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Development of a Standardized Reproduction Toxicity Test with the Earthworm Species *Eisenia fetida andrei* Using Copper, Pentachlorophenol, and 2,4-Dichloroaniline

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This article describes a standardized test method for determining the effect of chemical substances on the reproduction of the earthworm *Eisenia fetida andrei*. It is based on the existing guidelines for acute toxicity testing with earthworms, and for reasons of standardization the same artificial soil substrate and earthworm species were chosen as prescribed by these guidelines. After being preconditioned for one week in untreated soil, earthworms are exposed to the chemical substances for 3 weeks. The number of cocoons produced is determined, and cocoons are incubated in untreated artificial soil for 5 weeks to assess hatchability. Results are presented from toxicity experiments with pentachlorophenol, copper, and 2,4-dichloroaniline. For these compounds no-effect levels (NEL) for cocoon production were 32, 60–120, and 56 $mg \cdot kg^{-1}$ dry soil, respectively. Hatching of cocoons was influenced by pentachlorophenol (NEL, 10 $mg \cdot kg^{-1}$), but not by copper and dichloroaniline. Following exposure, earthworms were incubated in clean soil again to study the possibility of recovery of cocoon production. For copper and dichloroaniline earthworms did recover cocoon production to a level as high as the control level or even higher; in case of pentachlorophenol, cocoon production was still reduced after 3 weeks in clean soil. © 1989 Academic Press, Inc.

In recent years, two guidelines for determining the acute toxicity of chemicals for earthworms have been published (OECD, 1984; EEC, 1985). The objective of both guidelines is the determination of lethal concentrations of chemical substances, i.e., LC_{50} values. It is generally accepted, however, that mortality is a rather insensitive parameter and has only limited ecological significance. Reproduction seems to be of greater importance for the maintenance of populations (Moriarty, 1983). Some authors therefore determined the effect of chemicals on cocoon production by earthworms (Bengtsson *et al.*, 1986; Lofs-Holmin, 1982; Ma, 1984; Malecki *et al.*, 1982; Neuhauser *et al.*, 1984; Reinecke and Venter, 1985). Their test methods are, however, not standardized. For reasons of standardization the use of a well-defined substrate, such as an artificial soil, is recommended for standardized toxicity tests with earthworms (Dean-Ross, 1983).

This paper describes a reproduction toxicity test using the artificial soil substrate and the earthworm species *Eisenia fetida* prescribed by the two international guidelines (OECD, 1984; EEC, 1985). Results of tests with copper, pentachlorophenol, and 2,4-dichloroaniline are presented.

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MATERIAL AND METHODS

Earthworms

Earthworms of the species *E. fetida andrei* were grown in the laboratory on manure at an ambient temperature of 20–22°C. The worms used in this study adults with a well-developed clitellum (8–13 weeks old) and their average wei the start of the tests ranged between 248 and 425 mg.

Chemicals

Copper(II)chloride · $2H_2O$ (Cu), pentachlorophenol (PCP), and 2,4-dichloroan (DCA) were obtained from Merck and Fluka AG and were 95 to >99% pure.

Artificial Soil

The artificial soil comprised (by dry weight) 10% sphagnum peat, 20% kaolin 69% fine sand, and some calcium carbonate to adjust the pH to a level of 6.0 (pH in the test with Cu was a little higher: 6.3–7.1). In the case of cocoon produ tests 1 mm sieved, moist peat was used, and the substrate was moistened up to a of 55% (w/w) with demineralized water. For the incubation of cocoons finely gro peat was used, and the final substrate had a moisture content of 35% (w/w). Fi ground (<1.0 mm) cow dung was used as a food source.

Cocoon Production Toxicity Test

Cu was added to the artificial soil substrate as an aqueous solution. PCP and are not readily soluble in water and were therefore mixed with a small amount o artificial soil and subsequently added to the rest of the soil. The substrate was m intensively with a household mixer and the moisture content was raised to the de level by the addition of demineralized water. Amounts equivalent to 0.5 kg dry cial soil were placed in 1-liter glass jars. In the middle of the soil a hole was made filled with 2% (dry weight) finely ground cow dung. The cow dung was moistened to 55% with demineralized water before use. Ten adult earthworms were adde each jar. Jars were loosely covered with a glass petri dish to prevent moisture by evaporation and incubated at 20 ± 2°C in an illuminated climatic chamber 400 lux).

Before exposure earthworms were preconditioned for one week (phase A) in treated artificial soil with 2% cow dung. In the case of Cu, a second precondition period (phase A*) of one week in treated soil followed. After this earthworms w exposed for 3 weeks to the chemical substances (phase B). In this study the test w extended with a recovery period of 3 weeks in untreated soil (phase C). At the end each period the number of cocoons produced was determined by washing the conte of each jar through two sieves (2 and 1 mm) placed on top of each other. Soil p moisture content, and weights of all (surviving) worms (in groups of 10 per jar) we determined at the beginning and end of each phase of the test.

Each test was performed at five test concentrations and a control. The tests ha four jars per concentration, each jar containing 10 earthworms. Because earthworm are known to be able to avoid chemical substances (Edwards and Lofty, 1977), th might escape from the treated soil into the hole with untreated cow dung. For th

reason in the Cu test in two out of four jars per concentration cow dung was treated at the same Cu concentration as the soil (phase A* and B). And in the DCA test in phase A two out of four jars per concentration received only 1% cow dung instead of 2%. In phases B and C of this test 1% cow dung was applied at the beginning and another 1% after ca 1.5 weeks in order to reduce the amount of untreated material present.

Cocoon Incubation

To determine the influence of the chemical substances on cocoon hatchability, cocoons produced in phase B 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.

Statistics

EC_{50} values based on cocoon production data of phase B (in case of Cu also for phase A*) were determined according to a logit model. NEL were determined by comparing treated groups with controls using Student's *t* test. The relation between a treatment and effects on cocoon production and cocoon hatchability was tested by analysis of variance (ANOVA).

RESULTS

Cocoon Production

The results of the cocoon production tests are shown in Figs. 1, 2, and 3. In the Cu test there was no difference between the number of cocoons produced in jars with treated and untreated cow dung. And in the DCA test cocoon production was not affected by adding 1% (+1%) cow dung instead of 2%. In Figs. 1, 2, and 3 the numbers of cocoons produced are therefore averaged for all four jars per concentration.

EC_{50} values for the effect of PCP, DCA, Cu (phase A*), and Cu (phase B) on cocoon production by *E. fetida andrei* are 58 (46–75), 176 (150–206), 62 (21–181), and 191 (163–223) $mg \cdot kg^{-1}$ dry artificial soil, respectively. Using Student *t* tests NEL values were derived: for PCP, DCA, and Cu these values are 32, 56, and 60–120 (phases A* and B), respectively.

In phase A* of the test with Cu (Fig. 1) the increase in worm weights was affected in a dose-related manner: 19.9% increase in control and 0.3% at 300 mg $Cu \cdot kg^{-1}$. During phase B, however, the opposite occurred: control worms lost 4.4% of their weight, while at 300 mg $Cu \cdot kg^{-1}$ worm weights increased by 21.1%. There seemed to be a negative correlation between growth and cocoon production during phase B, but not during phase A*. In phase A* cocoon production was more affected than in phase B. In phase C worms did recover cocoon production to a level as high as or even exceeding control.

PCP (Fig. 2) (100 $mg \cdot kg^{-1}$) significantly inhibited cocoon production only at the highest concentration tested. During phase B three worms (7.5%) died at this concentration, and worm weights only increased by 0.9%. At the other concentrations weight increases ranged between 5.7 and 12.2%. In phase C worms exposed to the highest test concentration in phase B seemed to recover: weights increased by 40.0% (13.6–

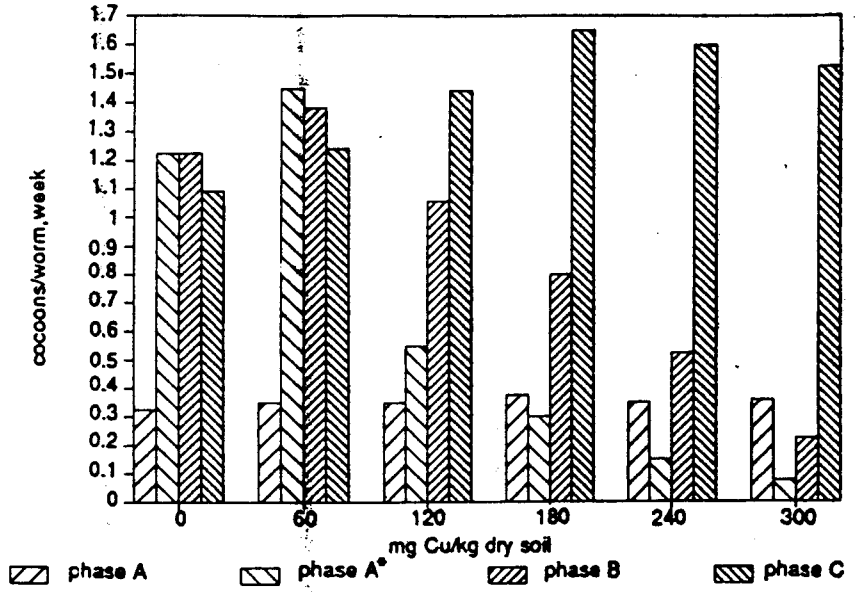

FIG. 1. The influence of copper on the cocoon production of *Eisenia fetida andrei* in artificial soil (mean of four replicates: phase A, preconditioning; phase A*, 1-week exposure; phase B, 3-weeks exposure; phase C, recovery).

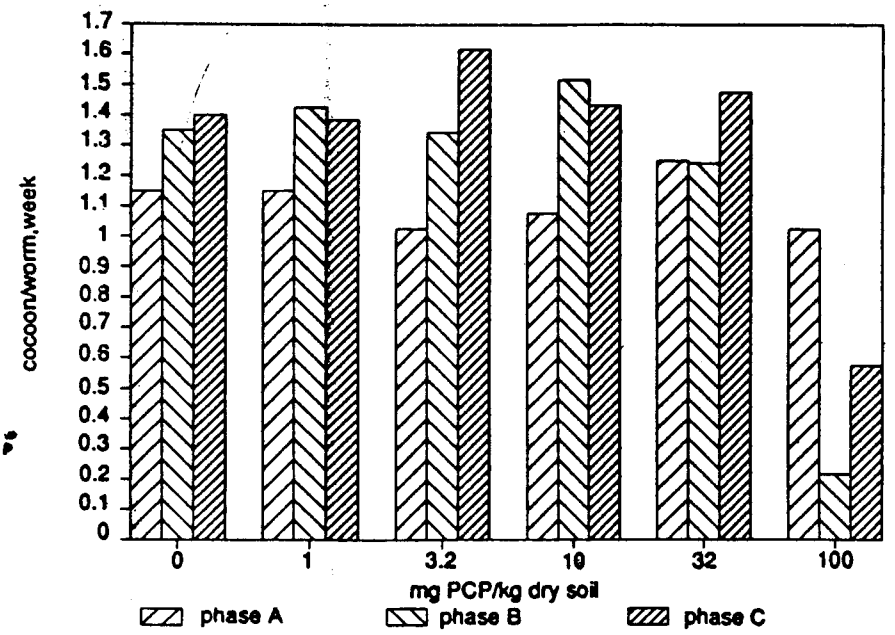

FIG. 2. The influence of pentachlorophenol on the cocoon production of *Eisenia fetida andrei* in artificial soil (mean of four replicates; phase A, preconditioning; phase B, exposure; phase C, recovery).

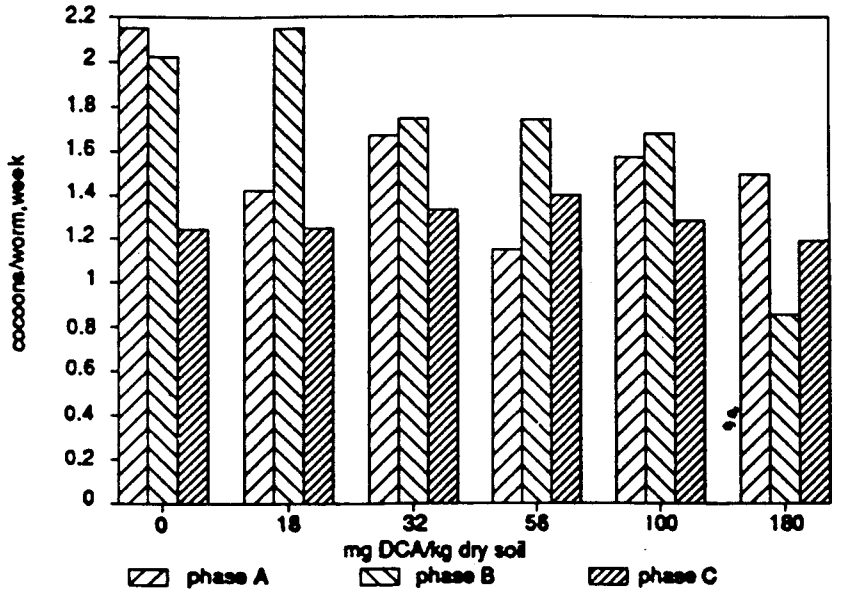

FIG. 3. The influence of 2,4-dichloroaniline on the cocoon production of *Eisenia fetida andrei* in artificial soil (mean of four replicates; phase A, preconditioning; phase B, exposure; phase C, recovery).

18.3% at the other concentrations). Cocoon production of these worms was, however, still significantly lower than control.

In the DCA test worm weights were not affected. Cocoon production was significantly reduced at 100 mg·kg^{-1}, but returned to control level when worms were transferred into untreated artificial soil (phase C).

Cocoon Hatchability

Table 1 shows the results of the cocoon incubation study. Cocoon hatchability was not affected by Cu. Upon ANOVA DCA seemed to affect the number of infertile cocoons in a dose-related manner. However, the number of infertile cocoons was significantly increased only at the highest test concentration. The number of juveniles per cocoon was not related to DCA-treatment.

PCP affected cocoon fertility: at 32 mg·kg^{-1} the number of infertile cocoons was significantly increased, and at 100 mg·kg^{-1} all cocoons were infertile. The number of juvenile worms per fertile cocoon was not affected by PCP. Based on these results the NEL for the effect of PCP on earthworm reproduction is 10 mg·kg^{-1} dry soil.

DISCUSSION

In this study, cow dung was used as a food source. Without food added, earthworms of the species *E. fetida* do not produce many cocoons. In this study, during phase B between 1.2 and 2.0 cocoons/worm/week were produced in controls. In artificial soil without cow dung, weekly cocoon production amounted to only ca. 0.2 cocoons per worm (van Gestel, unpublished data). And from the studies of Vonk *et al.* (1986), who also used artificial soil without food, an average value of 0.5 cocoons/worm/

TABLE 1

HATCHABILITY OF COCOONS PRODUCED BY *Eisenia fetida andrei* EXPOSED TO PENTACHLOROPHENOL, 2,4-DICHLOROANILINE, AND COPPER (MEAN OF FOUR REPLICATES)

Substance	Concentration ($mg \cdot kg^{-1}$ dry soil)	% Infertile cocoons	Number of juvenile worms per cocoon
Cu	0	42	1.7
	60	27	2.2
	120	36	1.9
	180	34	2.2
	240	32	1.8
	300	46	1.8
PCP	0	15	2.3
	1.0	18	2.4
	3.2	17	2.0
	10	15	2.5
	32	30**	2.0
	100	100*	—
DCA	0	6	3.5
	18	3	3.1
	32	7	3.1
	56	12	2.9
	100	6	2.9
	180	16**	2.9

* $P < 0.01$; ** $P < 0.05$.

week can be estimated. Results of the Cu test demonstrate that not treating the cow dung does not influence the test result. Neither is the test result influenced by using 1% (+1%) cow dung instead of 2% (DCA test). In order not to supply more food than necessary, it must be recommended to use 1% (+1%) cow dung for future tests.

Other authors also used a food source in reproduction tests with earthworms. Ma (1984) in a study with *Lumbricus rubellus* supplied dried alder leaves. Lofs-Holmin (1982) mixed soil with farmyard manure to study cocoon production by *Allolobophora caliginosa*, and Bengtsson *et al.* (1986) studying the effect of metals on cocoon production by *Dendrobaena rubida* mixed soil with cattle dung. Some authors even used pure cow dung for their studies: Venter and Reinecke (1987) and Reinecke and Venter (1985) in studies with *E. fetida*.

From Figs. 1, 2, and 3, it can be concluded that cocoon production is always higher in phase B than in phase A. This proves the necessity of a preincubation period to enlarge the number of cocoons produced and with this the reliability of the test.

From Figs. 1, 2, and 3, it can also be concluded that there is a large variation in the amount of cocoons produced in the different tests. In phase A of the Cu test, only 0.3–0.4 cocoons per worm per week were produced, in the PCP test 1.0–1.3, and in the DCA test 1.4–2.2. In controls of phase B the difference between Cu and PCP was reduced: 1.2 and 1.4 cocoons/worm/week, respectively. In the DCA test, however, much more cocoons were produced: 2.0 cocoons/worm/week. Finally, in phase C cocoon production was more or less similar for all three tests.

The number of juveniles per cocoon and the number of fertile cocoons also varies

greatly between the three tests described. In the Cu test, 42% of the cocoons in the control were infertile; in case of PCP this was 14.8%, and for DCA 5.7%. The number of juvenile worms per fertile cocoon were 1.7, 2.3, and 3.5 for the controls of the tests with Cu, PCP, and DCA, respectively. These differences are hard to explain. The tests were, however, not performed at the same time. It is possible that the reproductive performance of *E. fetida andrei* is influenced by the season. A similar phenomenon was found by Watanabe and Tsukamoto (1976). They sampled cocoons of *E. fetida* in field compost at different times of the year: hatchability ranged between 33.3 and 81.4%, and the number of juvenile worms per cocoon between 1.6 and 3.6. In our study, however, worms were used from a laboratory culture which is maintained under the same conditions throughout the year.

A period of 3 weeks was chosen for the cocoon production test (phase B), because after this time cocoons of *E. fetida* start to hatch: Hatching times of 29.7–30.9 days for cocoons of *E. fetida* incubated in distilled water at 25°C were reported by Reinecke and Venter (1987); Tsukamoto and Watanabe (1977) found an average incubation time of 25.2 (23–28) days for the incubation of cocoons of *E. fetida* on wet filter paper at 20°C. The test may be prolonged by incubating the adult worms for another 3 weeks in (freshly prepared) soil treated at the same concentrations. This procedure can be repeated as often as desired. Tests with aluminum and benomyl recently carried out in the authors' laboratory, however, did not indicate a significant influence on the test result (EC_{50} or NOEC) when extending the test with a second period of 3 weeks (van Gestel, unpublished data).

For the incubation of cocoons, a soil substrate was chosen. As described by van Gestel *et al.* (1988), incubation in water or on wet filter paper appeared to have some disadvantages, e.g., growth of fungi on wet filter paper or a delayed hatching in water. Using a soil substrate offers the possibility of extending the cocoon production toxicity test to a real reproduction toxicity test. By treating the cocoon incubation substrate at the same concentrations of the test substance, the influence on hatchability and juvenile worms can be tested. For PCP, such a test was carried out, and it was demonstrated that treating the cocoon incubation substrate led to a decreased number of juvenile worms per cocoon (van Gestel *et al.*, in preparation).

In this study, Cu at the lowest concentration tested (60 $mg \cdot kg^{-1}$) increased cocoon production. Since Cu is an essential element for many organisms, this concentration may have been close to the optimal level required for earthworms. Bengtsson *et al.* (1986) found an increased cocoon production for *D. rubida* at 100 $mg\ Cu \cdot kg^{-1}$ in soil at pH 6.5 (3 months). At this concentration and pH 5.5, many more cocoons were fertile than control; at pH 6.5, there was no difference with control. Cocoon hatching was, however, delayed at this concentration. Ma (1984) found a NEL of 54 $mg \cdot kg^{-1}$ for the effect of Cu on cocoon production of *L. rubellus* in a loamy sand; in a calcareous sandy loam cocoon production was already inhibited at the lowest concentration tested (63 $mg\ Cu \cdot kg^{-1}$). Growth of the earthworms during the 6-week testing period was not affected at concentrations $\geq$373 $mg\ Cu \cdot kg^{-1}$. Ma (1984) concludes that a test period of 2 weeks is sufficient to detect effects on cocoon production. Malecki *et al.* (1982) and Neuhauser *et al.* (1984) used a different method to study the effect of metals on the growth and reproduction of *E. fetida*. They incubated earthworms in a layer of untreated soil covered with a layer of treated horse manure. Cocoon production was affected at concentrations of 100 ($Cu(NO_3)_2$ or $CuSO_4$) or

2000 mg Cu·kg^{-1} ($CuCl_2$ or Cu-acetate)) in the manure. In the test with Cu-acetate, cocoon production was significantly increased at 500 mg Cu·kg^{-1}.

The effect of PCP on cocoon production by *E. fetida* was studied by Vonk *et al.* (1986). They found a NEL of 12 mg·kg^{-1} dry artificial soil (4 weeks). In this study, the number of infertile cocoons seemed to increase at higher PCP concentrations. Venter and Reinecke (1987) found a similar effect in a study with dieldrin. No data were found on the effect of DCA on earthworm reproduction.

CONCLUSIONS

The method proposed in this paper offers the possibility of studying effects of chemical substances on earthworm reproduction in a standardized way. Results obtained with this method are comparable with those from other literature.

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