

Does a Heterogeneous Distribution of Food or Pesticide Affect the Outcome of Toxicity Tests with Collembola?

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The reproduction of two closely related soil microarthropods, *Folsomia candida* Willem and *Folsomia fimetaria* L. (Insecta: Collembola), was tested under the influence of the insecticide dimethoate. Dimethoate had an adverse effect on the survival of adults and their reproduction in concentrations of about the recommended field dose, with *F. fimetaria* being more sensitive than *F. candida*. The experimental conditions were altered to evaluate the realism in the basic single species/single chemical reproductive test system. The importance of the spatial distribution of dimethoate was studied with food applied to the surface (original procedure), mixed homogeneously in the whole soil profile or only in the top layer, or mixed heterogeneously into the soil preserving the small granula of the yeast originally in the commercial formulation. Toxicity decreased significantly when exposure could be avoided in an uncontaminated bottom layer and even more if food was available in this soil horizon. But the results indicate that Collembola were not able to completely avoid dimethoate when they had the choice. For extrapolation purposes a simple test system may be sufficient as EC_{50} was changed less than one order of magnitude with the different test designs. In terms of EC_{50} the outcome of a toxicity test with a heterogeneous distribution of food and dimethoate was changed only slightly but the effects to suboptimally fed populations should be considered because they may be more vulnerable. © 1995 Academic Press, Inc.

Apart from the acute need for soil invertebrate tests, the development of tests should meet the additional requirement for minimizing uncertainty in the extrapolation to field condition. One important difference between field and the laboratory is the exposure conditions; therefore, this should be optimized in laboratory tests (OECD, 1992).

This paper presents a feasible way to perform laboratory studies on Collembola and it pursues questions about the importance of factors that are believed to influence the outcome of toxicity tests. The motivation for this lies in the fact that the recognized structural complexity of the biotic and abiotic compartments in the natural soil environment contrasts extremely with the simple design of laboratory test systems. Recently it was demonstrated that toxicity to Collembola in the field was related to the spatial distribution of pesticide (Krogh, 1991). This has stimulated the investigation of simple simulations in laboratory test systems of the aspects that play an important part in the toxicity under natural circumstances. Hence, the basic question to be clarified is whether manipulations of the distribution of food and pesticide result in significant changes of the toxicity in comparison with a simple test system.

INTRODUCTION

Laboratory test systems with species representative of the soil invertebrate community were demanded by an OECD workshop (OECD, 1989) and advocated by a European workshop (Eijsackers and Løkke, 1992). Present experience with single species reproductive test system counts for *Folsomia candida* (Collembola: Isotomidae) (Biologische Bundesanstalt (BBA), 1989), *Eisenia fetida andrei* (van Gestel *et al.*, 1989), *Platynothrus peltifer* (Acarina: Oribatida) (Denneman and van Straalen, 1991), *Orchesella cincta* (Collembola: Entomobryidae) (Badejo and Van Straalen, 1992), and *Hypoaspis aculeifer* (Acarina: Gamasida) (Krogh, in press). The present study uses the BBA procedure with *F. candida* as a starting point for tests and evaluates the protocol with respect to selected factors of primary importance to the ecotoxicity.

MATERIALS AND METHODS

Dimethoate

A commercial formulation of dimethoate available in Denmark, DLG Dimethoat 28, containing the organic solvents xylene and cyclohexanone was used. Dimethoate, *O,O*-dimethyl-*S*-(*N*-methylcarbomoyl-methyl) phosphorodithionate, is an inhibitor of cholinesterase. It has a half-life in soil of 5-9 days depending on soil texture (Kolbe *et al.*, 1991). It is a broad spectrum insecticide with pronounced effects on the arthropod fauna at the recommended dose level (e.g., Frampton, 1988; Goodwin, 1984; Hassan *et al.*, 1988; Vickerman and Sunderland, 1977; Powell *et al.*, 1985). Dimethoate has been selected in this laboratory as a reference chemical for toxicity studies.

Laboratory Cultures and Basic Test Protocol

F. candida and *F. fimetaria* are established in permanent cultures and test animals are produced according to the schedule in Table 1. Stimulation of oviposition is obtained by moving adults to newly produced plaster/charcoal substrate. When the Collembola have lived on the substrate for 5 weeks they are again stimulated to oviposit. If the culture is old or shows signs of decreasing health, it will be eliminated. The cultures are renewed by surplus synchronized offspring and from the eggs not collected for production of synchronized cultures.

A synchronized culture is produced by collecting about 1-week-old eggs which are allowed to hatch over the weekend from Friday to Monday. Tests with *F. candida* are conducted on the Wednesday when the animals are 9–12 days old and with *F. fimetaria* on the Wednesday when they are 16–19 days old.

In order to eliminate undesired fauna in the soil used in the tests, the soil was alternately dried (60°C), frozen (–35°C), and incubated (20°C) under wetted conditions. The soil was then sieved through a 2-mm mesh.

The soil was moistened and inoculated with a suspension of soil extract. The extract was produced from 0.5 kg fresh soil which had been stored at 5°C for no longer than 2 months, added 0.5 liter demineralized water, incubated for 24 hr for extraction of microbes, and then sieved through a 40- μ m mesh.

Concentrations of pesticides were prepared in an increasing geometric series with a factor of 1.8. Concentrations were calculated on the basis of the recommended dosage for the dimethoate, assuming an even distribution in the uppermost 5 cm of the soil resulting in a concentration of 0.389 mg commercial formulation/kg soil based on a density of 1.44 kg/liter of the actual sandy loam soil. The commercial formulation of dimethoate was suspended in water and the amount of water was adjusted to about 50% of water holding capacity (see Table 3 concerning soil characteristics). Thirty grams of moist soil was used per replicate.

The test scheme for the testing of chemicals is reflected

TABLE 1
Weekly Schedule for the Breeding of Collembolan Test Animals

Day	Operation
Monday	Removing unhatched eggs from synchrone culture. Feeding and watering synchrone culture.
Tuesday	Production of substrates (Gypsum/charcoal).
Wednesday	Changing the substrate with <i>F. candida</i> and <i>F. fimetaria</i> for stimulation of oviposition.
Thursday	Feeding and watering the cultures.
Friday	Collecting eggs from <i>F. fimetaria</i> and <i>F. candida</i> stimulated to oviposit last week. Feeding and watering synchrone culture.

in Table 2. Ten parthenogenitically reproducing *F. candida* females or 25 individuals of random mixture of roughly equal numbers of the sexes of *F. fimetaria* were added to each of the 4 replicates per concentration. A replicate thus consisted of contaminated soil in a closed container, i.e., a microcosm, with food and test Collembola. When the test was terminated after 4 weeks, the animals were extracted from the microcosms in a high gradient extractor (HGE) of the MacFadyen type (design developed from equipment at Mols Laboratory, see Petersen, 1978) collected into a cooled vessel (2°C) with plaster of paris/charcoal. The extraction was started at 25°C and the temperature was increased automatically every 12 hr. After 40°C the extraction was finished.

Spatial Distribution of Yeast and Dimethoate

Reproductive tests with *F. candida* were performed with dimethoate applied at 0, 0.4 (calculated field dosage), and 1.3 mg/kg. Food and dimethoate were distributed in the following ways with all treatments replicated five times.

TABLE 2
Test Scheme for the Testing of Chemicals with *F. candida* and *F. fimetaria*

Day	Day of week	Operation
1	Monday	1. Making inoculate from fresh undisturbed soil 2. Weighing of defaunated dry soil in portions for each test container
2	Tuesday	3. Pesticides and inoculate mixture are added to 100-ml beakers with automate pipet, mixed thoroughly into soil to obtain even humidity and distribution of chemical 4. Soil is incubated in fume cupboard until next day
3	Wednesday	5. Weighing of five beakers to adjust water content 6. Transferring soil to microcosm and weighing microcosms 7. Transferring test animals with exhaustor from synchronous culture to black lid for counting and addition to microcosm 8. Addition of 15 mg granulated dried baker's yeast to each microcosm, weighing of 5 microcosms 9. Incubation in climate chamber at 20° C
17	Wednesday	10. Aeration and addition of 15 mg granulated dried yeast to each microcosm, weighing of 5 microcosms to adjust for lost water
31	Wednesday	11. Termination of test. Extraction of soil in high gradient extractor
33	Friday	12. Removal of collection vessels from extractor. Storage at 5° C until counting

Control:

- a. Addition of yeast to the surface of the soil (Biologische Bundesanstalt, 1989)

Homogeneous distribution of yeast and dimethoate in the soil:

- b. 15 mg yeast
c. 60 mg yeast

The yeast was suspended in the dimethoate solution and mixed into the soil to obtain a homogeneous distribution. The same amount as in the base test system, 15 mg, was suspended. Furthermore, a set of replicates received a dose of 60 mg, as 15 mg was expected to be suboptimal because the availability would be considerably lower in the mixed form.

Homogeneous distribution of dimethoate in the top 1 cm of soil:

- d. Homogeneous distribution of yeast in the top layer
e. Homogeneous distribution of yeast in both top and bottom layer

An uneven vertical distribution of dimethoate was obtained by placing 30 g treated soil on top of 30 g untreated soil. The treatment consisted of an addition of a solution of dimethoate with 60 mg yeast in suspension to the soil. The bottom soil was either added demineralized water or a water suspension containing 60 mg yeast.

Heterogeneous distribution of yeast:

- f. Heterogeneous distribution of yeast in top layer
g. Heterogeneous distribution of yeast in both top and bottom layer

Fifteen milligrams yeast was distributed as intact granules by carefully mixing into the moist soil. This should resemble an intermediate distribution between completely homogeneous distribution and the confinement of yeast to the surface.

Statistical Analysis

Calculations of LC_{50} and EC_{50} were done by fitting a model to the data (Lacey and Mallett, 1991)

$$y = c \cdot (1 - a \cdot x^\beta)$$

$$y = c \cdot (1 - e^\alpha \cdot x^\beta)^{-1}$$

where

y = reproductive output,
 c = control reproduction,
 x = dose of chemical,
 a, α = "slope" parameter,
 β = curvature parameter.

These formulas were reparameterized by substitution of α with an expression including EC_x (Ann Gould, Shell Research Ltd, personal communication). This allows the calculation of 95% confidence intervals when estimation of the parameters including EC_x is done with the SAS procedure NLIN (SAS Institute Inc., 1988).

Statistical tests for NOEC and LOEC were made with a Williams' test (Gelber *et al.*, 1985) by the use of the program Toxstat (Gulley *et al.*, 1988).

RESULTS

Dose-Response Toxicity Data from Basic Test Protocol

Dimethoate had an adverse effect on survival and reproduction of the Collembola (Fig. 1). The derived toxicity measures are provided in Table 3. LC_{50} and EC_{50} were close to the calculated field dosage. *F. fimetaria* was more sensitive to dimethoate than *F. candida*. For

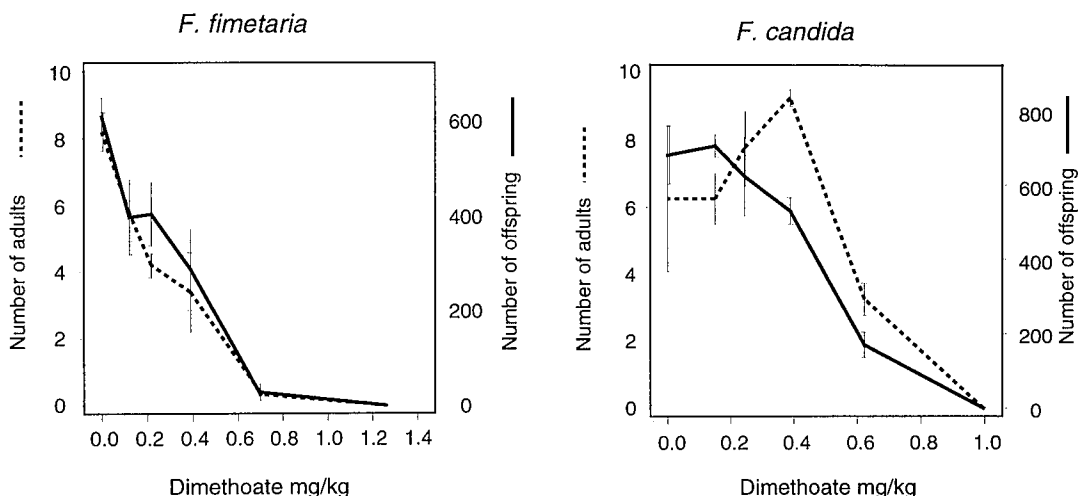


FIG. 1. The effects of a concentration series of dimethoate on the survival and reproduction of 25 adult *F. fimetaria* (♀ + ♂) and *F. candida* (♀) after 4 weeks. The calculated recommended field application dose is 0.4 mg/kg. Vertical bars, ± 1 SE.

TABLE 3
 LC₅₀, EC₅₀, NOAEC, and LOAEC Concentrations of Dimethoate Affecting Survival and Reproduction of Two
 Collembolan Species

	Adult mortality			Reproduction				
	LC ₅₀	95% CI		EC ₅₀	95% CI		NOAEC	LOAEC
		Lower	Upper		Lower	Upper		
<i>F. fimetaria</i>	0.2	0.2	0.3	0.3	0.1	0.5	<0.1	0.1
<i>F. candida</i>	0.6	0.6	0.6	0.5	0.4	0.6	0.4	0.6
<i>H. aculeifer</i>	0.8	0.7	1.0	0.9	0.8	1.0	0.7	1.3

Note. For comparison the gamasid mite *Hypoaspis aculeifer* Canestrini (Acarina: Gamasida) has been included. NOAEC, no observed adverse (reduction) effect concentration; LOAEC, lowest observed adverse effect concentration.

both species reproduction was reduced above 0.4 mg/kg, while concentrations higher than 1.0 led to high mortality of the adults.

A preliminary crude impression of the fertility rate (r_f) can be calculated under the assumptions that only surviving females have reproduced and all juveniles produced were unaffected by dimethoate. When this estimate of r_f is used to assess the incidence of a sublethal effect, violation of the first assumption does not affect the conclusion that a sublethal effect has occurred. It would only imply that the extra contribution to the number of adults makes the sublethal effect on fertility even more pronounced, i.e., as $r_f(\text{treatment})$ decreases the indication of a sublethal effect, $r_f(\text{control}) - r_f(\text{treatment})$, will increase.

Spatial Distribution of Yeast

The three concentrations run in this experiment allow only for a tentative estimation of the 4-week LC₅₀ for adults (P1 generation) and EC₅₀ (reproductive output/F1 generation) based on a linear decline in response from 0.4 to 1.3 mg dimethoate/kg. The following 4-week LC and EC values were estimated in this manner to make possible a direct comparison with the above-mentioned dose-response data.

The homogeneous distribution of yeast made it inaccessible to *F. candida* so the reproduction decreased to less than 10% of the control (Figs. 2a and 2b). The LC₅₀ and EC₅₀ values were about 0.6 mg/kg. At the highest concentration there was no reproduction although a few adults survived; hence, they have been subjected to a sublethal reproductive effect. Adding four times more yeast (Fig. 2c) increased the reproduction but the toxicity pattern was identical to the latter case. Confining the yeast and dimethoate to the top layer changed neither the toxicity nor the general control level of reproduction, thus Experiments c and d must be considered toxicologically identical.

When yeast was available in the bottom layer the survival of the adults increased resulting in an LC₅₀ in the order of 1 mg/kg (Fig. 2e). Again the toxicity pattern for

the reproduction was unchanged except for a small (15%) reproduction at the highest concentration. Because 50% of the adults had survived at the highest concentration, this implies that they were sublethally affected resulting in a fertility rate decreasing from 60 individuals in the control to 20 individuals per female at 1.3 mg/kg.

With yeast heterogeneously mixed into the soil the control survival and reproduction were at the same level as when yeast was on the top (Figs. 2f and 2g). The toxicity pattern was identical to Experiment e. In Experiment g adult survival became closer to that of the control resulting in the largest observed LC₅₀ of about 2.6 mg/kg and likewise the reproduction was the least affected with an EC₅₀ of 1.1 mg/kg. The fertility rate had increased to 100 in the control and 40 at 1.3 mg/kg (juveniles per female).

DISCUSSION

Dose-Response Toxicity Data from Basic Test Protocol

Numerous studies have dealt with dimethoate and for the most part the application led to high mortality. Only a few, however, are comparable to the present study covering well-defined and computable dosage levels.

Sublethal effects of dimethoate were indicated through a decrease in reproductive output per adult as determined here after 4 weeks or as LC₅₀ > EC₅₀. If a chemical had only a pure mortality effect, then the fertility rate per adult would have been constant, i.e., equal to the control.

Single species test systems of this simple kind can only reveal direct effects because the elements which could mediate indirect effects have been deliberately eliminated. The effects mediated through microorganisms where they act as an exposure route, i.e., food chain transfer, or if changes have occurred in quantity and composition are possible but the importance of the microorganisms to the Collembola have not been estimated in this study. The effects of repulsion could be registered with an uneven vertical distribution of pesticide but a particular experimental design would be needed to distinguish this effect from other effects.

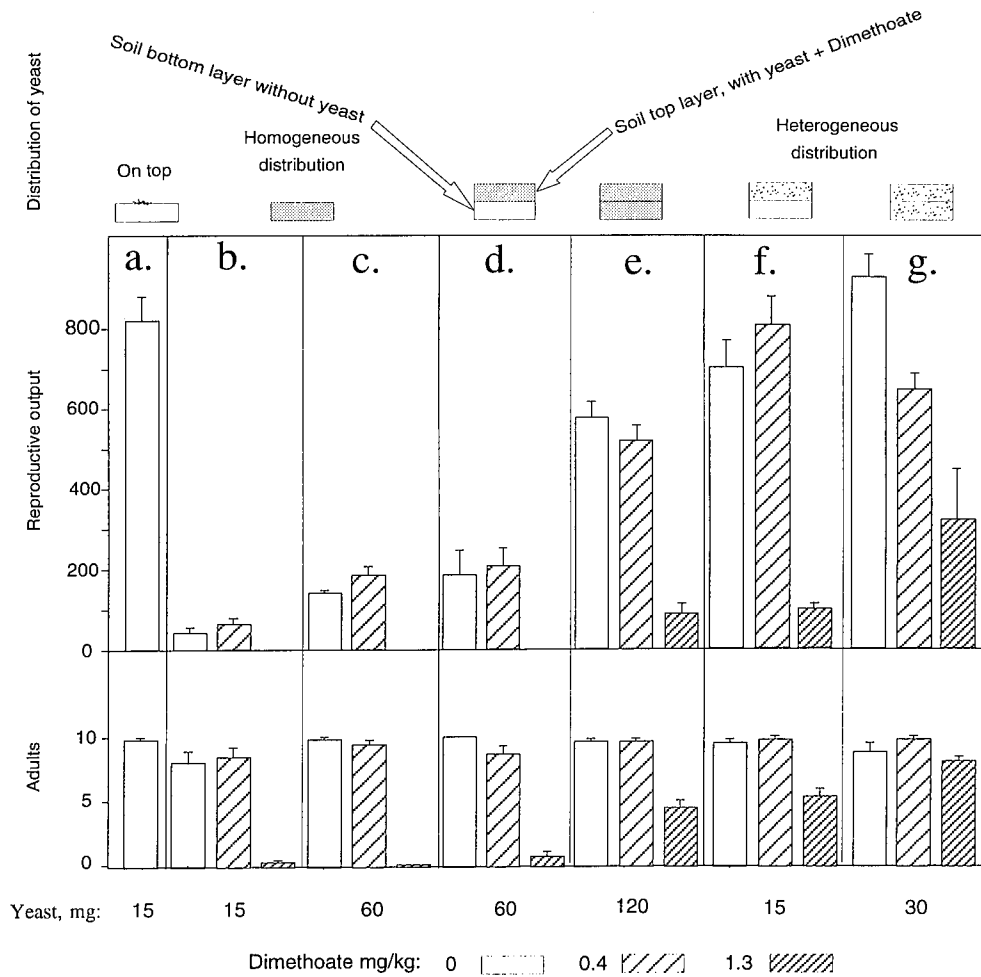


FIG. 2. The effect of spatial distribution of yeast and dimethoate on the reproduction of *F. candida*. Control reproduction with yeast on top (a). Homogeneous distribution of yeast and dimethoate: 15 mg yeast (b), 60 mg yeast (c). Homogeneous distribution of dimethoate in the top 1 cm of soil (d) and in both top and bottom layer (c). Heterogeneous distribution of yeast in top 1 cm of soil (f) and in both top and bottom layer (g). With two layers present (d through g) dimethoate was confined to the top layer. Vertical bars, ± 1 SE.

Spatial Distribution of Yeast and Dimethoate

The toxic effects on reproduction were stable and identical to the basic test in terms of EC_{50} despite the rather different conditions offered to *F. candida*. The toxicity to the reproduction was reduced only in the case of a heterogeneous distribution of yeast (Fig. 2g). Thus, *F. candida* apparently is not able to avoid dimethoate in the system. This may be due to its inability to detect dimethoate in the microenvironment and then to stay out of the contaminated habitat. Furthermore, *F. candida* prefers eating in a contaminated soil instead of starving in an uncontaminated zone without food. So, the uncontaminated bottom layer acting as a possible refuge seemed only useful to some extent. The food accessibility improved the survival (e.g., Figs. 2d and 2f) of the adults and resulted in higher reproduction.

The small reproduction level with the less accessible food indicates that a small and even starving population may be more easily wiped out than a large full population.

This must be considered when assessing the effects on natural populations. In other words, an EC_{50} dose may be devastating to a small population. Changing to EC_{10} , as it has been suggested on many occasions, may improve the chance of protecting these small populations that are for some reason in a susceptible state, e.g., starvation.

It may be expected that *F. candida* would stay in the bottom layer until the dimethoate was degraded to acceptable concentration levels. But in Experiment g they had the choice of staying down but they could not avoid the toxic effect. This may be due either to exposure through air which would traverse the soil or to their inability to detect the pesticide in the contaminated zone and thereby being unable to avoid it. Further investigations must explain the behavior of *F. candida* to dimethoate to determine if it actually makes complex decisions involving cost benefits of eating contaminated food and not eating but suffering from starvation.

The present investigation aimed at setting up different experiments including the vertical dimension along with

food accessibility. The outcome of toxicity tests under these different circumstances differed less than one order of magnitude in these artificial systems. Therefore, for extrapolation purposes the simple basic version of the toxicity test with *F. candida* may be sufficient.

CONCLUSIONS

F. candida was not able to avoid dimethoate when offered additionally an uncontaminated soil layer. In terms of EC₅₀ the outcome of a toxicity test with a heterogeneous distribution of food and dimethoate was changed only slightly but the effects to suboptimally fed populations should be considered because they may be more vulnerable.

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