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

C. A. M. van Gestel, W. A. van Dis, E. M. van Breemen, and P. M. Sparenburg

National Institute of Public Health and Environmental Protection,
P.O. Box 1, 3720 BA Bilthoven, The Netherlands

Received February 3, 1989

This article describes a standardized test method for determining the effect of chemical substances on the reproduction of the earthworm *Eisenia fetida andreii*. 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·kg^-1·dry soil, respectively. Hatching of cocoons was influenced by pentachlorophenol (NEL, 10 mg·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.

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., EC₅₀ 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; Reinecke and Venter, 1985). Their test methods are, however, not standardized. For reasons of standardization the use of a well-defined substrate, i.e., artificial soil, is recommended for standardized toxicity tests with earthworms (van Ross, 1983).

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

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

Chemicals

Copper(II)chloride-2H₂O (Cu), pentachlorophenol (PCP), and 2,4-dichlorophenoxyacetic acid (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% kaolinite, 69% fine sand, and some calcium carbonate to adjust the pH to a level of 6.0–7.1 (pH in the test with Cu was a little higher: 6.3–7.1). In the case of cocoon production tests 1 mm sieved, moist peat was used, and the substrate was moistened up to 55% (w/w) with demineralized water. For the incubation of cocoons finely ground peat was used, and the final substrate had a moisture content of 35% (w/w). Finely 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 DCA are not readily soluble in water and were therefore mixed with a small amount of artificial soil and subsequently added to the rest of the soil. 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 0.5 kg dry artificial 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 added to 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 preconditioning period (phase A*) of one week in treated soil followed. After this earthworms were exposed for 3 weeks to the chemical substances (phase B). In this study the test was extended with a recovery period of 3 weeks in untreated soil (phase C). At the end of each period the number of cocoons produced was determined by washing the contents of each jar through two sieves (2 and 1 mm) placed on top of each other. Soil pH, moisture content, and weights of all (surviving) worms (in groups of 10 per jar) were 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 had four jars per concentration, each jar containing 10 earthworms. Because earthworms are known to be able to avoid chemical substances (Edwards and Lofty, 1977), the chemicals might escape from the treated soil into the hole with untreated cow dung. For the
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

EC50 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.

EC50 values for the effect of PCP, DCA, Cu (phase A*), and Cu (phase B) on cocoon production by E. fetida andreii are 58 (46–75), 176 (150–206), 62 (21–181), and 191 (163–223) mg·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·kg−1. During phase B, however, the opposite occurred: control worms lost 4.4% of their weight, while at 300 mg Cu·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·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).
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 EI senia fetida andrei EXPOSED TO PENTACHLOROPHENOL, 2,4-DICHLOROANILINE, AND COPPER (MEAN OF FOUR REPLICATES)

<table>
<thead>
<tr>
<th>Substance</th>
<th>Concentration (mg·kg⁻¹ dry soil)</th>
<th>% Infertile cocoons</th>
<th>Number of juvenile worms per cocoon</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cu</td>
<td>0</td>
<td>42</td>
<td>1.7</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>27</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>36</td>
<td>1.9</td>
</tr>
<tr>
<td></td>
<td>180</td>
<td>34</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td>240</td>
<td>32</td>
<td>1.8</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>46</td>
<td>1.8</td>
</tr>
<tr>
<td>PCP</td>
<td>0</td>
<td>15</td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>18</td>
<td>2.4</td>
</tr>
<tr>
<td></td>
<td>3.2</td>
<td>17</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>15</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>32</td>
<td>30**</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>100*</td>
<td></td>
</tr>
<tr>
<td>DCA</td>
<td>0</td>
<td>6</td>
<td>3.5</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>3</td>
<td>3.1</td>
</tr>
<tr>
<td></td>
<td>32</td>
<td>7</td>
<td>3.1</td>
</tr>
<tr>
<td></td>
<td>56</td>
<td>12</td>
<td>2.9</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>6</td>
<td>2.9</td>
</tr>
<tr>
<td></td>
<td>180</td>
<td>16**</td>
<td>2.9</td>
</tr>
</tbody>
</table>

* 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 Alolobophora 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 (EC₅₀ 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·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·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·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·kg⁻¹). Growth of the earthworms during the 6-week testing period was not affected at concentrations >373 mg Cu·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(NO₃)₂ or CuSO₄) 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.

**REFERENCES**


