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OECD GUIDELINES FOR TESTING CHEMICALS

Collembolan Reproduction Test in Soil

INTRODUCTION

1. This Test Guideline is designed for assessing the effects of chemicals on the reproductive output of the collembolans in soil. It is based on existing procedures (1) (2). The parthenogenetic *Folsomia candida* and sexually reproducing *Folsomia fimetaria* are two of the most accessible species of Collembola, and they are culturable and commercially available. When specific habitats not covered by the two species need to be assessed the procedure is extensible also to other species of Collembola if they are able to fulfil the validity criteria of the test.

2. Soil-dwelling Collembola are ecologically relevant species for ecotoxicological testing. Collembolans are hexapods with a thin exoskeleton highly permeable to air and water, and represent arthropod species with a different route and a different rate of exposure compared to earthworms and enchytraeids.

3. Population densities of Collembola commonly reach 10^5 m^{-2} in soil and leaf litter layers in many terrestrial ecosystems (3) (4). Adults typically measure 0.5 - 5 mm, their contribution to total soil animal biomass and respiration is low, estimated between 1% and 5% (5)]. Their most important role may therefore be as potential regulators of processes through microbivory and microfauna predation. Springtails are prey animals for a wide variety of endogeic and epigeic invertebrates, such as mites, centipedes, spiders, Carabidae and rove beetles. Collembola contribute to decomposition processes in acidic soils where they may be the most important soil invertebrates besides enchytraeids, since earthworms and diplopods are typically absent.

4. *F. fimetaria* has a worldwide distribution and is common in several soil types ranging from sandy to loamy soils and from mull to mor soils. It is an eyeless, unpigmented collembolan. It has been recorded in agricultural soils all over Europe (6). It has an omnivorous feeding habit, including fungal hyphae, bacteria, protozoa and detritus in its food. It interacts through grazing with infections of plant pathogenic fungi (7) and may influence mycorrhiza, as is known to be the case for *F. candida*. As most collembolan species it reproduces sexually requiring the permanent presence of males for egg fertilization.

5. *F. candida* is also distributed worldwide. Although it is not common in most natural soils, it often occurs in very high numbers in humus rich sites. It is an eyeless, unpigmented collembolan. It has a well-developed furca (jumping organ) and an active running movement and jumps readily if disturbed. The ecological role of *F. candida* is similar to the role of *F. fimetaria*, but the habitats are more organic rich soils. It reproduces parthenogenetically. Males may occur at less than 1 per thousand.

PRINCIPLE OF THE TEST

6. Synchronous adult (*F. fimetaria*) or juvenile (*F. candida*) Collembola are exposed to a range of concentrations of the test substance mixed into a modified OECD artificial soil using a 5% organic matter content (or an alternative soil) (8). The test scenario can be divided into two steps:

- A range-finding test, in case no sufficient information on toxicity is available, in which mortality and reproduction are the main endpoints assessed after 2 weeks for *F. fimetaria* and 3 weeks for *F. candida*

- A definitive reproduction test in which the total number of juveniles produced by parent animals and the survival of parent animals are assessed. The duration of this definitive test is 3 weeks for *F. fimetaria* or 4 weeks for *F. candida*.

The toxic effect of the test substance on adult mortality and reproductive output is expressed as LC_x and EC_x by fitting the data to an appropriate model by non-linear regression to estimate the concentration that would cause x % mortality or reduction in reproductive output, respectively, or alternatively as the NOEC/LOEC value (9).

INFORMATION ON THE TEST SUBSTANCE

7. The physical properties, water solubility, the log Kow, the soil water partition coefficient and the vapour pressure of the test substance should preferably be known. Additional information on the fate of the test substance in soil, such as the rates of photolysis and hydrolysis and biotic degradation, is desirable. Chemical identification of the test substance according to IUPAC nomenclature, CAS-number, batch, lot, structural formula and purity should be documented when available.

8. This Guideline can be used for water soluble or insoluble substances. However, the mode of application of the test substance will differ accordingly. The Guideline is not applicable to volatile substances, i.e. substances for which the Henry's constant or the air/water partition coefficient is greater than one, or substances for which the vapour pressure exceeds 0.0133 Pa at 25 °C.

VALIDITY OF THE TEST

9. The following criteria should be satisfied in the untreated controls for a test result to be considered valid:
- Mean adult mortality should not exceed 20% at the end of the test;
- The mean number of juveniles per vessel should be at least 100 at the end of the test;
- The coefficient of variation calculated for the number of juveniles should be less than 30% at the end of the definitive test.

REFERENCE SUBSTANCE

10. A reference substance should be tested at its EC_{50} concentration for the chosen test soil type either at regular intervals or possibly included in each test run to verify that the response of the test organisms in the test system are responding within the normal level. A suitable reference substance is boric acid, which should reduce reproduction by 50% (10) (11) at about 100 mg/kg dry weight soil for both species.

DESCRIPTION OF THE TEST

Test vessels and equipment

11. Containers capable of holding 30 g of moist soil are suitable test vessels. The material should either be glass or inert plastic (non-toxic). However, using plastic containers should be avoided if the test substance exposure is decreased due to sorption. The test vessels should have a cross-sectional area allowing the actual soil depth within the test vessel to be 2-4 cm. The vessels should have lids (e.g. glass or polyethylene) that are designed to reduce water evaporation whilst allowing gas exchange between the soil and the atmosphere. The container should be at least partly transparent to allow light transmission.

12. Normal laboratory equipment is required, specifically the following:
- drying cabinet;
- stereo microscope;
- pH-meter and luxmeter;
- suitable accurate balances;
- adequate equipment for temperature control;
- adequate equipment for air humidity control (not essential if exposure vessels are covered by lids);
- temperature-controlled incubator or small room;
- forceps or a low-suction air flow device.

Preparation of the test soil

13. A modified OECD artificial soil (8) is used with an organic matter content of 5%. Alternatively a natural soil could be used, as the artificial soil does not resemble natural soils. The recommended composition of the artificial soil is as follows (based on dry weights, dried to a constant weight at 105 °C):

- 5% sphagnum peat, air-dried and finely ground (a particle size of 2 ± 1 mm is acceptable);
- 20% kaolin clay (kaolinite content preferably above 30%);
- approximately 74% air-dried industrial sand (depending on the amount of $CaCO_3$ needed), predominantly fine sand with more than 50% of the particles between 50 and 200 microns. The exact amount of sand depends on the amount of $CaCO_3$ (see below), together they should add up to 75 %.
- < 1.0% calcium carbonate ($CaCO_3$, pulverised, analytical grade) to obtain a pH of 6.0 ± 0.5; the amount of calcium carbonate to be added may depend principally on the quality/nature of the peat (see Note 1).

Note 1: The amount of $CaCO_3$ required will depend on the components of the soil substrate and should be determined by measuring the pH of pre-incubated moist soil sub-samples immediately before the test.

Note 2: It is recommended to measure the pH and optionally the C/N ratio, Cation Exchange Capacity (CEC) and organic matter content of the soil in order to enable a normalisation at a later stage and to better interpret the results.

Note 3: If required, e.g. for specific testing purposes, natural soils from unpolluted sites may also serve as test and/or culture substrate. However, if natural soil is used, it should be characterised at least by origin (collection site), pH, texture (particle size distribution), CEC and organic matter content and it should be free from any contamination. For natural soil it is advisable to demonstrate its suitability for a test and for achieving the test validity criteria before using the soil in a definitive test.

14. The dry constituents of the soil are mixed thoroughly (e.g. in a large-scale laboratory mixer). The maximum water holding capacity (WHC) of the artificial soil is determined in accordance with procedures described in Annex 5. The moisture content of the testing soil should be optimised to attain a loose porous soil structure allowing collembolans to enter into the pores. This is usually between 40-60% of the maximum WHC.

15. The dry artificial soil is pre-moistened by adding enough de-ionised water to obtain approximately half of the final water content 2-7 days before the test start, in order to equilibrate/stabilise the acidity. For the determination of pH a mixture of soil and 1 M potassium chloride (KCl) or 0.01 M calcium chloride ($CaCl_2$) solution in a 1:5 ratio is used (according to Annex 6). If the soil is more acidic than the required range, it can be adjusted by addition of an appropriate amount of $CaCO_3$. If the soil is too alkaline it can be adjusted by the addition of an inorganic acid harmless to collembolans.

16. The pre-moistened soil is divided into portions corresponding to the number of test concentrations (and reference substance where appropriate) and controls used for the test. The test compounds are added and the water content is regulated according to the paragraph 24.

Selection and preparation of test animals

17. The parthenogenetic *F. candida* is the recommended species, as in the ring testing of the method (11) this species met the validity criteria for survival more often than *F. fimetaria*. If an alternative species is used, it should meet the validity criteria outlined in paragraph 9. At the start of the test the animals should be well fed and the age between 23-26 days for *F. fimetaria* and 9-12 days for *F. candida*. For each replicate, the number of *F. fimetaria* should be 10 males and 10 females, and for *F. candida* 10 females should be used (see Annex 2 and Annex 3). The synchronous animals are selected randomly from the dishes and their health and physical condition is checked for each batch added to a replicate. Each group of 10/20 individuals is added to a randomly selected test container and the big females of *F. fimetaria* are selected to ensure a proper distinction from the *F. fimetaria* males.

Preparation of test concentrations

18. Four methods of application of the test substance can be used: 1) mixing the test substance into the soil with water as a carrier, 2) mixing the test substance into the soil with an organic solvent as a carrier, 3) mixing the test substance into the soil with sand as a carrier, or 4) application of the test substance onto the soil surface. The selection of the appropriate method depends on the characteristic of the compound and the purpose of the test. In general, mixing of the test substance into the soil is recommended. However, application procedures that are consistent with the practical use of the test substance may be required (e.g. spraying of liquid formulation or use of special pesticide formulations such as granules or seed dressings). The soil is treated before the collembolans are added, except when the test compound is added to the soil surface collembolans should be allowed to enter the soil.

Test substance soluble in water

19. A solution of the test substance is prepared in deionised water in a quantity sufficient for all replicates of one test concentration. Each solution of test substance is mixed thoroughly with one batch of pre-moistened soil before being introduced into the test vessel.

Test substance insoluble in water

20. For chemicals insoluble in water, but soluble in organic solvents, the test substance can be dissolved in the smallest possible volume of a suitable solvent (e.g. acetone) still ensuring proper mixing of the chemical in the soil and mixing it with a portion of the quartz sand required. Only volatile solvents should be used. When an organic solvent is used, all test concentrations and an additional solvent negative control should contain the same minimum amount of the solvent. Application containers should be left uncovered for a certain period to allow the solvent associated with the application of the test substance to evaporate, ensuring no dissipation of the toxic compound during this time.

Test substance poorly soluble in water and organic solvents

21. For substances that are poorly soluble in water and organic solvents, quartz sand, which should be a part of the total sand added to the soil, is mixed with the quantity of test substance to obtain the desired test concentration. This mixture of quartz sand and test substance is added to the pre-moistened soil and thoroughly mixed after adding an appropriate amount of deionised water to obtain the required moisture content. The final mixture is divided between the test vessels. The procedure is repeated for each test concentration and an appropriate control is also prepared.

Application of the test substance onto the soil surface

22. When the test substance is a pesticide, it may be appropriate to apply it onto the soil surface by spraying. The soil is treated after the collembolans are added. The test containers are first filled with the moistened soil substrate, and the animals added and then the test containers are weighted. In order to avoid any direct exposure of the animals with the test substance by direct contact, the test substance is applied at least half an hour after introducing the Collembola. The test substance should be applied to the surface of the soil as evenly as possible using a suitable laboratory-scale spraying device to simulate spray application in the field. The application should take place at a temperature within ± 2 °C of variation and for aqueous solutions, emulsions or dispersions at a water application rate according to the risk assessment recommendations. The rate should be verified using an appropriate calibration technique. Special formulations like granules or seed dressings could be applied in a manner consistent with agricultural use. Food is added after spraying.

PROCEDURE

Test conditions

23. The test mean temperature should be 20 ± 1 °C with a temperature range of 20 ± 2 °C. The test is carried out under controlled light-dark cycles (preferably 12 hours light and 12 hours dark) with illumination of 400 to 800 lux in the area of the test vessels.

24. In order to check the soil humidity, the vessels are weighed at the beginning, in the middle and at the end of the test. Weight loss > 2% is replenished by the addition of de-ionised water. It should be noted that loss of water can be reduced by maintaining a high air-humidity (> 80%) in the test incubator.

25. The pH should be measured at the beginning and the end of both the range-finding test and the definitive test. Measurements should be made in one extra control sample and one extra sample of the treated (all concentrations) soil samples prepared and maintained in the same way as the test cultures, but without addition of the collembolans.

Test procedure and measurements

26. For each test concentration, an amount of test soil corresponding to 30 g fresh weight is placed into the test vessel. Water controls, without the test substance, are also prepared. If a vehicle is used for application of the test substance, one control series containing the vehicle alone should be run in addition to the test series. The solvent or dispersant concentration should be the same as that used in the test vessels containing the test substance.

27. The individual springtails are carefully transferred into each test vessel (allocated randomly to the test vessels) and placed onto the surface of the soil. For efficient transfer of the animals, a low-suction air flow device can be used. The number of replicates for test concentrations and for controls depends on the test design used. The test vessels are positioned randomly in the test incubator and these positions are re-randomized weekly.

28. For the *F. fimetaria* test twenty adults, 10 males and 10 females, 23-26 days old should be used per test-vessel. On day 21 collembolans are extracted from the soil and counted. For *F. fimetaria* the gender are discriminated by size in the synchronised animal batch used for the test. Females are distinctively larger than the males (See Annex 3)

29. For the *F. candida* test, ten 9-12 days old juveniles per test vessel should be used. On day 28, the collembolans are extracted from the soil and counted.

30. As a suitable food source, a sufficient amount, e.g. 2-10 mg, of granulated dried baker's yeast, commercially available for household use, is added to each container at the beginning of the test and after about 2 weeks.

31. At the end of the test, mortality and reproduction are assessed. After 3 weeks (*F. fimetaria*) and 4 weeks (*F. candida*), collembolans are extracted from the test soil (see Annex 4) and counted (12). A collembolan is recorded as dead if not present in the extraction. The extraction and counting method should be validated. The validity includes extraction efficiency of juveniles greater than 95%, e.g. by adding a known number to soil.

32. Practical summary and timetable of the test procedure are described in Annex 2.

Test design

Range-finding test

33. When necessary, a range-finding test is conducted with, for example, five test substance concentrations of 0.1, 1.0, 10, 100, and 1000 mg/kg dry weight of soil and two replicates for each treatment and control. Additional information, from tests with similar compounds or from literature, on mortality or reproduction of Collembola may also be useful in deciding on the range of concentrations to be used in the range-finding test.

34. The duration of the range-finding test is two weeks for *F. fimetaria* and 3 weeks for *F. candida* to ensure one clutch of juveniles has been produced. At the end of the test, mortality and reproduction of the Collembola are assessed. The number of adults and the occurrence of juveniles should be recorded.

Definitive test

35. For determination of the EC_x (e.g. EC_{10}, EC_{50}), twelve concentrations should be tested. At least two replicates for each test concentration treatment and six control replicates are recommended. The spacing factor may vary depending on the dose-response pattern.

36. For determination of the NOEC/LOEC, at least five concentrations in a geometric series should be tested. Four replicates for each test concentration treatment plus eight controls are recommended. The concentrations should be spaced by a factor not exceeding 1.8.

37. A combined approach allows for determination of both the NOEC/LOEC and ECx. For this combined approach, eight treatment concentrations in a geometric series should be used. Four replicates for each treatment plus eight controls are recommended. The concentrations should be spaced by a factor not exceeding 1.8.

38. If no effects are observed at the highest concentration in the range-finding test (i.e. 1000 mg/kg), the reproduction test can be performed as a limit test, using a test concentration of 1000 mg/kg and the control. A limit test will provide the opportunity to demonstrate that there is no statistically significant effect at the limit concentration. Eight replicates should be used for both the treated soil and the control.

DATA AND REPORTING

Treatment of results

39. The reproductive output is the main endpoint (e.g. the number of juveniles produced per test vessel). The statistical analysis, e.g. ANOVA procedures, compares treatments by Student t-test, Dunnett's test, or Williams' test. 95% confidence intervals are calculated for individual treatment means.

40. The number of surviving adults in the untreated controls is a major validity criterion and should be documented. As in the range-finding test, all other harmful signs should be reported in the final report as well.

LC_x and EC_x

41. EC_x-values, including their associated lower and upper 95% confidence limits for the parameter, are calculated using appropriate statistical methods (e.g. logistic or Weibull function, trimmed Spearman-Karber method, or simple interpolation). An EC_x is obtained by inserting a value corresponding to x% of the control mean into the equation found. To compute the EC_{50} or any other EC_x, the complete data set should be subjected to regression analysis. LC_{50} is usually estimated by probit analysis or similar analysis that takes into account the binomially distributed mortality data.

NOEC/LOEC

42. If a statistical analysis is intended to determine the NOEC/LOEC, per-vessel statistics (individual vessels are considered replicates) are necessary. Appropriate statistical methods should be used according to OECD Document 54 on the Current Approaches in the Statistical Analysis of Ecotoxicity Data: a Guidance to Application (9). In general, adverse effects of the test substance compared to the control are investigated using one-tailed hypothesis testing at $p \leq 0.05$.

43. Normal distribution and variance homogeneity can be tested using an appropriate

statistical test, e.g. the Shapiro-Wilk test and Levene test, respectively ($p \leq 0.05$). One-way Analysis of Variance (ANOVA) and subsequent multi-comparison tests can be performed. Multiple comparisons (e.g. Dunnett's test) or step-down trend tests (e.g. Williams' test) can be used to calculate whether there are significant differences ($p \leq 0.05$) between the controls and the various test substance concentrations (selection of the recommended test according to OECD Document 54 (9)). Otherwise, non-parametric methods (e.g. Bonferroni-U-test according to Holm or Jonckheere-Terpstra trend test) could be used to determine the NOEC and the LOEC.

Limit test

44. If a limit test (comparison of control and one treatment only) has been performed and the prerequisites of parametric test procedures (normality, homogeneity) are fulfilled, metric responses can be evaluated by the Student test (t-test). The unequal-variance t-test (Welch t-test) or a non parametric test, such as the Mann-Whitney-U-test may be used, if these requirements are not fulfilled.

45. To determine significant differences between the controls (control and solvent control), the replicates of each control can be tested as described for the limit test. If these tests do not detect significant differences, all control and solvent control replicates may be pooled. Otherwise all treatments should be compared with the solvent control.

Test report

46. The test report should at least include the following information:

Test substance

- the identity of the test substance, batch, lot and CAS-number, purity;
- physico-chemical properties of the test substance (e.g. log Kow, water solubility, vapour pressure, Henry's constant (H) and preferably information on the fate of the test substance in soil) if available;
- the formulation of the test substance and the additives should be specified if not the pure chemical is tested;

Test organisms

- identification of species and supplier of the test organisms, description of the breeding conditions and age range of test organisms;

Test conditions

- description of the experimental design and procedure;
- preparation details for the test soil; detailed specification if natural soil is used (origin, history, particle size distribution, pH, organic matter content);
- water holding capacity of the soil;
- description of the technique used to apply the test substance to the soil;
- test conditions: light intensity, duration of light-dark cycles, temperature;
- a description of the feeding regime, the type and amount of food used in the test, feeding dates;
- pH and water content of the soil at the start and end of the test (control and each treatment);
- detailed description of the extraction method and extraction efficiency;

Test results

- the number of juveniles determined in each test vessel at the end of the test;
- number of adults and their mortality (%) in each test vessel at the end of the test;
- a description of obvious physiological or pathological symptoms or distinct changes in behaviour;
- the results obtained with the reference test substance;
- The NOEC/LOEC values, LC_x for mortality and EC_x for reproduction (mostly LC_{50}, LC_{10}, EC_{50}, and EC_{10}) together with 95% confidence intervals. A graph of the fitted model used for calculation, its function equation and its parameters (See (9));
- all information and observations helpful for the interpretation of the results;
- power of the actual test if hypothesis testing is done (9);
- deviations from procedures described in this Test Guideline and any unusual occurrences during the test;
- validity of the test;
- for NOEC, when estimated, the minimal detectable difference.

LITERATURE

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ANNEX 1

DEFINITIONS

The following definitions are applicable to this Guideline (in this test all effect concentrations are expressed as a mass of test substance per dry mass of the test soil):

NOEC (no observed effect concentration) is the test substance concentration at which no effect is observed. In this test, the concentration corresponding to the NOEC, has no statistically significant effect ($p < 0.05$) within a given exposure period when compared with the control.

LOEC (lowest observed effect concentration) is the lowest test substance concentration that has a statistically significant effect ($p < 0.05$) within a given exposure period when compared with the control.

ECx (Effect concentration for x% effect) is the concentration that causes an x% of an effect on test organisms within a given exposure period when compared with a control. For example, an EC_{50} is a concentration estimated to cause an effect on a test end point in 50% of an exposed population over a defined exposure period.

ANNEX 2

Main actions and timetable for performing a collembolan test

The steps of the test can be summarised as follows:

Time (day)	Action
-23 to -26	Preparation of synchronous *F. fimetaria* culture
-14	Prepare artificial soil (mixing of dry constituents)
	Check pH of artificial soil and adjust accordingly
	Measure max WHC of soil
-9 to -12	Preparation of synchronous *F. candida* culture
-2 to -7	Pre-moist soil
–1	Distribute juveniles into batches
	Prepare stock solutions and apply test substance if solvent required
0	Prepare stock solutions and apply test substance if solid compound, water soluble or surface application is required.
	Measure soil pH and weigh the containers.
	Add food. Introduce collembolans.
14	Range-finding test *F. fimetaria*: Terminate test, extract animals, measure soil pH and loss of water (weight)
	Definitive tests: Measure moisture content and replenish water and add 2-10 mg yeast
21	Definitive *F. fimetaria* test: Terminate test, extract animals, measure soil pH and loss of water (weight)
	Range-finding *F. candida*: Terminate test, extract animals, measure soil pH and loss of water (weight)
28	Definitive *F. candida* test: Terminate test, extract animals, measure soil pH and loss of water (weight)

ANNEX 3

GUIDANCE ON REARING AND SYNCHRONISATION OF *F. fimetaria* and *F. candida*

The time and durations given in this guidance should be checked for each specific collembolan strain to ensure that timing will allow for sufficient synchronized juveniles. Basically, the incidence of oviposition after the adults are transferred to fresh substrate and egg hatching determines the appropriate day for egg collection and collection of synchronous juveniles.

It is recommended to have a permanent stock culture consisting of e.g. 50 containers/Petri dishes. The stock culture should be kept in a good feeding condition by weekly feeding, watering and removal of old food and carcasses. Too few collembolans on the substrate may result in inhibition by more fungal growth. If the stock culture is used for egg production too often, the culture may get fatigue. Signs of fatigue are dead adults and mould on the substrate. The remaining eggs from the production of synchronous animals can be used to rejuvenate the culture.

In a synchronous culture of *F. fimetaria*, males are distinguished from females primarily by size. Males are clearly smaller than females, and the walking speed of the males is faster than for females. Correct selection of the gender requires little practice and can be confirmed by microscopic inspection of the genital area (13).

1. Rearing

1.a. *Preparation of culturing substrate*

The culturing substrate is plaster of Paris (calcium sulphate) with activated charcoal. This provides a moist substrate, with the function of the charcoal being to absorb waste gases and excreta (14) (15). Different forms of charcoal may be used to facilitate observations of the Collembola. For example, powdered charcoal is used for *F. candida* and *F. fimetaria* (producing a black/grey plaster of Paris):

Substrate constituents:

- 20 ml of activated charcoal
- 200 ml of distilled water
- 200 ml of plaster of Paris

or

- 50 g of activated pulverized charcoal
- 260-300 ml of distilled water
- 400 g plaster of Paris.

The substrate mixture is allowed to set before use.

1.b. *Breeding*

Collembolans are held in containers such as Petri dishes (90 mm x 13 mm), with the bottom covered by a 0.5 cm layer of plaster /charcoal substrate. They are cultured at 20 ± 1 °C at a light-dark cycle of 12-12 hours (400-800 Lux). Containers are kept moist at all times ensuring that the relative humidity of the air within the containers is 100%. This can be guaranteed by presence of free water within the porous plaster, but avoiding generating a water film on the plaster surface. Water loss can be prevented by providing a humid ambient air. Any dead individuals should be removed from the containers, as should any mouldy food. To stimulate production of eggs it is necessary to transfer the adult animals to Petri dishes with newly prepared plaster of Paris/charcoal substrate.

1.c. *Food source*

Granulated dried baker's yeast is used as the sole food supply for both *F. candida* and *F fimetaria*. Fresh food is provided once or twice a week, to avoid moulding. It is placed directly on the plaster of Paris in a small heap. The mass of baker's yeast added should be adjusted to the size of the collembolan population, but as a general rule 2-15 mg is sufficient.

2. Synchronisation.

The test should be performed with synchronized animals to obtain homogeneous test animals of the same instar and size. Furthermore, the synchronisation enables discrimination of *F. fimetaria* males and females from the age of 3 weeks and onwards based on sexual dimorphism, i.e. size differences. The procedure below is a suggestion on how to obtain synchronized animals (the practical steps are optional).

2.a. Synchronisation.

- Prepare containers with a 0.5 cm layer of plaster of Paris/charcoal substrate.
- For egg laying transfer 150-200 adult *F. fimetaria* and 50-100 *F. candida* from the best 15-20 containers of the stock culture with 4-8 weeks old substrate to the containers and feed them 15 mg baker's yeast. Avoid bringing juveniles together with adults as presence of juveniles may inhibit egg production.
- Keep the culture at 20 ± 1 °C (the mean should be 20 °C) and a light-dark cycle of 12-12 hours (400-800 Lux). Ensure that fresh food is available and the air is water saturated. Lack of food may lead the animals to defecate on the eggs resulting in fungal growth on the eggs or *F. candida* may cannibalize its own eggs.-- After 10 days the eggs are carefully collected with a needle and spatula and moved to “egg-paper” (small pieces of filter paper dipped in plaster of Paris/charcoal slurry) which is placed in a container with fresh plaster/charcoal substrate. A few grains of yeast are added to the substrate to attract the juveniles and make them leave the egg-paper. It is important that the egg-paper and substrate are humid, or the eggs will dehydrate. As an alternative, adult animals may be removed from the synchronization culture boxes after producing eggs for 2 or 3 days.
- After three days most of the eggs on the egg-paper will have hatched, and some juveniles may be found under the egg-paper.
- To have evenly aged juveniles, the egg-paper with un-hatched eggs is removed from the Petri dish with forceps. The juveniles, now 0-3 days, stay in the dish and are fed baker's yeast. Un-hatched eggs are discharged.
- Eggs and hatched juveniles are cultured in the same manner as the adults. In particular for *F. fimetaria* the following measures should be taken: ensuring sufficient fresh food, old moulding food is removed, after 1 week the juveniles are divided into new Petri dishes provided that the density is above 200.

2.b. Handling collembolans at test initiation

- 9-12 days old *F. candida* or the 23-26 days old *F. fimetaria* are collected, e.g. by suction, and released into a small container with moist plaster/charcoal substrate and their physical condition is checked under the binocular (injured and damaged animals are disposed). All steps should be done while keeping the collembolans in a moist atmosphere to avoid drought stress, e.g. by using wetted surfaces etc.
- Turn the container up-side down and knock on it to transfer the collembolans to the soil. Static electricity should be neutralized, otherwise the animals may just fly into the air, or stick to the side of the test container and dry out. An ionizer or a moist cloth below the container may be used for neutralisation.
- The food should be spread all over the soil surface and not just in one lump.
- During transportation and during the testing period it should be avoided to knock or otherwise physically disturb the test containers, as this may increase the compaction of the soil, and hamper the interaction between the collembolans.

3. Alternative Collembolan species

Other collembolan species may be selected for testing according to this guideline such as *Proisotoma minuta*, *Isotoma viridis*, *Isotoma anglicana*, *Orchesella cincta*, *Sinella curviseta*, *Paronychiurus kimi*, *Orthonychiurus folsomi*, *Mesaphorura macrochaeta*. A number of prerequisites should be fulfilled in advance before using alternative species:

- They should be unequivocally identified;
- The rationale for the selection of the species should be given;
- It should be ensured that the reproductive biology is included in the testing phase so it will be a potential target during the exposure;
- The life-history should be known: age at maturation, duration of egg development, and instars subject to exposure;
- Optimal conditions for growth and reproduction should be provided by the test substrate and food supply;
- Variability should be sufficiently low for precise and accurate toxicity estimation.

ANNEX 4

Extraction and counting of animals

1. Two methods of extraction can be performed.

1.a. First method: A controlled temperature gradient extractor based on principles by MacFayden can be used (1). The heat coming from a heating element at the top of the extraction box (regulated through a thermistor placed on the surface of the soil sample). The temperature in the cooled liquid surrounding the collecting vessel is regulated through a thermistor situated at the surface of the collection box (placed below the soil core). The thermistors are connected to a programmable controlling unit which raises the temperature according to a pre-programmed schedule. Animals are collected in the cooled collecting box (2 °C) with a bottom layer of plaster of Paris/charcoal. Extraction is started at 25 °C and the temperature is increased automatically every 12 h by 5 °C and has a total duration of 48 hours. After 12 h at 40 °C the extraction is finished.

1.b. Second method: After the experimental incubation period the number of juvenile Collembola present is assessed by flotation. For that purpose the test is performed in the vessels of approximately 250 ml volume. At the end of the test approx. 200 ml of distilled water are added. The soil is gently agitated with a fine paintbrush to allow Collembola to float to the water surface. A small amount, approx. 0.5 ml, of black Kentmere photographic dye may be added to the water to aid counting by increasing the contrast between the water and the white Collembola. The dye is not toxic to Collembola.

2. Counting: Counts of numbers may be carried out by eye or under a light microscope using a grid placed over the floatation vessel or by photographing the surface of each vessel and later counting the Collembola on enlarged prints or projected slides. Counts may also be performed using digital image processing techniques (12). All techniques should be validated.

ANNEX 5

DETERMINATION OF THE MAXIMUM WHC OF THE SOIL

The following method for determining the maximum water holding capacity (WHC) of the soil has been found to be appropriate. It is described in Annex C of the ISO DIS 11268-2 (Soil Quality - Effects of pollutants on earthworms (*Eisenia fetida*). Part 2: Determination of effects on reproduction).

Collect a defined quantity (e.g. 5 g) of the test soil substrate using a suitable sampling device (auger tube etc.). Cover the bottom of the tube with a wet piece of filter paper and then place it on a rack in a water bath. The tube should be gradually submerged until the water level is above to the top of the soil. It should then be left in the water for about three hours. Since not all water absorbed by the soil capillaries can be retained, the soil sample should be allowed to drain for a period of two hours by placing the tube onto a bed of very wet finely ground quartz sand contained within a covered vessel (to prevent drying). The sample should then be weighed, dried to constant mass at 105 °C. The water holding capacity (WHC) should be calculated as follows:

$$\text{WHC (in \% of dry mass)} = \frac{S - T - D}{D} \times 100$$

Where:

S = water-saturated substrate + mass of tube + mass of filter paper

T = tare (mass of tube + mass of filter paper)

D = dry mass of substrate

ANNEX 6

DETERMINATION OF SOIL pH

The following method for determining the pH of a soil is based on the description given in ISO DIS 10390: Soil Quality – Determination of pH.

A defined quantity of soil is dried at room temperature for at least 12 h. A suspension of the soil (containing at least 5 grams of soil) is then made up in five times its volume of either a 1 M solution of analytical grade potassium chloride (KCl) or a 0.01 M solution of analytical grade calcium chloride ($CaCl_2$). The suspension is then shaken thoroughly for five minutes and then left to settle for at least 2 hours but not for longer than 24 hours. The pH of the liquid phase is then measured using a pH-meter that has been calibrated before each measurement using an appropriate series of buffer solutions (e.g. pH 4.0 and 7.0).