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Background report on pre-validation of an OECD springtail test guideline

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Indhold

Summary and conclusions

The aim of the present project was to develop a draft proposal for an OECD test guideline for long-term toxicity-testing of Collembola (see Annex A). The draft guideline was based on two existing standardised test methods, one for ***Folsomia fimetaria*** (Wiles & Krogh, 1998) and one for ***Folsomia candida*** (ISO, 1999), which were combined and updated.

The test on ***F. candida*** employs an asexually reproducing species typically living in the organic matter of compost heaps or pot-plants, whereas the test on ***F. fimetaria*** employs a sexually reproducing species living in garden and agricultural soils. Accordingly for the test on ***F. fimetaria*** the effect on sexual reproduction is obtained. This latter may be seen as a more ecologically relevant endpoint than effects on asexual reproduction, as sexual reproduction is the most frequent reproductive strategy of soil dwelling organisms. In addition, effects on the sexual reproduction of ***F. fimetaria*** would include effects caused by chemicals that act via a disturbance of the sex-determination system in arthropods. In the present project the possibility of including a simple test for effects on the sex-determination was investigated, but this was not successful. Given distinct life strategies (and sizes) initial studies were performed investigating possible general sensitivity differences between the two species. The results indicate a difference in chemical sensitivity both according to species and to life-stage, but the differences could only partly be explained by size-differences.

To prepare for a ring-test of the draft test guideline three compounds were tested fipronil, 3,5-dichlorophenol and boric acid. Future test concentration ranges were identified for these compounds.

Sammendrag og konklusioner

Formålet med projektet var at udvikle et udkast til en OECD test-guideline for langtids toksicitetstest af springhaler (se Annex A). Udkastet til test-guidelinen blev baseret på to eksisterende testmetoder, en for ***Folsomia fimetaria*** (Wiles & Krogh, 1997) og en for ***Folsomia candida*** (ISO 1999), hvilke blev kombineret og opdateret.

Testen med ***F. candida*** baseres på en aseksuel reproducerende art, der typisk lever i organisk materiale som kompost eller potteplante muld, hvorimod testen med ***F. fimetaria*** benytter en seksuelt reproducerende art, der lever i have- og landbrugsjorde. Deraf følger, at testen med ***F. fimetaria*** vil medtage effekter på den seksuelle reproduktion, hvilket synes at være et mere økologisk relevant mål, idet seksuel reproduktion er den mest forekommende strategi for jordlevende organismer. Ydermere vil effekter på ***F. fimetarias*** seksuelle reproduktion inkludere effekter forårsaget af kemikalier, der virker via en forstyrrelse af kønsbestemmelses-systemet hos leddyr. I det nuværende projekt blev det undersøgt, hvorvidt det var muligt at inkludere en simpel test for effekter på kønsbestemmelsen, men dette lykkedes ikke. Givet de to forskellige livsstrategier (og størrelser) for de to arter blev det undersøgt, om der var en generel forskel i følsomheden imellem arterne. Resultaterne indikerede en forskel i kemikaliefølsomheden som følge af arten og som følge af livsstadiet, men forskellen kunne delvist forklares ved hjælp af størrelsesforskellen.

For at forberede en ring-test til test-guidelineudkastet blev tre kemiske stoffer testet fipronil, 3,5-dichlorophenol og borsyre. Testkoncentrations områder til brug i ringtesten blev identificeret for disse stoffer.

1 Introduction

This report provides a background report for a draft OECD test guideline for long-term toxicity to Collembola, commonly known as springtails (see Annex A). This report aim at providing a background for the draft guideline and to discuss relevant questions related to this guideline.

Collembolans are the most numerous and widely occurring insects in terrestrial ecosystems. This is the one of the main reasons for why they have been widely used as bioindicators and test organisms for detecting the effects of environmental pollutants and different agricultural (especially pesticide) regimes on biodiversity in soils (e.g. Paoletti ***et al.*** 1991; Crossley ***et al.*** 1992; Filser 1995; Filser ***et al.*** 1995; Frampton 1997; Paoletti and Bressan 1996). A variety of species have been employed in terrestrial ecotoxicological test. For the present draft OECD guideline ***Folsomia candida*** and ***Folsomia fimetaria*** have been select as previous standard tests are available (for further discussion on this see "results and discussion").

A springtail test employing the species ***Folsomia candida*** (ISO, 1999[3]) is already in use for pesticide evaluation within Europe and USA. It is used where Environmental Agencies require an evaluation of the soil quality, for example when evaluating the potential problems related to spreading sewage-sludge or plant protection products on agricultural land. The test is well suited as a standard-test as the test organisms are easily cultured, the test easily accomplished and the demand for equipment is minimal. The springtail is representative for arthropods that is characterised by an aerobiontic cuticle compared to the soft-bodied semi-aquatic organisms viz. the earthworm test. On these grounds the springtail test is recommended for development in the OECD TG in OECD Monograph no. 11 (Detailed review Paper on Aquatic Testing methods for Pesticides and Industrial Chemicals (Paris 1998)[1]). The method is also recommended by SETAC[2] and in the EU Guidance Document for risk assessment for the soil environment for pesticides[3], biocides and industrial chemicals[4].

An alternative to the ***F. candida*** test is a standard test with the near relative ***Folsomia fimetaria*** (Wiles & Krogh, 1998). The two species differ in their habitat choice, ***F. candida*** resides primarily in habitats rich in organic matter such as pot-plants and compost heaps, whereas ***F. fimetaria*** is common in agricultural soils. This make the toxicity effects estimated based on ***F.***

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[1] Danish publication (sponsored by DG ENV, Danish EPA 14th Office and the Danish EPA Pesticide Research Programme)

[2] Cf. "Test Methods to determine hazards of Sparing Soluble Metal Compounds in Soils" 2002

[3] Cf. EU Guidance Document on terrestrial Ecotoxicology Under Council Directive 91/414/EEC, SANCO/10329/2002 rev.2 final

[4] jfr. EU´s rev. TGD: "Technical Guidance Document on Risk Assessment in support of Commission Directive 93/67/EEC on Risk Assessment for new notified substances and Commission Regulation (EC) No. 1488/94 on Risk Assessment for Existing Substances and Directive 98/8/EC of the European Parliament and of the Council Concerning the placing of Biocidal Products on the Market" (May 2002)

fimetaria more applicable to agricultural land and nature areas. Further, ***Folsomia fimetaria*** has a sexual mode of reproduction, which is in contrast to ***F. candida*** consist of females only with a parthenogenetic (asexual) reproductive mode. It is expected that when utilising a sexually reproducing species the results will have a broader application, since the majority of species are assumed to have sexual reproduction.

The two species can appear alike from a visual point-of-view, but they differ in size with ***F. candida*** being the larger. It is unknown whether this difference is important for their general sensitivity toward chemicals. The literature is inconclusive on whether the two species differ in sensitivity. For example, Krogh (1995) exposed the two species to dimethoate under identical conditions and observed significant ($P<0.05$) differences in the EC_{10} values with regard to reproduction. On the other hand, no significant differences were observed for the copper EC_{50} values for the two species when exposed under identical conditions (Pedersen et al 1999). Form a pure theoretical point-of-view it could be argued that the two species are likely to differ in sensitivity as the surface-to-volume ratio [this is probably important for toxicity of chemicals that have a passive uptake] is related to the size.

As ***F. fimetaria*** have a sexual mode of reproduction it may in theory be possible to identify chemicals with sex-determination effects. There are several ways in which to identify a compound that can influence the sex-determination, these includes biochemical assays, morphological tests and sex-ratio tests (deFur et al 1999). Since, the mode-of-action is not known a test of sex-ratio of the juvenile outcome seem the most appropriate test. In addition, a determination of the sex-ratio of the offspring, if successful, could be a simple addition to the overall test design of the draft guideline.

There is at present no clear evidence for which compounds that primarily influence sex-determination of invertebrates. The vertebrate sex hormones have been suggested, but this is uncertain. Testosterone has been reported to have some androgenlike activity in some crustaceans (LeBlanc 1999) and endogenous concentrations of testosterone and other vertebrate-type androgens have been reported in ***Daphnia magna*** and ***Neomysis integer*** (LeBlanc and MacLachlan 2000, Verslycke et al 2002, 2004). Estradiol has been recorded in the silk moth ***Bombyx mori*** (Keshan and Ray 2001). However, neither estradiol nor testosterone affected the sex-ratio in exposed ***Daphnia magna*** (Kashian and Dodson 2004). Nevertheless, two chemicals, methyl-testosterone and ethynyl-estradiol, have been suggested as reference chemicals for evaluating potential sex-endocrine-disrupting compounds in invertebrates by the participants of "Workshop on Endocrine Disruption in Invertebrates: Endocrinology, Testing, and Assessment (EDIETA)", held in The Netherlands in 1997 (Ingersoll et al 1999).

1.1 Aims

To support the draft guideline two main activities were pursued.

1. As the two species differ with respect to ecology, reproduction biology and physiology (size). The importance of this in relation to the guideline will be reviewed and shortly discussed. These issues will partly be pursued via literature review and through laboratory test, with emphasis on possible chemical sensitivity differences due to age/size differences between the two species.

2. In connection with the test-guideline and a future ring-test it will be necessary to have positive control compounds to ensure the stability of the test-system. Hence, it is an aim to identify the future test concentration ranges for potential positive controls. The selection of the three compounds will be based on previous ring-test programmes.

2 Materials and methods

According to the aims the studies were divided in two parts. The first part deals with studies on the importance of ecological, biological and physiological for choice of species. These issues will be dealt with though specific aspects (see 2.1). Second, to identify test-concentration ranges for future positive control compounds three compounds will be tested based on previous ring-test programmes and test guidelines (see 2.2).

2.1 Background - species identification and comparison

2.1.1 Literature search - Ecology and biology of species

Based on a literature search and experience a short description of the ecology and biology was performed. Emphasis was put on background for choice of the species included in the guideline (see 3.1).

2.2 Sensitivity comparison

The two selected species differ in various aspects and may hence in theory differ in sensitivity to chemical toxicity. This issue was studied via two approaches.

2.2.1 General sensitivity comparison

To study the general chemical sensitivity of the species a literature survey was performed. In this review two compounds were selected for further analysis. The first compound was an choline-esterase inhibiting pesticide, dimethoate. For this compound there was already considerable knowledge available. The second compound was a heavy metal, copper, with a more broad mode-of-action. For this group relevant information is also available in the published literature. Hence, in the present report a comparison was made between the two species, ***F. fimetaria*** and ***F. candida,*** with regard to the toxicity of Copper and Dimethoate. The data has been obtained via searches in international databases such as WOS, BIOSIS, SCIC and ECOTOX. Only original literature has been cited (see 3.2.1).

2.2.2 Laboratory experiments - Comparison of age/size and toxicity

One of the notable differences between the two species is the size difference. In theory the two species may differ in sensitivity to chemicals due to such a size difference, i.e. the surface area compared to volume is larger for smaller animals (***F. fimetaria***), which will influence uptake chemicals taken up via outer cuticle.

In accordance, an experiment was set up to study whether the toxicity differed between the two species and to see if this could be attributed to size differences. The experiment was based on exposing a range of age-classes (hence also size classes) to one concentration of a chemical and observing differences in mortality between the age-classes (see Table 1). Due to the

limited time-span of the project it was decided to use a compound, dimethoate, already tested for both species and to use the LC_{50} for ***Folsomia fimetaria*** reported by Krogh (1995) (see 3.2.2).

2.2.2.1 Test compound

1. Dimethoate (purity: 400 g L^{-1} Cheminova Agro A/S) (CAS no. 60515)

Table 1. Age of animals in size classes employed for both *Folsomia fimetaria* and *Folsomia candida*.

Size class	1	2	3	4	5	6	7	8	9
Age/ days	0-2	3-5	6-8	9-11	12-14	15-17	18-20	21-23	24-26

Hence, the mortality after exposure for one week to dimethoate 2 mg kg^{-1} and control was tested for 9 size/age-classes of ***Folsomia fimetaria*** and ***Folsomia candida*** in 5% OECD soil. They were fed 15 mg Baker's yeast in the beginning of the experiment. Ten individuals were added to each replicate container and extracted after 1 week. There were 2 replicates per species and size-class and treatment.

2.2.2.2 Test soil, counting and statistics

See section 2.2.1.3-2.2.1-5

2.2.3 Sex-ratio effects on offspring

Experiments were conducted to see if it was possible to include offspring sex-ratio as an effect parameter in the draft guideline. To study this adult ***Folsomia fimetaria*** were exposed for 42 days to soil contaminated with two potential sex-ratio disrupting compounds, ethynyl-estradiol and methyl-testosterone. The experiment was continued for a total of 42 days to allow the first clutch of the juveniles to become adult (Table 2). Allowing the first clutch of the juveniles to become adult made it potentially possible to identify the sexes on the offspring. The maximum concentrations used were 1000 mg a.s. kg^{-1} soil corresponding to the limit-testing for plant protection products (see 3.2.3).

Since the compounds may bind to the soil particles and hence not be available to the organism a water- exposure test was conducted to ensure exposure. Hence, adult Collembola were exposed for 48 hours to 12 mg testosterone L^{-1} or 2 mg estradiol L^{-1} after which the adults were allowed to produce eggs over a period of 7 days. The hatched juveniles were allowed to growth for 23 days prior to size measuring.

Table 2. Test conditions of the sex-determination toxicity tests with *Folsomia candida*.

Test species	*Folsomia fimetaria*
Life stage	23-26 days (synchronised)
Exposure duration	42 days
Test volume	30 g wet weight
Temperature	20 °C
Light cycle	16:8 light/dark
Feeding	every 14 days
Initial organisms per replicates	20

2.2.3.1 Test compounds

1. Methyl-testosterone (purity: 97%, Sigma Aldrich) (CAS no. 58-18-4)
2. 17β-Ethynyl-testosterone (purity: 85%, Sigma Aldrich) (CAS no. 57-63-6)

2.2.3.2 Test soil, counting and statistics

See section 2.2.1.3-2.2.1-5

2.3 Identifying test concentration ranges for ring-testing

Experiments were performed in order to identify the test concentrations used in a future ring-test programme and to identify test concentrations used for a positive control. The experiments included three compounds fipronil, 3,5-dichlorophenol and boric acid. The experiments were performed according to conditions stated in table 3.

2.3.1 Test conditions

2.3.1.1 Test organisms

The Collembolans ***Folsomia fimetaria*** and ***Folsomia candida*** (Collembola, Isotomidae) were used as test organisms. For fipronil and 3,5-dichlorophenol tests were performed with ***F. fimetaria*** and for boric acid tests were performed with both species.
Cultures of these species were kept on moist substrate of plaster of Paris and pulverised activated charcoal (as described in the guideline). Granulated yeast was added weekly as a food source. The cultures were maintained at the Department of Terrestrial Ecology, National Environmental Research Institute.

Table 3: Test conditions of the range determination toxicity tests with *Folsomia candida* and *Folsomia fimetaria*.

Test species	*Folsomia candida*	*Folsomia fimetaria*
Life stage	10-12 days (synchronised)	23-26 days (synchronised)
Exposure duration	14/21 days	14/21 days
Test volume	30 g wet weight	30 g wet weight
Temperature	20 °C	20 °C
Light cycle	16:8 light/dark	16:8 light/dark
Feeding	every 14 days	every 14 days
Initial organisms per replicates	10	20

2.3.1.2 Test compounds

All compound used were of analytic grade and obtained from Sigma-Aldrich (Germany) or Cheminova.

1. Boric Acid (purity: 100%, Sigma Aldrich) (CAS no. 10043-35-3)
2. Fipronil (purity: 97%, Sigma Aldrich) (CAS no. 1200068-37-3)
3. 3,5-Dichlorophenol (DCP) (purity: 97%, Sigma Aldrich) (CAS no. 591-35-5)

2.3.1.3 Test soil

The test soil was an artificial mixed soil according to OECD guideline. Compared to the "standard" OECD soil, which has a 10% organic matter content, it was chosen in the present test to use a 5% organic matter soil. This latter choice was made to mimic field condition and reduce binding of test chemicals. Soil composition: Organic matter - 5%; clay - 21% and sand - 74% [the clay and sand content has been changed, compared to the original OECD soil, to keep the relative composition the same].

2.3.1.4 Counting

At the end of each experiment the animals were heat-extracted and counted with a computerised system employing a digital image processing (DIP) approach (Krogh et al. 1998). The guideline alternatively suggests to extract the animals by flotation and to manually count the organisms floating on the surface. It was chosen in this context to use the heat extraction and the automated counting since this is considered more precise than the flotation and manually counting.

2.3.1.5 Statistics

the data were checked for normality using a χ^2 test, and for homogeneity of variance by Barlett's test. Effect concentrations, EC_{10} and EC_{50}, and confidence intervals were estimated by fitting a logistic model to the data (Lacey and Mallet 1991). The formulae were reparametrized by incorporation of the EC_{10} and EC_{50} into the equation (Krogh 1995). The estimates with a 95% confidence interval were performed with the SAS procedure PROC NLIN (Enterprise Guide 1.3)

3 Results and Discussion

3.1 Background

3.1.1 Ecology and Biology of Species

The following summary is based on the chapter written by Wiles and Krogh (1998). The Collembola, commonly known as springtails, are the most numerous and widely occurring insects in terrestrial ecosystems. This is one of the main reasons why they have been widely used as bioindicators for detecting the effects of environmental pollutants and different agricultural (especially pesticide) regimes on biodiversity in soils (e.g. Paoletti ***et al.*** 1991; Crossley ***et al.*** 1992; Filser 1995; Filser ***et al.*** 1995; Frampton 1997; Paoletti and Bressan 1996, Fountain and Hopkin 2004 a, b). A wealth of literature exists concerning the systematics, evolution, physiology, behaviour and ecology of these organisms (see Hale 1967; Wallwork 1970; Fjellberg 1985; Eisenbeis and Wichard 1987; Greenslade 1991; Christiansen 1990; Curry 1994 and Hopkin 1997).

Population densities of Collembola commonly reach 10^5 m^{-2} (e.g. Burges and Raw 1967; Peterson and Luxton 1982) in soil and leaf litter layers in many terrestrial ecosystems. Because of their small size however, adults typically measure 0.5 - 5 mm long, their contribution to total soil animal biomass and respiration is low, estimated at being between 1 and 5% (see Petersen 1994). 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 and carabid and rove beetles. The role of Collembola in humus formation is not well known, but it is commonly accepted that they may contribute significantly to the decomposition processes on acidic stands, where earthworms and diplopods are absent.

3.1.2 Ecotoxicology

Collembola possess many attributes of the ideal experimental organism for ecotoxicological research. They are ecologically important, widely distributed, often highly abundant, easily sampled in the field, can be cultured or maintained in the laboratory and have a relatively rapid life-cycle with a high reproductive rate (Spahr 1981). They lend themselves to studies over a wide range of spatio-temporal scales including field monitoring of population trends (e.g. Filser 1995), field bioassays (e.g. Wiles and Frampton 1996), meso- and microcosm studies (e.g. Parmelee ***et al.*** 1993) and a wide variety of laboratory investigations (e.g. Crommentuijn ***et al.*** 1993; Krogh 1995, Scott-Fordsmand et al 2000; van Gestel and Koolhaas 2004, da Luz et al 2004). They have been used in studies to investigate the environmental effects of a wide range of environmental contaminants.

One of the earliest laboratory studies was undertaken by Sheals (1956) who studied effects of organochlorine compounds on microarthropod communities and used supplementary tests with filter paper to screen for species

differences in susceptibility to DDT. In a later study Scopes and Lichtenstein (1967) used 6 week old ***F. fimetaria*** in an acute test, also using exposure through filter paper. Their results showed that the LD_{50} for adults was greater than twice the LD_{50} for juveniles, a fact that has since been used to explain interspecific differences between collembolan species of different sizes. In 1972 Thompson and Gore described an acute test on filter paper and in soil for a sexual population of ***F. candida***. They were one of the first authors to promote the use of ***F. candida*** as a test species for laboratory bioassays of pesticides. A large number of laboratory studies with Collembola followed in the 1970's, 80's and early 90's. In these studies a range of ten to fifteen different species of Collembola were used, of which four species have been by far the most common; ***Folsomia candida***, ***Folsomia fimetaria***, ***Onychiurus armatus*** (***Protaphorura armata***), ***Orchesella cincta***. Some of the first standard acute toxicity tests with Collembola were suggested by Huang (1992) and Kiss and Bakonyi (1992) and more recently by Houx ***et al.*** (1996).

The first initiative to develop a standard sub-lethal test was taken at the Institute for Chemical Examination of the German Federal Biological Research Centre for Agriculture and Forestry. A description of the background for the development of the resulting draft test method of sub-lethal testing with ***F. candida*** has been given by Riepert and Kula (1996). The development of a test with ***F. fimetaria*** was started in Denmark in 1990 in a project on side-effects of pesticides funded by the Danish Ministry of the Environment (Løkke 1995). The choice of ***F. fimetaria*** was based on its wide distribution in Danish agricultural soil and the ease with which it can be cultured in the laboratory (Petersen and Gjelstrup 1995). The procedures for culturing and conducting tests with ***F. fimetaria*** were described in Krogh and Pedersen (1995) and Krogh (1995). Based on the above consideration these two species, ***F. fimetaria*** and ***F. candida***, were chosen for the draft OECD guideline.

3.1.3 Species inclusion in draft guideline

The draft OECD guideline proposal includes a choice between two species (see Annex A). The most prominent reason for choosing a particular species for the toxicity test is the reason of relevance to the compartment or life-characteristic which this toxicity study is supposed to represent, e.g. use a species present in agricultural soils when the test is used for agricultural risk assessment.

F. fimetaria is widely distributed and common in several soil types ranging from sandy to loamy soils and from mull to mor soils. It has been recorded in agricultural soils in Denmark (Christensen ***et al.*** 1987; Krogh 1994; Petersen and Gjelstrup 1995), Sweden (Andrén and Lagerlöf 1983), Finland (Huldén 1984), and Germany (Heimann-Detlefsen ***et al.*** 1994). 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 (Ulber 1983) and may influence mychorrhiza, as is known to be the case for ***F. candida***.
Folsomia fimetaria has a sexual mode of reproduction, whereas ***F. candida*** consists of females only with a parthenogenetic (asexual) reproductive mode. It is expected that when utilising a sexually reproducing species the results will have a broader application, since the majority of species are assumed to have sexual reproduction.

F. candida is widely distributed throughout Europe. Although it is not common in most natural soils, it often occurs in very high numbers in humus rich sites. It is a blind, unpigmented collembolan which reproduces parthenogenetically. It has a well developed furca (spring organ) and an active running movement and springs readily if disturbed.

3.2 Sensitivity of species

3.2.1 Ecotoxicology - General sensitivity differences between the two species

When deciding which of the two species have to be tested a relevant question is whether one of the two species proposed in the guideline can be assumed generally more sensitive than the other species. There has hitherto been few studies dealing with differences in sensitivity between ***F. fimetaria*** and ***F. candida***, and it is unknown whether this difference is important for their general sensitivity toward chemicals. To study this a literature was performed and an array of experiments performed (see also 3.2.2).

With regard to the literature study two compounds were selected, i.e. the specific acting dimethoate (acetylcholinesterase inhibitor) and the toxicologically more broad acting metal Copper. Based on the literature data for the two compounds no differences in sensitivity between the two species could be detected (see Table 4).

For dimethoate the mean EC_{50} for ***F. fimetaria*** was 1.8 [±1.1] dimethoate kg^{-1} and for ***F. candida*** it was 4.0 [±2.6] dimethoate kg^{-1}, but a t-test showed no significant difference ($P>0.05$). As observed the means were more than 2-fold different, although not significant, which indicate a possible trend in ***F. fimetaria*** being more sensitive. This is in line with the observation by Krogh (1995) who exposed the two species under identical conditions and observed significant ($P<0.05$) differences in the EC_{10} values with regard to reproduction. They observed that for ***F. fimetaria*** the EC_{50} (reproduction) was 0.3 [0.14-0.47, 95% C.I.] mg kg^{-1} soil and for ***F. candida*** the EC_{50} (reproduction) was 0.5 [0.43-0.57, 95% C.I.] mg kg^{-1} soil. Significant differences were also observed on the mortality level, with ***F. fimetaria*** being the most sensitive. However, curiously the current laboratory data (see Fig 1) indicate that ***F. candida*** is more sensitive than ***F. fimetaria*** on a mortality level. Studying the pooled literature data (Table 3) in detail reveals that much of the variation between the different reporting can probably be explained by differences in experimental conditions, such as differences in the soil organic matter content. Differences in the exposure conditions, such as variations in soil type, may render the "bioavailable" fraction different under such conditions. To eliminate differences due to exposure condition (especially soil type) the soil-water concentration could be calculated for K_{OW} (Martikainen and Krogh 1999). Calculating the soil-water concentration by the equation reported by Martikainen and Krogh (1995) it is observed that the ECx values are relatively closer for the two species, when expressed on mg dimethoate L^{-1} soil water (see Table 4) than when based on soil concentrations.

Table 4. The EC_{50} values (reproduction) for Copper and dimethoate exposure of *Folsomia fimetaria* and *Folsomia candida* based on studies reported in the literature and recalculated to soil water concentrations after Martikainen and Krogh 1999).

REFERENCE	SPECIES	COMPOUND	EC_{50}-soil (mg kg^{-1})	Average (stdev)	EC_{50}-soil-water (mg L^{-1})	Average (stdev)
Sandifer and Hopkin (1996)	Folsomia candida	Copper	700			
Sandifer and Hopkin (1996)	Folsomia candida	Copper	710			
Sandifer and Hopkin (1996)	Folsomia candida	Copper	1480			
Sandifer and Hopkin (1997)	Folsomia candida	Copper	700			
Sandifer and Hopkin (1997)	Folsomia candida	Copper	640			
Pedersen et al (2000)	Folsomia candida	Copper	657			
Herbert et al (2004)	Folsomia candida	Copper	813			
Scott-Fordsmand et al (1997)	Folsomia fimetaria	Copper	129			
Pedersen and van Gestel (2001)	Folsomia fimetaria	Copper	1244			
Pedersen et al (2000)	Folsomia fimetaria	Copper	519			
Scott-Fordsmand et al (2000)	Folsomia fimetaria	Copper	994			
Martikainen (1996)	Folsomia candida	Dimethoate	6.3		7,5	
Martikainen (1996)	Folsomia candida	Dimethoate	5.5		8,6	
Martikainen (1996)	Folsomia candida	Dimethoate	3.8		4,4	
Krogh (1995)	Folsomia candida	Dimethoate	0.5	4.0(±2.6)	2,4	5.7(±2.8)
Martikainen and Krogh (1999)	Folsomia fimetaria	Dimethoate	0.8		3,7	
Martikainen and Krogh (1999)	Folsomia fimetaria	Dimethoate	1.7		4,8	
Martikainen and Krogh (1999)	Folsomia fimetaria	Dimethoate	3.2		5,2	
Martikainen and Krogh (1999)	Folsomia fimetaria	Dimethoate	2.5		4,6	
Martikainen and Krogh (1999)	Folsomia fimetaria	Dimethoate	2		6,0	
Krogh (1995)	Folsomia fimetaria	Dimethoate	0.3	1.8(±1.1)	1,4	4.2(±1.5)

For Cu the t-test comparison between the two species also exhibited no difference between the means with regard to reproduction ($P>0.05$). The

mean for ***F. fimetaria*** was 722 [±496] Cu kg^{-1} and ***F. candida*** was 814 [±299] Cu kg^{-1}. This is in line with observations by Pedersen et al (1999) who observed no differences in the copper EC_{50} values for the two species when exposed under identical conditions. As for dimethoate soil type models could be considered resolving the differences between the soil types, for example the biological ligand-model (BLM). The latter conversion was not performed as the models are presently under development and analysis of the experiments showed that for example temperature and pH may be as important for toxicity as soil type (Sandifer and Hopkin 1996, 1997).

In conclusion no difference in toxicity between the two species could be identified, but a possible difference in toxicity may be blurred by confounding factors.

3.2.2 Age- and size-toxicity relationships using dimethoate

Folsomia fimetaria and ***F. candida*** can appear quite alike from a visual point-of-view, but they differ in size with ***F. candida*** being the larger. From a pure theoretical point-of-view it could be argued that the two species are likely to differ in sensitivity as the surface-to-volume ratio is related to the size. Such sensitivity differences could be very relevant when choosing which species to test.

Based on an experimental study (see 2.1.2.2) growth of ***F. candida*** was faster than ***F. fimetaria*** as would be expected from previous knowledge of this species (data not shown). The toxicity decreased with age with juveniles being most sensitive and it decreased faster for juvenile ***F. fimetaria*** than for ***F. candida*** (Fig 1)**.** Furthermore, there is a clear indication of increased sensitivity of the reproductive instars about age 15 and 22 days (every second instar is reproductive and occurs approx. weekly). Thus, juvenile ***F. fimetaria*** was the least sensitive to dimethoate, which is different from a previous studying showing adult ***F. fimetaria*** to be the most sensitivity (Krogh 1995). Hence, it seems that the sensitivity is life-stage dependent as confirmed on a refined level in this study (Fig 1). In fact it is seen that both species have very contrasting sensitivities in the days around the age for which they are selected in the draft OECD test. For example, in the ***F. candida*** test the start age should be 10-12 days, but if the organisms tend to rather be 9-11 days than 12-14 days the may change the sensitivity two fold (see Fig 1). It is important to realise such small differences may not only be due to a selection based on number of days (age) for the organisms, but a slight increase or drop in temperature may also cause the organisms to be in a different life-stage at the start of the test. In fact it is seen that both species have very contrasting sensitivities around the age for with they are selected for the OECD test. For example, in the F. candida test the start age should be 10-12 days, but if the organisms tend to be 9-11 days rather than 12-14 days this may increase the sensitivity two fold (see Fig 1). It is important to realise such small differences may not only be due to a selection based on number of days (age) for the organisms, but a slight increase or drop in temperature may also cause the organisms to be in a different life-stage.

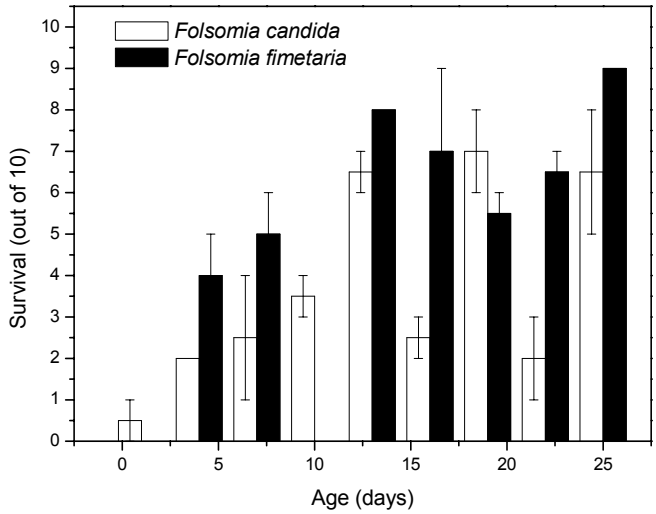

Fig 1. Survival (out of 10, mean ± standard error) of nine age-/size-classes for *Folsomia candida* and *Folsomia fimetaria* exposed for 7 days to dimethoate, 2 mg kg^{-1} in 5% OECD soil.

In conclusion, difference in toxicity between the species could be detected, and a pronounce life-stage toxicity dependency was present for both species.

3.2.3 Additional differences: Sex-ratio of off-spring

Given that ***F. fimetaria*** is a sexually reproducing species it is possible that compounds which introduces changes in the sex-ratio of the offspring can be identified when using this species. Measurements of the offspring sex-ratio, if successful, would be a simple addition to, and involve a minor change of, the overall test design of the general guideline for long-term Collembola toxicity tests. Hence, based on these considerations it was decided to attempt the sex-ratio approach.

The simplest way, and the approach taken, to identify a bias in the offspring sex-ratio was to extend the exposure period ensuring that the offspring would be of sufficient age (19-23 days) for sex identification. Given the excessive man-power that would be required if each individual should be sexed under the microscope it was decided to use the size characteristics to class animals into sexes, this latter could be done by employing the data produced by the digital image processing counting (see Fig 2). In performing this it is necessary to have an synchronous culture partly enabling the addition for females and males at the beginning of the experiment, partly to ensure synchronised clutches and thus avoiding overlap of size classes. An overlap in size-classes will make sex-determination extremely difficult by the termination of the experiment. It was decided to extend the exposure period to 42 days, which would allow for the first clutch to become adult, hence allowing for sex-ratio determination of this.

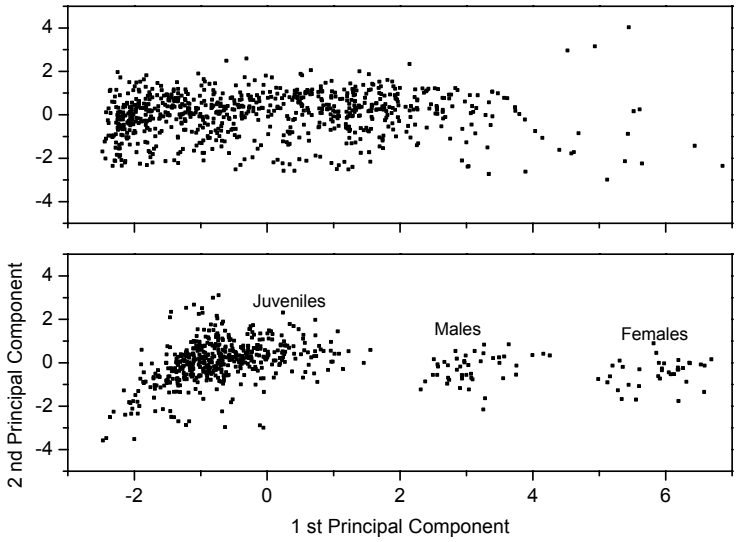

Fig 2. PCA plot of the first and second principal component scores of the original 5 dimension data: area, length, width, slimness and grey density. Lower Fig: three *Folsomia fimetaria* clusters can be identified: females, males and juveniles following exposure to copper (21, days of exposure, Scott-Fordsmand et al 1997). Upper Fig: No clusters can be identified following exposure to testosterone (42 days of exposure).

Two chemicals, methyl-testosterone and ethynyl-estradiol, are suggested as reference chemicals for evaluating potential endocrine-disrupting compounds in invertebrates (Ingersoll et al 1999). There is at present no clear evidence for whether these compounds are involved in the sex-determination of invertebrates. Testosterone has been reported to have some androgen-like activity in some crustaceans LeBlanc (1999) and endogenous concentrations of testosterone and other vertebrate-type androgens have been reported in ***Daphnia magna*** and ***Neomysis integer*** (LeBlanc and MacLachlan 2000, Verslycke et al 2002, 2004). Estradiol has been recorded in the silk moth ***Bombyx mori*** (Keshan and Ray 2001). However, neither estradiol nor testosterone affected the sex-ratio in exposed ***Daphnia magna*** (Kashian and Dodson 2004). No studies have previously been reported for Collembola.

Upon counting and sizing the offspring from the conducted soil-tests it was realised that with the employed approach it was not possible to identify the sex-ratio of the offspring. The problem seemed to be that the juvenile growth was not sufficient synchronous under the present conditions and the individual clutches overlapped, hence a "small" male from the first clutch had the same size as a "large" female from the second clutch (see Fig 2). It could then have been resolved to identifying the sex of the individual animals, but as the tests resulted in more than 18,000 animals per test this would have been an almost impossible task. The present result indicates that the sex-ratio identification is not applicable with the present approach (see Fig 2). Other approaches may be considered in the future.

In fact another approach was partly followed using short-term exposure to an aquatic solution. Based on aquatic solutions adult Collembola were exposed for 24 hours to 12 mg testosterone L^{-1} or 2,0 mg estradiol L^{-1} after which the adults were allowed to produce eggs over a period of 7 days. The hatched juveniles were allowed to growth for 23 days prior to size measuring. No bias

in the sex-ratio could be identified (Fig 3). No comparable literature data was available.

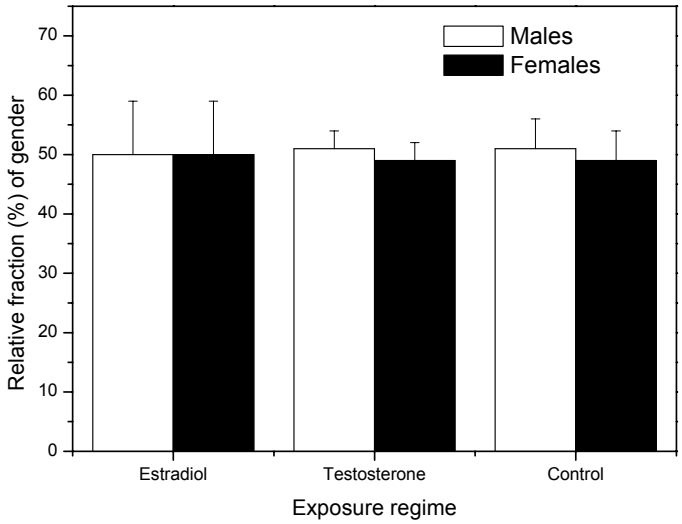

Fig 3 Relative fraction of gender ***Folsomia fimetaria*** following juvenile exposed to water, estradiol (12 mg L^{-1}) and testosterone (2 mg L^{-1}) following exposure of parent generation for 48 hours in an aqueous solution. Columns represent mean ± standard deviation.

3.3 Test concentration ranges of possible ring-test compounds

In connection to the test-guideline and a ring-test programme "standardized" test compounds should be available. Such compounds can be selected on a wide range of criteria, such as how non-toxic it is to humans, how easy it is to store etc. In the present project three compounds were tested, fipronil, 3,5-Dichlorophenol and boric acid. The three compounds were selected on basis of the inclusion in similar test programmes. Two of these compounds, fipronil and 3,5-DCP are currently used in the Copepod ring-test and included here upon the request from the Danish Environmental Protection Agency. Boric acid is suggested as positive control in soil test in North America. Previously, dimethoate has been suggested as a positive control in Collembola tests, but considering that this compound may be taken off the marked it is suggested to avoid using this.

As the above sensitivity studies indicated that the difference between the two species would be less than what is important for determining future test ranges, it was decided to test two of the compounds on ***F. fimetaria*** only. In other words, the ***F. fimetaria*** test ranges is supposed also to cover the ***F. candida*** test ranges. For boric acid both species were tested, confirming that the test range covering ***F. fimetaria*** also covered ***F. candida***.

3.3.1 Fipronil

Fipronil has been suggested as a positive control in the Copepod ring-test programme. It is an insecticide, mainly used on pets for flea elimination (for a detailed review on this see Tingle et al 2003). As such it should be highly toxic to arthropods, as also confirmed with the present results. For ***F. fimetaria***

exposed through the soil fipronil had EC_{10} and EC_{50} (based on nominal concentrations) values of 0.58 mg fipronil kg^{-1} and 1.89 mg fipronil kg^{-1}, respectively. Based on these tests, the ring-test test ranges should be 0-10 mg fipronil kg^{-1} (see Fig 4).

Many data are available with regard to effects of fipronil on invertebrates, but few with soil exposed organisms. Soil toxicity data were only available for earthworms showing LC_{50} values above 8550 mg kg^{-1} (Mostert et al 2002). As fipronil is an insecticide it is of little surprise that it is relatively non-toxic to earthworms. No soil toxicity data were found for Collembolans.

In conclusion: Based on these tests, the ring-test test ranges should be 0-15 mg kg^{-1} for fipronil.

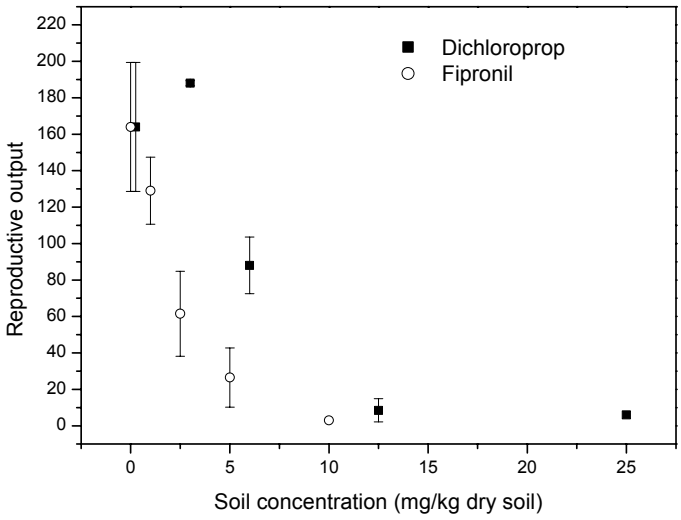

Fig 4. Number of juveniles (mean ± standard deviation) *Folsomia fimetaria*, following exposure of adult Collembola to fipronil or 3,5-chlorophenol for 21 days.

3.3.2 3,5-dichlorophenol

3,5-Dichlorophenol (3,5-DCP) has been suggested as a positive control in the Copepod ring-test programme. For ***F. fimetaria*** exposed through the soil 3,5-DCP had EC_{10} and EC_{50} (based on nominal concentrations) values of 3.07 mg 3,5-DCP kg^{-1} and 5.53 mg 3,5-DCP kg^{-1}, respectively (see Fig 5). Many data are available with regard to chlorophenol effects on invertebrates, but no 3,5-DCP toxicity data for soil organisms were available. For ***F. candida*** Weigmann et al (1985) reported an LC_{50} value (24 hr exposure) for PCP at 32 ppm. Being weak acids, chlorophenol dissociate at a given pH in accordance with their pKa values. Hence, it can be inferred that the solubility alters much with environmental changes in pH if the pKa is close to the environmental pH. The pKa of 3,5-DCP is 8.54 indicating that at a soil pH around 6 (as in the present test) more than 99% is on the non-ionized state.

In conclusion: Based on these tests, the ring-test test ranges should be 0-15 mg 3,5-DCP kg^{-1} using the reproduction test.

3.3.3 Boric acid

Boric acid is suggested as positive control in North America and has been used in many plant toxicity studies. The toxicity of boric acid was studied for both species, first in a short-term test (***F. fimetaria***) and second in a reproduction study (***F. fimetaria*** and ***F. candida***). According to the guideline the two test should last 21 and 28 days respectively. It was, however, chosen to use a 21-day exposure for both species in order to be able to compare the sensitivity between the two species.

The present studies revealed LC_{10} and LC_{50} values of 394 mg boric acid kg^{-1} and 648 mg boric acid kg^{-1} for ***F. fimetaria*** in a 14-day experiment (see table 5). Based on this it was decided to run the reproduction test with concentrations ranging for 0 to 1000 mg boric acid kg^{-1} for both species. In the reproduction test, which lasted 7 days more than the preliminary test, the mortality proved higher resulting in lower LCx values. The LC_{10} was below 200 mg boric acid kg^{-1} for both species. Applying dose-response modelling the EC_{10} and EC_{50} values were 24 and 117 mg boric acid kg^{-1} for ***F. fimetaria*** and 22 and 111 mg boric acid kg^{-1} for ***F. candida***. The latter ECx values should be considered with great care since no test concentration below 200 mg boric acid kg^{-1} was employed. Time did not allow for a repetition of the experiment, besides the experiment is valid for a test-range identification. Becker-van Slooten (2004) reports ECx values corresponded with results obtained in the present study (see Table 5).

Table 5. LC_x and EC_x concentrations (mg kg^{-1}) for *Folsomia fimetaria* (14-days and 21-day) and *Folsomia candida* (21-day) exposed to boric acid in soil.

Reference	Species	LC_{10}	LC_{50}	EC_{10}*	EC_{50}*
14-day test					
This report	*F. fimetaria*	394 [225-563]	648 [477-819]		
Becker van Slooten (2004)	*F. candida*		800 [633-1012]		
21-day test					
This report	*F. fimetaria*	< 200	>200	24±1 [21-26]	117±3 [107-1289]
This report	*F. candida*	<200	<200	22±1 [21-229]	111±3 [103-121]
28-day test					
Becker van Slooten (2004)	*F. candida*				147 [137-156]

* Effect concentrations, EC_{10} and EC_{50}, and bootstrapping intervals (equivalent to 95 % confidence intervals, ICP Nordberg-King, 1993). The studies by Becker van Slooten et al. (2004) is based on a 10% OECD soil

In conclusion: Based on the given data the following test concentration ranges are suggested for a ring-test: 0 to 300 mg boric acid kg^{-1} dry soil. The EC_{50} (reproduction) should be in the range of 100-150 mg boric acid kg^{-1} and the LC_{50} needs to be better defined in the ring test.

3.3.4 Proposed test ranges for a ring-test

Based on the three above tests, the ring-test test ranges should be 0-10 mg kg^{-1} for fipronil, 0-15 mg kg^{-1} for 3,5-dichlorophenol and 0-300 mg kg^{-1} for boric acid using reproductive output as a test endpoint.

4 Conclusion

Searching the literature a variety of collembolan species have been used in terrestrial ecology. For the draft OECD guideline two collembolan species, ***Folsomia fimetaria*** and ***Folsomia candida***, were chosen as these two species have been much used in terrestrial ecotoxicology and standard tests are available. The two selected species represents two ecological and biological approaches. ***F. candida's*** habitat is primarily high organic soils, whereas ***F. fimetaria*** is present in most agricultural soils. ***F. candida*** is parthenogenetic (asexual) reproducing, whereas ***F. fimetaria*** is sexually reproducing as are most other species. It was attempted to include juvenile sex-determination as an additional endpoint for the test, but due to overlapping juvenile clutches (within one test) this was not possible. Given the differences between the two species these were reviewed with respect to differences in sensitivity to chemicals, with particular emphasis on possible alterations caused by differences in their size. Neither the literature study nor the laboratory studies resulted in conclusive differences in sensitivity between the species. A comparison in size-class related differences between the two species revealed that there was a slight difference in sensitivity for animals of the same size, but a pronounced stage related difference in toxicity. Hence, the life-stage at which the organisms are exposed determines the sensitivity of the test.

In connection with the test guideline (and a ring-test) it will be necessary to have a positive control to ensure the stability of the test-system. Three compounds were tested in order to identify future test ranges. The compounds were fipronil, 3,5-dichlorophenol and boric acid. The tests performed with these chemicals indicated that the test range for fipronil should be 0 to 10 mg fipronil kg^{-1}, for 3,5-Dichlorophenol 0 to 15 mg 3,5-dichlorophenol kg^{-1} soil and for Boric acid 0 to 300 mg boric acid kg^{-1}.

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OECD GUIDELINES FOR THE TESTING OF CHEMICALS

PROPOSAL FOR A NEW GUIDELINE

Collembola Reproduction Test (Folsomia fimetaria and Folsomia candida)

INTRODUCTION

1. This Test Guideline is designed for assessing the effects of chemicals on the reproductive output of the collembolans, ***Folsomia fimetaria*** and ***Folsomia candida*** in soil**.** It is principally based on a method developed by the National Environmental Research Institute, Denmark [1] and the [2]. When more information is available it should be considered including more Collembola species in a test-programme.

2. Soil-dwelling Collembola are ecologically relevant species for ecotoxicological testing. Compared to for example earthworms the collembolans represent organisms with an exoskeleton, indicating a different route (or at least different rate) of exposure.

3. Population densities of Collembola commonly reach 10^5 m^{-2} In soil and leaf litter layers in many terrestrial ecosystems [3,4]. Because of their small size however, adults typically measure 0.5 - 5 mm their contribution to total soil animal biomass and respiration is low, estimated at being 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 and carabidae and rove beetles. Collembola contribute to decomposition processes in soil and on acidic stands they may be the most important soil invertebrates as earthworms and diplopods are absent.

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 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 mychorrhiza, as is known to be the case for ***F. candida***. It is a sexually reproducing species.

F. candida is worldwide distributed. Although it is not common in most natural soils, it often occurs in very high numbers in humus rich sites. It is a blind, unpigmented collembolan, which reproduces parthenogenetically. It has a well-developed furca (jumping organ) and an active running movement and jumps readily if disturbed. It's a parthenogenetic species.

PRINCIPLE OF THE TEST

4. Synchronous adult Collembola are exposed to a range of concentrations of the test substance mixed into an artificial soil (or alternative soil). The test scenario can be divided into two steps:

4.a A range-finding test, in case no sufficient information is available, in which mortality is the main endpoint assessed after two weeks exposure

4.b A definitive reproduction test in which the total number of juveniles produced by parent animal and the survival of parent animals are assessed. The duration of this definitive test is three (***F. fimetaria***) /four (***F. candida***) weeks.

The toxic effect of the test substance on the reproductive output of the animals is expressed as ECx (e.g. EC_{10}, EC_{50}) by using a sigmoid-regression model to estimate the concentration that would cause x % reduction in reproductive output.

INFORMATION ON THE TEST SUBSTANCE

5. The following information relating to the test substance should be available to assist in the design of appropriate test procedures:
- water solubility
- the log K_{ow},
- the soil water partition coefficient
- 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 is desirable.

6. 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

7. The following criteria should be satisfied in the controls for a test result to be considered valid:

7.a Adult mortality should not exceed 20% at the end of the range-finding test and after the first three/four weeks of the reproduction test.

7.b ***F. candida***: assuming that 10 adults per vessel were used in setting up the test, an average of at least 100 juveniles per vessel should have been produced at the end of the four-week test.

7.c ***F. fimetaria***: assuming that 20 adults (10 females and 10 males) per vessel were used in setting up the test, an average of at least 200 juveniles per vessel should have been produced at the end of the three-week test.

7.d The coefficient of variation of juveniles should be less than 25% at the end of the reproduction test.

Where a test fails to meet the above validity criteria the test should be terminated unless a justification for proceeding with the test can be provided. The justification should be included in the test report.

REFERENCE SUBSTANCE

8. A reference substance must be tested at its EC_{50} concentration for the chosen test soil type either at regular intervals or possibly included in each test to verify that the response of the test organisms has not changed significantly over time and that the test system is responding at is normal level. A suitable reference substance is -TO BE DECIDED-, which has been shown to affect survival and reproduction of Collembola [8, 9].

DESCRIPTION OF THE TEST

Equipment

9. Containers capable of holding 30 gram of soil are suitable test vessels. The material should either be glass or inert plastic (non-toxic). The vessels should have a cross-sectional area allowing the soil depth to be between 3-4 cm. The vessels should have transparent 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.

10. Normal laboratory equipment is required, specifically the following:

- drying cabinet;
- stereomicroscope;
- pH-meter and photometer;
- suitable accurate balances;
- adequate equipment for temperature control;
- adequate equipment for humidity control (not essential if exposure vessels have lids);
- incubator or small room with air-conditioner;
- tweezers, hooks or loops.

Preparation of the artificial soil

11. The artificial soil is prepared according to OECD guideline 207 [10] and consist of the below components. It is highly preferable to use, in addition to the OECD soil, a natural standard soil such as Lufa Speyer. With regard to the artificial soil its recommended composition is as follows (based on dry weights, dried to a constant weight at 105 °C):

- 5 or 10% sphagnum peat, air-dried and finely ground (a particle size of 2 ± 1 mm is acceptable); it is recommended to check that a soil prepared with a fresh batch of peat is suitable for containing the Collembola before it is used in a test. It is recommended to measure the C/N ratio, pH and CEC of the peat.

- 20% kaolin clay (kaolinite content preferably above 30%);
- approximately 0.3 to 1.0% calcium carbonate ($CaCO_3$, pulverized, 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;
- approximately 69% 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.

It is advisable to demonstrate the suitability of the test soil for use in the test and for achieving the test validity criteria before using the soil in a definitive test

11. The dry constituents of the soil are mixed thoroughly (e.g. in a large-scale laboratory mixer). This should be done at least one week before starting the test. The mixed soil should be stored for two weeks 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 3). 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 more of the mixture comprising, but excluding the $CaCO_3$.

12. The maximum water holding capacity (WHC) of the artificial soil is determined in accordance with procedures described in Annex 2. At the start of the test, the pre-moistened soil is divided into portions corresponding with the number of test concentrations (and reference substance where appropriate) and controls used for the test. The moisture content is adjusted to about 50% of the maximum WHC (corresponding to 50 ± 10% moisture dry mass) by the addition of the test substance solution and/or distilled or de-ionised water. However, moisture content should be optimized to attain a loose porous texture to allow animals to enter into the pores. The weight of the containers is determined at the beginning, in the middle and at the end of the test.

<u>Selection and preparation of test animals</u>

13. The recommended test species are ***Folsomia fimetaria*** and ***Folsomia candida***. These two species are some of the most accessible species of Collembola, with specimens sizes of up to 2.5 mm in length. These collembolans are culturable and commercially available.

13.a ***Preparation of culturing substrate***
The culturing substrate is plaster of Paris (calcium sulphate) with activated charcoal. This provides a moist substrate, the function of the charcoal being to absorb waste gases and excreta [11, 12]. 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.

13.c ***Breeding***

Collembolans are held in Petri dishes (90 mm x 13 mm) with the bottom covered by a 0.5 cm layer of plaster of Paris/charcoal substrate. They are cultured at 20 ± 1 °C at a light:dark cycle of 12:12 hours.

Every time the Petri dishes are 4-8 weeks old, it is necessary to transfer the animals to Petri dishes with newly prepared plaster of Paris/charcoal substrate, and they will start producing eggs again.

Cultures are kept in an incubator at a temperature of 20±0.5 °C, under a 16:8 hours light regime (<1000 Lux). Containers are kept moist at all times. Any dead individuals should be removed from the containers immediately, as should any stale food. Stock cultures of both species should be periodically moved (e.g. every 2-3 months) to fresh plaster of Paris.

13.d ***Food source***

Granulated dried baker's yeast is used as the principal food supply for ***F. candida***. Fresh food is provided either once or twice a week, to avoid spoilage by fungal growth. It is placed on filter paper discs, which are removed together with the food when necessary. The mass of baker's yeast added should be tailored to the size of collembolan population, but as a general rule 10-30 mg is sufficient.

14. The animals used in the tests are adult individuals. Synchronisation of the breeding culture is necessary, especially so for the ***F. fimetaria***.

14.a Synchronising and culturing ***F. fimetaria***.

1) Prepare Petri dishes with a 0.5 cm layer of plaster of Paris/charcoal substrate.
2) For egg laying transfer 150-300 adult ***F. fimetaria*** from a 4-8 weeks old
substrate to the containers and feed with 15 mg baker's yeast.
3) Keep the culture at 20±1 °C (means should be 20 °C) and a light:dark cycle 12:12 hours (<1000 Lux).
4) After 9 days the eggs are carefully collected with a needle and spatula and moved to an “egg-paper” (small pieces of filter paper dipped in plaster of Paris/charcoal substrate) which is placed in a fresh Petri dish with plaster of Paris/charcoal substrate. It is important that the egg-paper and substrate are humid, or else the eggs will dehydrate.
5) Eggs and hatched juveniles are cultured in same manner as the adults.
6) After three days most of the eggs on the egg-paper have hatched, and the juveniles tend to stay under the egg-paper.
7) To have evenly aged juveniles the egg-paper with unhatched eggs is removed from the Petri dish with a tweezer. The juveniles stay in the dish. Age of the juveniles are 0-3 days.
8) Label the container with date of hatching and provide baker's yeast.

14.b. Synchronising and culturing ***F. candida***.

1) Prepare new breeding containers with a 1 cm layer of plaster of Paris.
2) Transfer several hundred adult Collembola from stock cultures into each container, and supply with baker's yeast.
3) Keep the culture at 20±1 °C (means should be 20 °C) and a light:dark cycle 12:12 hours (<1000 Lux).
4) Remove adults after 24 to 48 hours.
5) Eggs and hatched juveniles are cultured in same manner as the adults.
6) Observe daily and record date of hatching. Provide food immediately after hatching.
7) After three days most of the eggs on the egg-paper have hatched, and the juveniles tend to stay under the egg-paper.
8) To have evenly aged juveniles the egg-paper with unhatched eggs is removed from the Petri dish with a tweezer. The juveniles stay in the dish. Age of the juveniles are 0-3 days.
9) Label the container with date of hatching and provide baker's yeast.

15. Other Collembolan species may also be suitable, e.g. ***I. viridis or O. folsomi***. If other species of Collembola are used, they must be clearly identified and the rationale for the selection of the species should be reported.

Preparation of test concentrations

16. Two methods of application of the test substance can be used: mixing the test substance into the soil or application to the soil surface . The selection of the appropriate method depends on the purpose of the test. In general, mixing of the test substance into the soil is recommended. However application procedures that are consistent with normal agricultural practice may be required (e.g. spraying of liquid formulation or use of special pesticide formulations such as granules or seed dressings).

Mixing the test substance into the soil

Test substance soluble in water

17. 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

18. For chemicals insoluble in water but soluble in organic solvents, the test substance can be dissolved in the smallest possible volume of a suitable vehicle (e.g. acetone). Only volatile solvents should be used. When such vehicles are used, all test concentrations and an additional control should contain the same minimum amount of the vehicle. The vehicle is sprayed on or mixed with a small amount, for example 2.5 g, of fine quartz sand. The vehicle is eliminated by evaporation under a fume hood for at least one hour. This mixture of quartz sand and test substance is added to the pre-moistened soil and thoroughly mixed after adding an appropriate amount of de-ionised water to obtain the moisture required. The final mixture is introduced into the test vessels.

Test substance insoluble in water and organic solvents

19. For substances that are poorly soluble in water and organic solvents, the equivalent of 2.5 g of finely ground quartz sand per test vessel 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 de-ionised 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 to the soil surface

20. The soil is treated after the Collembolans are added. The test containers are first filled with the moistened soil substrate and the weighed. The test substance is applied. It should not be added to the soil within half an hour of introducing the Collembola so as to avoid any direct exposure to the test substance by skin contact. When the test substance is a pesticide it may be appropriate to apply it to the soil surface by spraying. 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. Before application the cover of the test container should be removed and replaced by a liner, which protects the side walls of the container from spray. The liner can be made from a test container with the base removed. 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 of between 600 and 800 µl/m^2. 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.
Test containers should be left uncovered for a period of one hour to allow any volatile solvent associated with the application of the test substance to evaporate**.**

PERFORMANCE OF THE TESTS

Test groups and controls

21. For each test concentration, an amount of test soil corresponding to 25 g dry weight is placed into the test vessel. Controls, without the test substance, are also prepared. Food is added to each vessel. Ten (***F. candida***) or twenty (***F. fimetaria***) collembolans are randomly allocated to each test vessel. The individuals are carefully transferred into each test vessel and placed on the surface of the soil using. For efficient transfer of the animals a low-suction air flow devise 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-randomised weekly.

22. If a vehicle is used for application of the test substance, one control series containing quartz sand sprayed or mixed with solvent 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. A control series containing additional quartz sand (2.5 g per vessel) should be run for substances requiring administration.

Test conditions

23. The test temperature is 20 ± 2 °C. To discourage collembolans from escaping from the soil, the test is carried out under controlled light-dark cycles (preferably 16 hours light and 8 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 of the test and thereafter once a week. Weight loss is replenished by the addition of an appropriate amount of deionised 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 moisture content and 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 control and treated (all concentrations) soil samples prepared and maintained in the same way as the test cultures. Food should be added at the top of the soil samples at the start of the test and after 2 weeks. The amount of food added should be the same as that added to the test cultures.

Feeding

26. A suitable source is granulated dried Bakers's yeast, commercially available for household use.

27. At the beginning of the test and after each 14-day interval, add about 15 mg yeast to each container.

Design for the range-finding test

28. 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). Two replicates for each treatment and control are sufficient.

29. The duration of the range-finding test is two weeks. At the end of the test, mortality of the Collembola is assessed. A Collembola is recorded as dead if not present in the extraction. Additional information to mortality may also be useful in deciding on the range of concentrations to be used in the definitive test.

30. The LC_{50} can be determined using Probit analysis. In order for an accurate determination of the LC_{50} performing the test it should be ensured that the number of replicates and test concentrations matches the power requirements for the test.

Design for the definitive reproduction test

31. For determination of the ECx (e.g. EC_{10}, EC_{50}), an adequate number of concentrations to cause at least four statistically significantly different mean responses at these concentrations is recommended.. At least four replicates for each test concentration and four control replicates are recommended. The spacing factor should ensure that the majority of test concentrations are on the slope of the ECx curve. It should be considered that the power requirements of the test should be maintained.

30. Number of test individuals and duration (three test designs).

30 a. For the ***F. fimetaria*** test twenty adults per test vessel should be used. Food is added to the test vessels at the beginning of the test and then after 14 days up to and including Day 21. On Day 21 the soil samples should be extracted and counted.

30 b. For the ***F. candida*** test ten adults per test vessel should be used. Food is added to the test vessels at the beginning of the test and then after 14 days up to and including Day 28. On Day 28 the soil samples should be extracted and counted.

Limit test

31. If no effects are observed at the highest concentration in the range-finding test (i.e. 1000 mg/kg), the reproduction test could be performed as a limit test, using a test concentration of 1000 mg/kg. A limit test will provide the opportunity to demonstrate that the NOEC for reproduction is greater than the limit concentration whilst minimising the number of Collembola used in the test. Eight replicates should be used for both the treated soil and the control.

Power of the test

32. For all test designs it is advised that the Type I error is set at a 5% level and the type II error is set at maximum 20%. This should be ensured for each test.

Counting animals

33. Two methods of extraction can be performed.

33.a. First method: A controlled temperature gradient extractor based on principles by MacFayden can be used [1]. The heat coming from a heathing element at the top of the extraction box (regulated though a thermistor placed ion 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 the 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. After 12 h at 40 °C the extraction is finished.

33.b. Second method: After the experimental incubation period the number of juvenile Collembola present is assessed by floatation. This involves emptying the tube of soil into a 250 ml vessel and adding approx. 200 ml of distilled water. 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 the Collembola.

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 Collembola on the enlarged prints or projected slides. Count may also be performed using digital image processing techniques [13].

Summary and timetable for the test

34. The steps of the test can be summarised as follows:

Purpose	Range-finding test	Definitive Reproduction test - 1	Definitive Reproduction test - 2
Species	F. fimetaria/ F. candida	F. candida	F. fimetaria
Aim	Mortality	Reproduction	Reproduction
Time (day)			
-9-12 (F. candida) -13-26 (F. fimetaria)	Preparation of synchronous culture	Preparation of synchronous culture	Preparation of synchronous culture
Day –5 or earlier	- Prepare artificial soil (mixing of dry constituents)	- Prepare artificial soil (mixing of dry constituents)	- Prepare artificial soil (mixing of dry constituents)
Day –3	- Check pH of artificial soil - Measure max WHC of soil	- Check pH of artificial soil - Measure max WHC of soil	- Check pH of artificial soil - Measure max WHC of soil
Day –2 to –1	- Sort Collembola for testing	Sort Collembola for testing	- Sort Collembola for testing
Day –1	- Pre-moisten artificial soil and distribute into batches - Prepare stock solutions - Apply test substance if solvent required	- Pre-moisten artificial soil and distribute into batches - Prepare stock solutions - Apply test substance if solvent required	- Pre-moisten artificial soil and distribute into batches - Prepare stock solutions - Apply test substance if solvent required
Day 0	- Prepare stock solutions - Apply test substance - Weigh test substrate into test vessels - Mix in food - Introduce	- Prepare stock solutions - Apply test substance - Weigh test substrate into test vessels - Mix in food - Introduce	Prepare stock solutions - Apply test substance - Weigh test substrate into test vessels - Mix in food - Introduce

Day 7	Collembola - Measure soil pH and moisture content - Check soil moisture content - Determine adult mortality	Collembola - Measure soil pH and moisture content - Check soil moisture content - Check soil moisture content	Collembola - Measure soil pH and moisture content - Check soil moisture content - Check soil moisture content
Day 14	- Estimate number of juveniles - Measure soil pH and moisture content	- Feed	-Feed
Day 21		- Check soil moisture content	-Extract all individuals - Check soil moisture content -Measure pH
Day 28		Extract all individuals - Check soil moisture content -Measure pH	

DATA AND REPORTING

Treatment of results

35. An overview is in the following but more detailed statistical guidance for analysing test results is given in the "OECD draft statistical report 2003".

36. In the range finding test, the main endpoint is mortality. Probit analysis [14] should normally be applied to determine the LC_{50}. However, in cases where this method of analysis is unsuitable (e.g., if less than three concentrations with partial kills are available), alternative methods can be used. These methods could include moving averages [14], the trimmed Spearman-Karber method [15] or simple interpolation (e.g., geometrical mean of LC_0 and LC_{100}, as computed by the square root of LC_0 multiplied by LC_{100}).

ECx estimation (preferred method)

37. To compute any ECx value, the per-treatment means data are used for regression analysis (linear or non-linear), after an appropriate dose-response function has been selected [16]. Among suitable functions are the normal sigmoid, logistic or Weibull functions, containing two to four parameters, some of which can also model hormetic responses. If a dose-response function was fitted by linear regression analysis a significant r^2 (coefficient of determination) and/or slope should be found with the regression analysis before estimating the ECx by inserting a value corresponding to x% of the control mean into the equation found by regression analysis. 95%-confidence limits are calculated according to [17].

38. Alternatively, one may express the treatment results as percentages of the control result or as percent inhibitions relative to control. In these cases, the normal (logistic, Weibull) sigmoid curve can often be easily fitted to the results using the probit regression procedure [17]. In these cases the weighting function has to be adjusted for metric responses as given by [18]. However, if hormesis has been observed, regression analysis should be replaced by a four-parameter logistic or Weibull function, fitted by a non-linear regression procedure [19]. If a suitable dose-response function cannot be fitted to the data, one may use alternative methods to estimate the ECx, and its confidence limits, such as Moving Averages after Thompson [14] and the Trimmed Spearman-Karber procedure [15].

NOEC estimation (alternative method)

39. If an analysis of variance has been performed, the standard deviation, s, and the degrees of freedom, df, may be replaced by the pooled variance estimate obtained from the ANOVA and by its degrees of freedom, respectively – provided variance does not depend on the concentration. In this case, use the single variances of control and treatments. Those values are usually calculated by commercial statistical software using the per-vessel results as replicates. If pooling data for the negative and solvent controls appears reasonable rather than testing against one of those, they should be tested to see that they are not significantly different (for the appropriate test consider Annex 4). Further statistical analysis and inferences depend on whether the replicate values are normally distributed and are homogeneous with regard to their variance.

41. NOEC estimation: Kolmogoroff-Smirnov's [20] and Bartlett's-test procedures [21] are used respectively for testing for normality and homogeneity of variance homogeneity. With normally distributed and homogeneous data, multiple t-tests such as Dunnett´s test [22, 23] or William´s test (= 0.05, one-sided) [24, 25] should be performed. It should be noted that, in the case of unequal replication, the tabulated t-values must be corrected as suggested by Dunnett and Williams. Sometimes, because of large variation the responses do not increase/decrease regularly. In this case Dunnett´s test does not lead to reasonable NOEC/LOEC values and analysis by Williams-test is to be preferred. Alternatively, Otherwise, a multiple U-test, e.g. the Bonferroni-U-test according to Holm [26], could be used.

42. If a limit test has been performed and the prerequisites of parametric test procedures (normality, homogeneity) are fulfilled, the pair-wise Student t-test can be used or otherwise the Mann-Whitney-U-test procedure [20].

40. The test report should include the following information:

40.a. Test substance:
- physical nature and, where relevant physical-chemical properties (e.g. water solubility, vapour pressure);
- chemical identification of the test substance according to IUPAC nomenclature, CAS-number, batch, lot, structural formula and purity;
- expiry date of sample.

40.b. Test species:
- test animals used: species, scientific name, source of organisms and breeding conditions.

40.c. Test conditions:

- ingredients and preparation details of the artificial soil; (minimum: C/N ratio, pH, WHC, CEC)
- method of application of the test substance (data verifying homogeneity of application);
- description of the test conditions, including temperature, moisture content, pH, etc.;
- full description of the experimental design and procedures.

40.d. Test results:

- Actual concentration of the tested compound in the test media (at minimum for control exposures)
- mortality of adult Collembola after two weeks and the number of juveniles at the end of the range-finding test;
- mortality of adult Collembola after three weeks exposure and the full record of juveniles at the end of the definitive test;
- any observed physical or pathological symptoms and behavioural changes in the test organisms;
- the LC_{50}, the NOEC and/or ECx (e.g. EC_{50}, EC_{10}) for reproduction if some of them are applicable with confidence intervals, and a graph of the fitted model used for its calculation, the slope of the dose-response curve and its standard error;
- all information and observations helpful for the interpretation of the results.
- Power of the actual test.

Deviations from procedures described in this guideline and any unusual occurrences during the test.

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ANNEX 1

THE FOLLOWING DEFINITIONS ARE APPLICABLE TO THIS GUIDE-LINE

LC_{50} (Median lethal concentration) is the concentration of a test substance that is statistically likely to kill 50% of exposed test organisms within a given time period. In this test the LC_{50} is expressed as a mass of test substance per dry mass of the test soil or as a mass of test substance per unit area of soil.

LOEC (Lowest Observed Effect Concentration) is the lowest test substance concentration that has a statistically significant effect ($p < 0.05$) In this test the LOEC is expressed as a mass of test substance per dry mass of the test soil or as a mass of test substance per unit area of soil. All test concentrations above the LOEC should normally show an effect that is statistically different from the control. Any deviations from the above must be justified in the test report.

NOEC (No Observed Effect Concentration) is the highest test substance concentration immediately below the LOEC 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.

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. In this test the effect concentrations are expressed as a mass of test substance per dry mass of the test soil or as a mass of the test substance per unit area of the soil.

Reproduction rate: Mean number of juvenile Collembola produced per a number of adults over the test period.

ANNEX 2

DETERMINATION OF THE MAXIMUM WATER HOLDING CAPACITY OF THE SOIL

The following method for determining the maximum water holding capacity 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 (3)).

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 piece of filter paper fill with water 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) can then 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 3

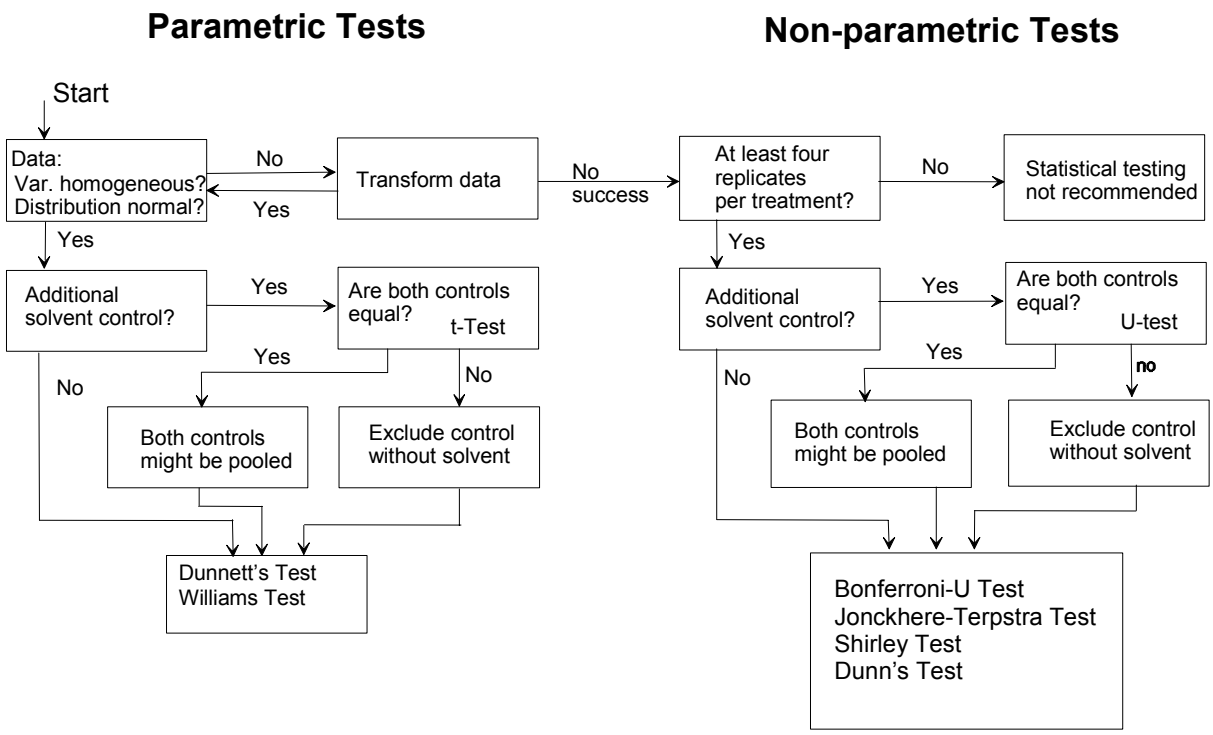
DETERMINATION OF SOIL pH

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 (15).

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).

ANNEX 4

OVERVIEW OF THE STATISTICAL ASSESSMENT OF DATA (NOEC DETERMINATION)



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