 summarizes the results of this test.

At the highest concentration tested, high mortality occurred: the LC₅₀ was 129 mg a.i. · kg⁻¹ dry soil. Growth was significantly affected at the two highest concentrations. Cocoon production was reduced at all concentrations tested: the EC₅₀ was 52 (39-69) mg a.i. · kg⁻¹ dry soil. Phenmedipham did not influence the number of juveniles per fertile cocoon, but cocoon fertility was reduced at 51.8 mg a.i. · kg⁻¹ dry soil. And as a result of the effect on cocoon production, the total number of juveniles/worm/week was significantly reduced at all concentrations tested.

One important aspect to note is that in this study worms did not grow: even in the control group they lost weight. No explanation can be given for this. No literature data were found on the earthworm toxicity of phenmedipham.

TABLE 9

EFFECT OF PENTACHLOROPHENOL ON THE GROWTH AND COCOON PRODUCTION OF *Eisenia andrei* IN ARTIFICIAL SOIL (MEAN OF FOUR REPLICATES)

Concentration (mg PCP · kg ⁻¹ dry soil)	% growth	Cocoons/worm/week
0 ^d	20.9 ± 3.5	0.91 ± 0.25
5	25.9 ± 17.7	0.79 ± 0.27
10	30.9 ± 25.9	0.33 ± 0.47
20	19.1 ± 7.8	0.30 ± 0.35
40	21.0 ± 14.9	0.64 ± 0.18
60	51.2 ± 9.2*	0.39 ± 0.08*

^d 12 replicates.
* P < 0.05.

TABLE 9
EFFECT OF PENTACHLOROPHENOL ON THE GROWTH AND COCOON PRODUCTION OF *Eisenia andrei* IN ARTIFICIAL SOIL (MEAN OF FOUR REPLICATES)

Juveniles/fertile cocoon	Juveniles/worm/week
90 ± 0.16	1.27 ± 0.43
38 ± 0.29	0.94 ± 0.18
44 ± 0.13*	0.18 ± 0.12*
—	—

Pentachlorophenol

Table 9 shows the effect of PCP on the growth and cocoon production of *E. andrei*; the results of the cocoon incubation test with PCP are shown in Table 10. From Table 9, it can be concluded that PCP at the highest concentration tested (60 mg · kg⁻¹) reduced cocoon production, but stimulated earthworm growth. The EC₅₀ for the effect of PCP on cocoon production was 55 (39–77) mg · kg⁻¹ dry soil.

Two-week LC₅₀ values for *E. fetida* or *E. andrei* in artificial soil of 50–87 mg PCP · kg⁻¹ dry soil can be found in the literature (Heimbach, 1984; Van Gestel and Ma, 1990; Vonk *et al.*, 1986). From the study of Vonk *et al.* (1986) a 4-week LC₅₀ of 33 mg PCP · kg⁻¹ and an NOEC of 12 mg PCP · kg⁻¹ dry soil can be derived for *E. fetida* in an artificial soil. Van Gestel *et al.* (1989) reported an NOEC of 32 mg · kg⁻¹ for the effect of PCP on the cocoon production of *E. andrei* in a test similar to this study.

Incubation in treated artificial soil of cocoons produced in untreated soil affected the number of juveniles per fertile cocoon at concentrations of 40 and 60 mg PCP · kg⁻¹ dry soil, but did not affect cocoon fertility. Incubation in untreated soil of cocoons produced in treated soil had no influence on the number of juveniles per fertile cocoon, but significantly reduced the number of fertile cocoons at 60 mg PCP · kg⁻¹ dry soil. Incubation in treated soil of cocoons produced in treated soil did affect both cocoon fertility and the number of juveniles hatching from fertile cocoons. It can therefore be concluded that treatment of the cocoon incubation substrate affected the number

TABLE 10

EFFECT OF PENTACHLOROPHENOL ON THE HATCHABILITY OF COCOONS OF *Eisenia andrei* IN ARTIFICIAL SOIL (MEAN OF TWO REPLICATES)

Concentration (mg · kg ⁻¹)				
Exposure of worms	Cocoon incubation	% fertile cocoons	Juveniles/ferale cocoon	Juveniles/worm/week
0	0	91 ± 6	2.35 ± 0.21	1.86 ± 0.55
0	5	73 ± 5	2.30 ± 0.28	1.72 ± 0.34
0	10	90 ± 4	2.15 ± 0.07	1.90 ± 0.70
0	20	65 ± 3	1.90 ± 0.28	1.37 ± 0.50
0	40	66 ± 48	0.55 ± 0.50*	0.17 ± 0.08*
0	60	82 ± 0	0.25 ± 0.07*	0.19 ± 0.01*
5	0	65 ± 21	2.20 ± 0.14	1.04 ± 0.81
10	0	94 ± 4	2.40 ± 0.00	2.68 ± 0.05
20	0	92 ± 1	2.05 ± 0.21	1.18 ± 0.51
40	0	68 ± 12	2.45 ± 0.64	1.41 ± 0.86
60	0	44 ± 23*	2.50 ± 0.28	0.48 ± 0.30*
5	5	87 ± 1	2.25 ± 0.07	1.77 ± 0.22
10	10	92 ± 12	2.30 ± 0.14	1.97 ± 0.61
20	20	85 ± 2	1.05 ± 0.78*	0.93 ± 0.71
40	40	32 ± 2*	1.25 ± 0.35*	0.20 ± 0.00*
60	60	21 ± 18*	0.15 ± 0.21*	0.02 ± 0.02*

* $P < 0.05$.

of juveniles, whereas incubation of cocoons produced by exposed worms in untreated soil did not have an effect on the number of juveniles, but led to a reduced number of fertile cocoons at 40 and 60 mg PCP · kg⁻¹ dry soil.

The reduced number of juveniles produced when cocoons are incubated in treated soil may be caused by the toxicity of PCP for juveniles hatching from the cocoons. Van Gestel *et al.* (1991) reported a 5-week LC₅₀ of 28 mg PCP · kg⁻¹ dry soil for juveniles of *E. andrei*.

Van Gestel *et al.* (1989) also found an effect of PCP on the fertility of cocoons: they incubated cocoons produced in treated soil in untreated soil and found an NOEC of 10 mg · kg⁻¹ dry soil. And at the highest concentration without effect on cocoon fertility (32 mg PCP · kg⁻¹ dry soil) they did not find an effect on the number of juveniles per fertile cocoon.

Comparison of Effects on Growth and Reproduction with Acute Toxicity

In Table 11 NOEC values for the effect of the nine test chemicals on growth and reproduction are summarized and compared with LC₅₀ values for the acute toxicity for earthworms. For this purpose 2-week LC₅₀ values obtained from the literature or 3-week LC₅₀ values determined in the experiments described above were used.

From Table 11, it can be concluded that for most chemicals growth is less sensitive than reproduction: the only exception is parathion, for which growth was the most sensitive parameter. Parathion, PCP, and carbendazim especially affect cocoon fertility. Some chemicals induced the formation of abnormally shaped cocoons; this effect always led to a reduced number of fertile cocoons and therefore is implicitly included in the NOEC values for cocoon fertility. For the other chemicals cocoon production is more or equally sensitive compared to cocoon fertility. In the case of PCP the number of juveniles per fertile cocoon was most sensitive when cocoons were incubated in treated soil, probably because of a direct toxic effect of PCP on the juveniles. As

TABLE 11
NOEC VALUES FOR THE EFFECT OF NINE CHEMICALS ON GROWTH AND REPRODUCTION OF *Eisenia andrei*, AND COMPARISON WITH LC₅₀ VALUES

Chemical	NOEC (mg · kg ⁻¹ dry soil) for effects on						
	Growth	Cocoon production	% fertile cocoons	Juveniles/ fertile cocoon	Juveniles/ worm/ week	LC ₅₀ (mg · kg ⁻¹ dry soil)	Ratio (LC ₅₀ / NOEC)
Cadmium	100	<10	100	100	10	>1000	>100
Chromium	320	320	320	1000	32	>1000	>30
Paraquat	1000	450	1000	1000	450	>1000	>2
Parathion	<10	56	10	13	32	>180	>18
Fenitn	10	≤10	32	32	10	57	≥6
Benomyl	1.0	1.0	1.0	1.0	1.0	5.7	6
Carbendazim	1.9	1.9	0.6	0.6	0.6	5.7	10
Phenmedipham	16	<1.6	16	52	<1.6	129	>80
PCP	40 ^a	40	20	10	20	50-87	5-9

^a Stimulation of growth at higher concentrations.

production of *E. andrei* in Table 10. From Table on tested (60 mg · kg⁻¹) h. The EC₅₀ for the effect soil. initial soil of 50-87 mg n. 1984; Van Gestel and *al.* (1986) a 4-week LC₅₀ dry soil can be derived reported an NOEC of 32 *E. andrei* in a test similar

in untreated soil affected (40 and 60 mg PCP · kg⁻¹ untreated soil of cocoons juveniles per fertile cocoon, 0 mg PCP · kg⁻¹ dry soil. did affect both cocoon cocoons. It can therefore rate affected the number

ITY OF COCOONS
(D REPLICATES)

Juveniles/ cocoon	Juveniles/ worm/week
0.21	1.36 ± 0.55
0.28	1.72 ± 0.84
0.07	1.90 ± 0.70
0.28	1.37 ± 0.50
0.50*	0.17 ± 0.08*
0.07*	0.19 ± 0.01*
0.14	1.04 ± 0.87
0.00	2.68 ± 0.05
0.21	1.18 ± 0.51
0.64	1.41 ± 0.86
0.28	0.48 ± 0.30*
0.07	1.77 ± 0.22
0.14	1.97 ± 0.61
0.73*	0.93 ± 0.71
0.35*	0.20 ± 0.00*
0.21*	0.02 ± 0.00*

an overall parameter for the effect of chemicals on earthworm reproduction, the number of juveniles produced per worm per week seems to be a good parameter. For most chemicals tested it is not more sensitive than cocoon production or fertility of the cocoons; for chromium it is, however, the most sensitive parameter, being a factor of 10 more sensitive than cocoon production.

Differences between NOEC values for the effect of chemicals on earthworm reproduction and LC_{50} values show a great variation. Ma (1983) reported a factor of 15-50 for the difference between 6-week LC_{50} 's and NOEC values for four heavy metals and *L. rubellus*. In our study for cadmium and chromium the factors are somewhat higher. This is also the case for pnenamedipham. For the other organic chemicals differences between acute toxicity and reproduction toxicity are about one order of magnitude. In the study of Ma (1983) for three pesticides differences between 6-week LC_{50} values and NOEC was a factor of 5-20, whereas it was a factor of 50 for lindane.

From data of Adema *et al.* (1981), Slooff and Canton (1983), and Slooff *et al.* (1986) it can be concluded that also for aquatic organisms differences between acute LC_{50} or EC_{50} values and NOEC's for sublethal parameters are not constant. For eight chemicals tested on three fish species, Adema *et al.* (1981) found ratios for LC_{50} /NOEC between 1.7 and 450. Slooff and Canton (1983) concluded that for most chemicals differences between acute and chronic toxicity values were less than a factor of 100; for some chemicals differences may, however, be as high as a factor of 3200.

Standardization

For reasons of standardization, in the test method used in this study, the same earthworm species and artificial soil were chosen as described for acute toxicity testing by the OECD (1984) and EEC (1985). The results of this study demonstrated that the method offers opportunities to determine the effects of chemical substances on earthworm reproduction in a standardized way. A test duration of 3 weeks seems to be sufficient to adequately assess effects on reproduction and growth. The study with benomyl confirms this. For some persistent chemicals, however, a longer test period might be advisable, as is shown by Fischer (1989) for paraquat.

As noted when comparing the different tests, cocoon production and worm growth can fluctuate substantially. This fluctuation does not affect the number of fertile cocoons nor the number of juveniles produced per fertile cocoon. It does, however, influence the total number of offspring produced in a test. Comparing the results on PCP in this study and that described by Van Gestel *et al.* (1989), it can be concluded that the differences in cocoon production and worm growth did not influence the test results to a great extent. The difference between NOEC values in these two studies was at most a factor of 4. The problem of fluctuating growth and cocoon production is discussed by Van Gestel *et al.* (in press).

The application of cow dung as a food source in a hole in the middle of the soil gives satisfactory results. Mixing the food homogeneously through the soil is not advisable as in that case more food is needed to reach the same level of growth (Van Gestel *et al.*, 1991) or cocoon production (Van Gestel, unpublished results). The test with cadmium presented here and that with copper described by Van Gestel *et al.* (1989) demonstrate that treatment of the cow dung is not necessary.

If the only objective is to study the effect on reproduction, incubation of cocoons in untreated substrate is sufficient. When cocoons are incubated in treated substrate,

it is not possible to distinguish effects on reproduction from those on juvenile worms hatching from the cocoons.

4. CONCLUSIONS

The method for determining the effect of chemicals on reproduction of the earthworm *E. andrei* in an artificial soil as proposed by Van Gestel *et al.* (1989) is proven to be sensitive and it can be used in a standardized way. Both earthworm growth and reproduction can be assessed, and besides cocoon production and cocoon fertility, the total number of juveniles produced per worm seems to be a useful parameter to determine the effect of chemicals on the reproductive performance of earthworms.

The difference between lethal and sublethal toxicities of chemicals can vary considerably, probably depending on the mode of action of the chemical tested. In this study, the difference between the acute LC₅₀ and NOEC values for the effect on earthworm growth and reproduction was highest for cadmium (a factor of >100) and lowest for benomyl, fentin, and pentachlorophenol (a factor of 5-6).

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