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Influence of cadmium, copper, and pentachlorophenol on growth and sexual development of *Eisenia andrei* (Oligochaeta; Annelida)

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Summary. In the existing guidelines for earthworm toxicity testing, mortality is the only test criterion. Mortality is, however, not a very sensitive parameter, and from an ecological point of view growth and reproduction are more important for a proper risk assessment of chemicals in soil. In this study the growth and sexual development of juvenile earthworms were considered as test criteria in a standardized earthworm toxicity test. The effect of Cd, Cu, and pentachlorophenol on the growth and sexual development of juveniles of the species *Eisenia andrei* was studied in an artificial soil substrate. Two tests with Cd were carried out to study the effects of the mode of application of the food source (cow dung). EC_{50} (50% effective concentration) values for the effect of Cd, Cu and pentachlorophenol on the growth of *E. andrei* were 33–96, >100, and >32 mg kg^{-1} dry soil, respectively, and there was no observed effect at 18–32, 56, and ≥32 mg kg^{-1} dry soil, respectively. Sexual development of the earthworms was inhibited at 10 mg Cd kg^{-1} and 100 mg Cu kg^{-1} dry soil, but was not affected at the highest pentachlorophenol concentration tested (32 mg kg^{-1} dry soil). The results were the same whether the food was applied in a hole in the middle of the soil or mixed homogeneously through the soil.

Key words: *Eisenia andrei* – Sublethal effects – Cadmium – Copper – Pentachlorophenol (PCP) – Growth – Sexual development

Some years ago two international guidelines for the determination of acute toxicity of chemicals to earthworms were published (OECD 1984; EEC 1985). The main purpose of these guidelines was the determination of 50% lethal concentrations (LC_{50} values). It is generally accepted, however, that survival is rather intensitive and, from an ecological point of view, not the most interesting parameter. Growth and reproduction are much more important for the maintenance of populations (Moriarty 1983).

Lofs-Holmin (1980) described a test for determining the effect of chemicals on the growth and sexual development of earthworms. Different earthworm species were used, and tests were carried out in a substrate of sand or clay mixed with farmyard manure. To standardize the tests, however, the use of standard soil and standard earthworm species has been recommended (Dean-Ross 1983). The preferred soil and earthworm species are those prescribed for acute toxicity tests by OECD (1984) and EEC (1985).

To further develop a standardized toxicity test with growth and sexual development of juvenile earthworms as test criteria, we studied the effects of three chemicals on these parameters using the earthworm species *E. andrei* in an artificial soil substrate.

Materials and methods

Earthworms

Juvenile earthworms of the species *E. andrei* (Bouché 1972) were obtained from our own culture; the worms were grown on horse dung as a food source at an ambient temperature of 20±5 °C. Generally, the worms used were between 0 and 3 weeks old, and their individual weights varied between 4 and 30 mg. For the growth test with pentachlorophenol not enough small worms were available, and some juveniles aged 3–6 weeks and weighing between 30 and 51 mg were used.

Chemicals

Cadmium chloride and copper chloride were obtained from Baker Chemical Co. and Merck, and were ≥99% pure; the pentachlorophenol was obtained from Merck and was 95% pure.

Toxicity tests

All tests were carried out in an artificial soil, with a dry weight of 10% peat, 20% kaolin clay, ca. 69% sand, and 0.5–1% $CaCO_3$. Test conditions differed slightly between the various experiments, since improvements to the test procedures were made in the course of the research.

For the Cd test, the artificial soil was made up with finely ground (<0.5 mm) peat and a moisture content of 35% (w:w). For the tests

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with Cu and pentachlorophenol 1 mm sieved peat was used, and the soil moisture content was 55% (w : w). Upon analysis the untreated artificial soil appeared to contain 6.1 mg Cu kg^{-1} and 0.3 mg Cd kg^{-1} dry soil.

The chemicals were mixed homogeneously through the soil. Soil equivalent to 400 (for the pentachlorophenol test) or 500 g dry weight was placed in 1-litre glass jars. Finely ground (<0.5 mm) cow dung was used as a food source, and 10 juvenile worms were added to each jar. Once a week the moisture loss was adjusted by reweighing the jars. At the start of the test the individual weights of the worms were determined. At different intervals all worms were sorted out from the soil, washed with tap water, dried on a filter paper, and weighed individually; they were checked for development of tubercula pubertata (sub-adult) and clitella (adult), and returned to the soil again. Each test included four replicates per concentration, with each replicate jar containing 10 earthworms.

Two tests were carried out with Cd. The soil pH was 6.7–6.8 at the start of the tests and did not change by more than 0.2–0.3 units during the test. In the first test, concentrations of 0, 10, 32, 100, 320 and 1000 mg Cd kg^{-1} dry soil were used. About 3 g finely ground cow dung was added to each jar, in a hole in the middle of the soil, having been moistened up to 50% with distilled water. Worm weights and sexual development were determined after 2, 4, 6, 8, 10, and 12 weeks. At these times another 3 g moistened cow dung was added to a hole in the middle of the soil. In the second test with Cd, 5 g cow dung was mixed homogeneously through the artificial soil, and Cd concentrations of 0, 10, 18, 32, 56, and 100 mg kg^{-1} dry soil were tested. After 4 weeks another 5 g cow dung was added to the soil. Worm weights and sexual development were assessed after 6, 8, 10, and 12 weeks. At these times another 5 g cow dung was mixed through the soil before the worms were returned to it. Both tests with Cd were incubated in the dark at a temperature of 23±1 °C.

In the test with Cu, 10 g finely ground cow dung was mixed homogeneously through the artificial soil. The soil pH was 6.2 at the start of the test and did not change by more than 0.2–0.3 units during the test. Concentrations of 0, 10, 18, 32, 56, and 100 mg Cu kg^{-1} dry soil were tested. Worm weights and sexual development were determined after 5, 8, 10, and 12 weeks, and at these times another 5 g cow dung was mixed through the soil before returning the worms to it. The incubation was performed at 20±1 °C under continuous illumination (200–400 lux) to stop the worms escaping from the test containers.

To determine the desired test concentrations for the test with pentachlorophenol, an acute toxicity test was performed with juvenile worms. These juveniles were exposed for 5 weeks to concentrations of 0, 10, 20, 40, 80, and 160 mg pentachlorophenol kg^{-1} dry artificial soil. For this test, 0.5-litre jars were filled with 150 g (dry weight) artificial soil, 10 juvenile worms were placed in each jar, and 1.5 g cow dung was mixed through the soil. Two replicate jars were used for each concentration. At the start and the end of the test the individual weights of the worms were determined, and mortality was assessed after 7, 14, and 35 days. Soil pH increased during the test from 5.8 to 6.3. In the growth test, concentrations of 0, 0.32, 1, 4, 8, 16, and 32 mg pentachlorophenol kg^{-1} dry soil was used. Worm weights and sexual development were assessed after 3, 6, 8, and 10 weeks. At the start of the test and after each time interval, artificial soil was freshly prepared and treated with pentachlorophenol. Cow dung (4 g) was added in a hole in the middle of the soil. During the first test interval, soil pH increased from 5.7–5.8 to 6.0–6.1, during the second interval, from 5.9–6.0 to 6.4–6.6, and during the latter two test intervals, from 5.7–5.9 to 6.2–6.4. Both tests with pentachlorophenol were incubated at 20±3 °C under continuous illumination (200–400 lux).

Statistics

To determine the no-observed-effect concentrations, an analysis of variance and the Williams test (Williams 1971, 1972) were applied. EC_{50} values were calculated using a logit model, and LC_{50} values using the trimmed Spearmann-Kärber method (Hamilton et al. 1977/1978).

Results

In all tests, the small size of the earthworms at the start made it almost impossible and rather time-consuming to recover all the worms from the soil, especially during the first 4–6 weeks.

Cd

Figure 1 shows the results of the first test with Cd. At 320 mg Cd kg^{-1} only 13 worms survived after 12 weeks, and at 1000 mg Cd kg^{-1} all worms died within 4 weeks. The LC_{50} values were 588 (525–658), 552 (417–733), 421 (354–502), 303 (246–373), and 253 (210–305) mg Cd kg^{-1} dry soil after 4, 6, 8, 10, and 12 weeks, respectively. The worm weights were very variable, with standard deviations for the average weight of 40 worms ranging between 21 and 79% after 12 weeks. The analysis of variance showed that after 4 weeks growth was significantly ($P<0.01$) reduced at all concentrations. At 10 and 32 mg Cd kg^{-1} the worms recovered, and the weights were no longer different from controls after 6 and 12 weeks, respectively. The EC_{50} and no-observed-effect val-

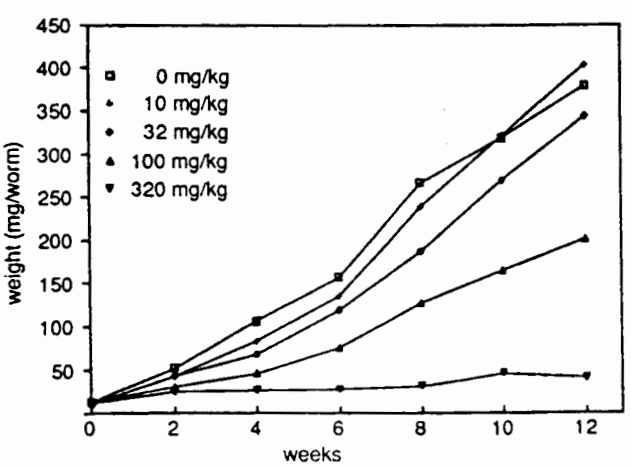

Fig. 1. Influence of Cd on the growth of *Eisenia andrei*, with cow dung supplied in a hole in the middle of an artificial soil

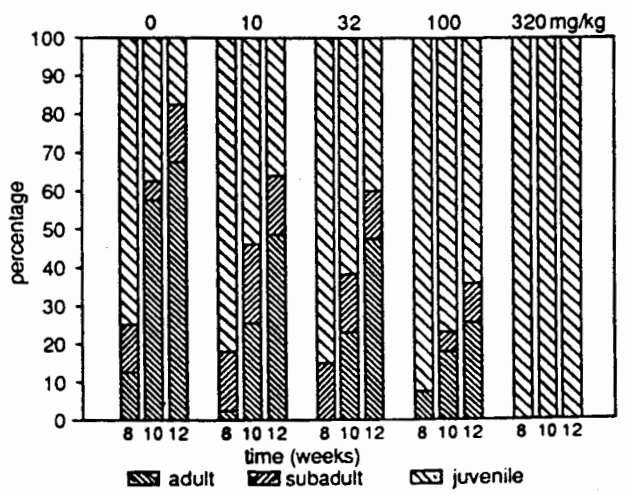

Fig. 2. Influence of Cd on the sexual development of *Eisenia andrei* with cow dung supplied in a hole in the middle of an artificial soil

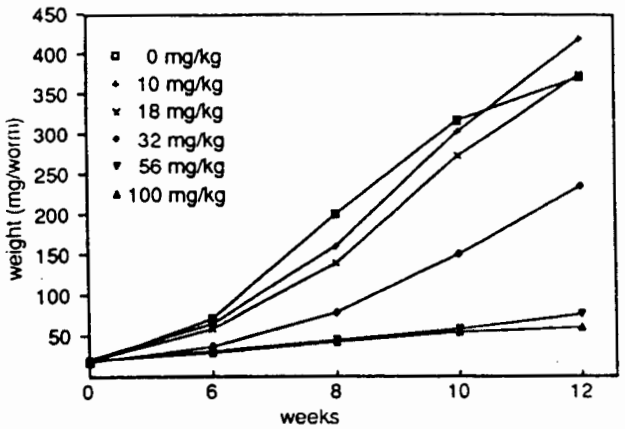

Fig. 3. Influence of Cd on the growth of *Eisenia andrei* with cow dung mixed homogenously through an artificial soil

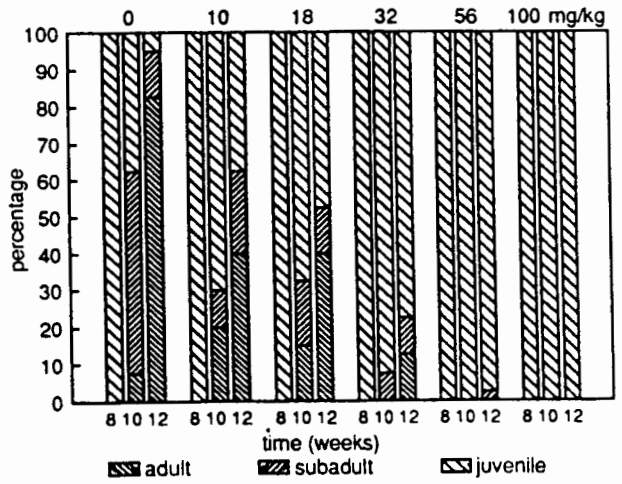

Fig. 4. Influence of Cd on the sexual development of *Eisenia andrei* with cow dung mixed homogenously, through an artificial soil

ues for the effect of Cd on the growth of *E. andrei* were 96 (65–140) and 32 mg Cd kg^{-1} dry soil, respectively.

Figure 2 shows the effect of Cd on the sexual development of the earthworms. The number of adult and subadult worms was significantly reduced ($P<0.01$) at 100 and 320 mg Cd kg^{-1} dry soil. The EC_{50} and no-observed-effect values for the effect of Cd on the sexual development of *E. andrei* were 108 (85–138) and <10 mg Cd kg^{-1} dry soil, respectively.

Figure 3 shows the results of the second experiment with Cd. After 12 weeks one worm was missing at 100 mg Cd kg^{-1}. After 8 weeks growth was significantly reduced at all concentrations tested. As in the first experiment with Cd, the worms subject to the two lower concentrations seemed to grow somewhat faster than controls during the last 4 weeks. After 12 weeks, the weights of worms exposed to Cd were significantly higher than controls at 10 mg Cd kg^{-1}. In this experiment also, the worm weights were highly variable, with standard deviations for the average weight of 40 worms ranging between 20 and 71% after 12 weeks. In this second experiment, the EC_{50} and no-observed-effect values for the effect of Cd on the growth of *E. andrei* were 33 (22–51) and 18 mg Cd kg^{-1} dry soil, respectively.

Figure 4 shows the effect of Cd on the sexual development of the earthworms in the second experiment. The number of adult and subadult worms was significantly reduced at all concentrations tested after 12 weeks. The EC_{50} and no-observed-effect values in this experiment were 27 (25–29) and <10 mg Cd kg^{-1} dry soil, respectively.

Cu

Figure 5 shows the results of the experiment with Cu. At 10 mg Cu kg^{-1} dry soil, high mortality occurred after 8 weeks in one jar; only two worms survived. As this mortality was not dose-related and could not be explained, this jar was discarded. The data in Fig. 5 for 10 mg Cu kg^{-1} after 8, 10, and 12 weeks are therefore means of 30 worms. After 10 weeks at 32 mg Cu kg^{-1}, six worms were missing in one jar; the data for this concentration after 10 and 12 weeks are therefore means of 34 worms.

Figure 5 indicates that Cu stimulated earthworm growth at the lower concentrations of 10 and 18 mg kg^{-1}. At 10 mg Cu kg^{-1}, worm weights were significantly ($P<0.01$) increased during the entire test period. At 18 mg Cu kg^{-1}, the weights were also increased, but the increase was only significantly different from control after 8 and 10 weeks. There was somewhat less variation in worm weight compared to the tests with Cd. Standard deviations for the average weight of 40 worms ranged between 17 and 32%. The EC_{50} and no-observed-effect values for the effect of Cu on the growth of *E. andrei* were >100 and 56 mg Cu kg^{-1} dry soil, respectively.

Figure 6 shows the effect of Cu on the sexual development of the earthworms. The number of adult worms was increased (not significantly) at 10 and 18 mg Cu kg^{-1} dry soil. The EC_{50} and no-observed-effect values for the number of adult worms were >100 and 56 mg Cu kg^{-1} dry soil, respectively.

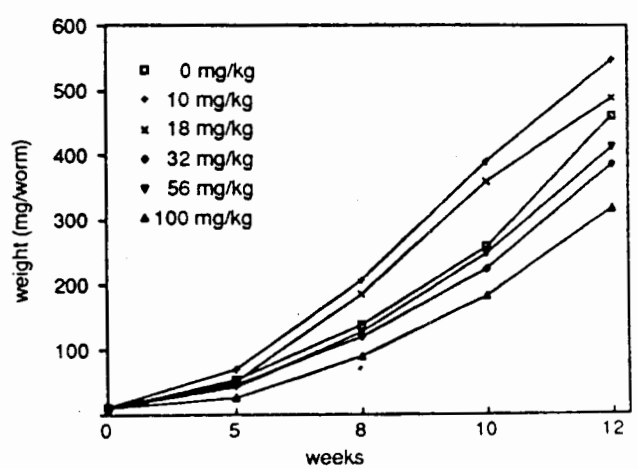

Fig. 5. Influence of Cu on the growth of *Eisenia andrei* with cow dung mixed homogenously through an artificial soil

Table 1. Influence of pentachlorophenol (PCP) on the growth of *Eisenia andrei*, with cow dung supplied in a hole in the middle of the artificial soil

PCP (mg kg^{-1} dry soil)	Average worm weights (mg worm^{-1}) after weeks				
	0	3	6	8	10
0	22 ± 8	65 ± 17	213 ± 44	337 ± 71	340 ± 64
0.32	19 ± 8	62 ± 19	212 ± 55	339 ± 80	350 ± 71
1	22 ± 12	71 ± 23	244 ± 59	398 ± 74	396 ± 69
4	22 ± 11	70 ± 21	240 ± 55	371 ± 86	356 ± 93
8	21 ± 11	67 ± 21	230 ± 53	360 ± 71	374 ± 71
16	21 ± 11	65 ± 23	233 ± 65	344 ± 101	351 ± 104
32	23 ± 9	67 ± 20	240 ± 61	380 ± 82	372 ± 82

Means ± SD, $n = 40$

Pentachlorophenol

In the acute toxicity test with juvenile worms, the LC_{50} values were >160, 48 (37–62), and 28 (23–34) mg pentachlorophenol kg^{-1} dry soil after 1, 2, and 5 weeks, respectively. In a similar study with adult worms, the LC_{50} values were >160, 106 (83–134), and 72 (62–84) mg pentachlorophenol kg^{-1} dry soil, respectively. Table 1 shows the effect of pentachlorophenol on the growth of juvenile worms.

The variation in worm weights was similar to that in the test with Cu, with standard deviations for the average weight of 40 worms ranging between 17 and 30% after 10 weeks. Table 1 indicates that pentachlorophenol did not influence earthworm growth at the concentrations tested, neither was there an influence on the maturation of the worms. Between 70 and 100% of the worms had developed tubercula pubertata after 10 weeks of exposure, and between 70 and 100% of the worms were adult, except for one jar at 4 mg kg^{-1}, in which only 40% of the worms were adult.

Upon analysis, the actual pentachlorophenol concentrations in the soil appeared to be much lower than the nominal concentrations. At the start of the 2nd 3-week incubation period, soil concentrations of 0.32, 0.48, 1.3, 4.2, 5.7, and 7.1 mg pentachlorophenol kg^{-1} dry soil were determined, corresponding to nominal concentrations of 0.32, 1, 4, 8, 16, and 32 mg kg^{-1}, respectively. At the end of the incubation period, the soil concentrations had decreased to 0.10, 0.48, 0.16, 1.1, 1.6, and 5.0 mg kg^{-1} dry soil, respectively. Unfortunately, the soil samples had to be stored for 8 months before they could be analysed. For that reason and because no storage experiments were performed, the analysed concentrations must be considered less reliable. In previous studies on pentachlorophenol (Van Gestel and Ma 1990), the analysed concentrations at initiation ranged between 73 and >100% of the nominal concentrations. In the present study, therefore, the no-observed-effect concentrations are based on nominal concentrations.

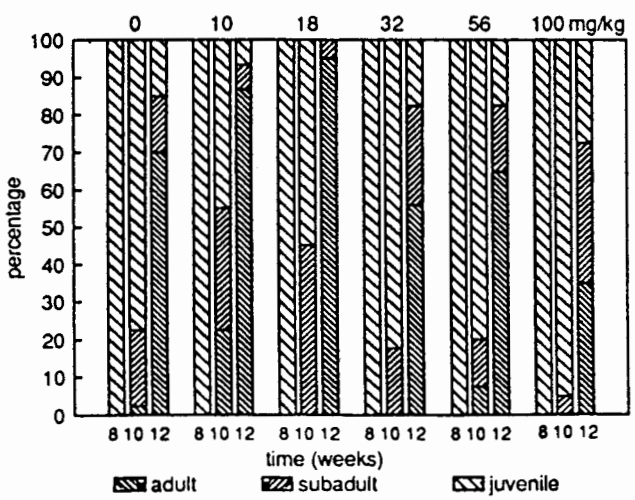

Fig. 6. Influence of Cu on the growth of *Eisenia andrei* with cow dung mixed homogenously through an artificial soil

Discussion

In all four studies, worm weights showed considerable variation (up to 79%) at all concentrations tested. In the second test with Cd (Fig. 3) and the test with Cu (Fig. 5) the worms did not grow very fast during the 1st 6 weeks, compared to the first test with Cd and the test with pentachlorophenol. As even more food was given in the first (25 g per 10 worms) compared to the latter two tests (16–18 g per 10 worms), the worms apparently had to spend more energy in finding food when cow dung was mixed homogeneously through the soil than when it was placed in a hole in the middle of the soil. In the second Cd experiment the food was mixed homogeneously through the soil to prevent the worms from escaping in the hole with the untreated cow dung, as this was considered the main reason for the considerable variation in worm weights observed in the first experiment. Comparing the results of all four studies, however, we concluded that the way the food was supplied did not influence the variation in worm weights. Cu stimulated the growth and sexual development of the worms at 10 and 18 mg kg^{-1}. As Cu is an essential element, its concentration in the untreated artificial soil (6.2 mg kg^{-1}) was apparently suboptimal for *E. andrei*. These test results therefore indicate that 16–24 mg Cu kg^{-1} dry artificial soil is optimal for the growth of *E. andrei*.

Ma (1983) reported EC_{50} values of 50 and 150 mg kg^{-1} dry soil, respectively, for the effect of Cd and Cu on the growth of juveniles of *Lumbricus rubellus* in a sandy loam soil. These values are in agreement with those found in our study. Bengtsson et al. (1986) studied the influence of Cu and Cd (as nitrate salts) on the growth of *Dendrobaena rubida* at different levels of soil pH. At pH 6.5, the growth of *D. rubida* was stimulated at 100 mg Cu kg^{-1} or 10 and 100 mg Cd kg^{-1} dry soil. At pH 4.5 and 5.5, worms died at 100 mg Cu kg^{-1}, while growth was significantly reduced at 10 and 100 mg Cd kg^{-1}. In the second test with Cd, we also noted that earthworm growth was stimulated by low Cd concentrations; at these concentrations the maturation of the worms was, however, significantly reduced.

In studies using a substrate of untreated soil covered with a layer of treated horse dung, Malecki et al. (1982) found that concentrations of Cd and Cu (both as acetate) of 50 and 500 mg kg^{-1} horse dung, respectively, represented the no-observed-effect limit on the growth of *E. fetida*. When copper chloride was used, the no-observed-effect limit was 100 mg Cu kg^{-1} horse dung. Hartenstein et al. (1981), using a substrate of untreated soil covered with a layer of treated activated sludge, found that the growth of *E. fetida* was significantly reduced at 1100–11000 mg Cu kg^{-1} and 1800–18000 mg Cd kg^{-1} in the sludge. As earthworms are capable of avoiding toxic substances (Edwards and Lofty 1977), it is unlikely that the worms in these studies were exposed continuously. The results reported by Hartenstein et al. (1981) and Malecki et al. (1982) therefore cannot be compared with those presented here.

Pentachlorophenol did not influence the growth or the sexual development of *E. andrei* at the concentrations tested. This finding conflicts with the 5-week LC_{50} of 28 mg kg^{-1} found for juvenile worms. Since soil pH values for the acute toxicity test and the growth test were more or less similar, this factor cannot explain the observed difference. Apparently, the difference between the LC_{50} and the no-observed-effect limit on the growth and sexual development is small in the case of pentachlorophenol.

We found no published studies on the effect of pentachlorophenol on the growth and/or sexual development of earthworms. When the results of the present study were compared with acute toxicity data, especially for Cd, there appeared to be a great difference between LC_{50} values for adult worms and the no-observed-effect limits to the growth of juvenile worms determined in the present study. Van Gestel and Van Dis (1988) reported a 2-week LC_{50} of > 1000 mg Cd kg^{-1} for *E. andrei* in artificial soil. Thus, for Cd, the difference between the LC_{50} and the no-observed-effect limit is a factor of > 100. For Cu this difference is smaller. In a reproduction study with Cu, a high mortality among adult *E. andrei* occurred at 1000 mg Cu kg^{-1} artificial soil, and the LC_{50} was 626 mg Cu kg^{-1} dry soil (Van Gestel, unpublished data). Ma (1983) reported a 6-week LC_{50} of 1000 mg Cu kg^{-1} dry soil for *Lumbricus rebullus* in a sandy loam soil. So, for Cu, the difference between the LC_{50} and the no-observed-effect limit is a factor of 11–18. For pentachlorophenol, the 2-week LC_{50} for adult *E. andrei* was 106 mg kg^{-1}, and the no-observed-effect limit was $\geq$ 32 mg kg^{-1} dry artificial soil. Therefore the difference between the LC_{50} and the no-observed-effect limit for pentachlorophenol is a factor of ≤ 3. It is therefore concluded that the inclusion of sublethal parameters (growth, sexual development) in toxicity tests used to evaluate the risks of chemicals in soil may significantly improve the ecological relevance of the tests.

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