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Toxicity testing with the collembolans *Folsomia fimetaria* and *Folsomia candida* and the results of a ringtest

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1 Preface

Collembolans have been used for ecotoxicological testing for about 4 decades now but they have not yet had the privilege to enter into the OECD test guideline programme. Thus, as a proposal for OECD, two different collembolans, namely *Folsomia fimetaria* and *Folsomia candida* are introduced, and the results of a ringtest and a draft test guideline are presented. *F. candida* is already a well-established testing species and is extensively used for ecotoxicological testing as a representative for soil arthropods, with an ISO standard available since 1999. For *F. fimetaria* a testing protocol was published in 1998 as an outcome of DK-EPA and EU projects. International guidelines for chemical testing have occasionally included more than one species or included optional species that may be preferred for various reasons, such as representability and target habitat. In the case of alternatives to *F. candida* we include *F. fimetaria* due to its sexual mode of reproduction and worldwide distribution in natural and agricultural habitats in contrast to the asexually reproducing *F. candida*, which is not present in many types of natural and agricultural habitats. Furthermore, additional details needed to perform testing with *F. fimetaria* are provided.

With *F. fimetaria* as an optional testing species, the complete biology of the sexual reproduction, lacking for *F. candida*, will now be included as a potential target for any chemical being tested, including sex hormone disrupting chemicals. As stated in OECD Monograph No. 21 (OECD, 2002), progress in the field of endocrine disrupters is limited by the absence of chemicals accepted as suitable for use as references. Although this is the case too with *F. fimetaria*, its introduction as a test species nevertheless is needed as no other arthropods, i.e. *F. candida*, are suitable as a test species in this respect.

2 Biology and ecotoxicology of *F. fimetaria* and *F. candida*

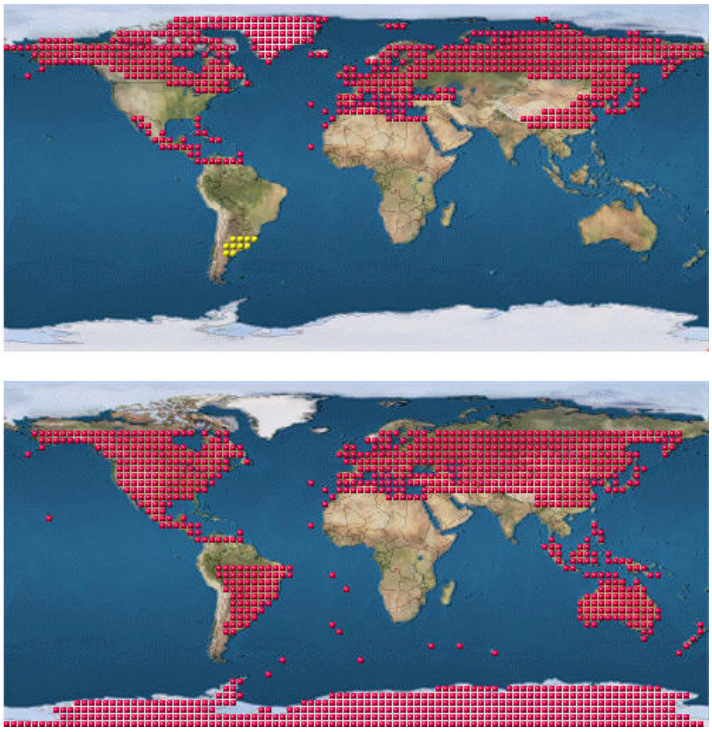

Fig. 1. Biogeographically distribution of *F. fimetaria*, upper, and *F. candida,* lower, (Bellinger *et al.*, 1996-2008)[1]. The dotted areas indicate that the species have been found in the corresponding biogeographically region.

2.1 Introduction to *F. fimetaria* and *F. candida*

The use of *F. candida* and *F. fimetaria* for ecotoxicological testing purposes has been covered in various publications including: (Riepert and Kula, 1996; Wiles and Krogh, 1998; Fountain and Hopkin, 2005; Scott-Fordsmand and Krogh, 2005; Environment-Canada, 2007). As *F. fimetaria* is not yet included in internationally approved standards and is less studied than *F. candida*, it is briefly introduced here. A bibliographic search in Science Citation Index (ISI Web of Knowledge/Web of Science accessed Jan 2008) revealed about 400 papers referring to *F. candida* and 74 papers referring to *F. fimetaria*. Of the *F. fimetaria* papers, about 35 deal with ecotoxicology and some 27 originate from the NERI Soil Fauna laboratory or authors affiliated to this laboratory.

The selection of *F. fimetaria* for ecotoxicological testing was done by curator, senior researcher, Henning Petersen, Mols Laboratory, Natural History Museum, Aarhus Denmark, in a project supported by the Danish Environmental Protection

[1] Maps reproduced with permission from the authors

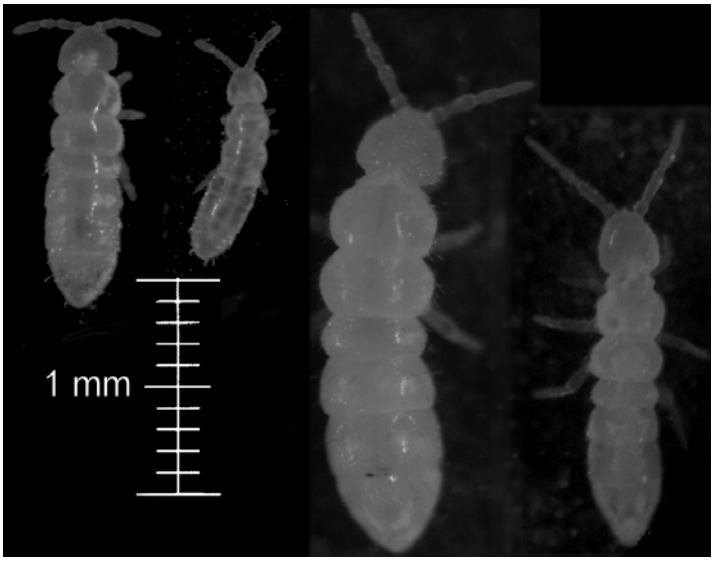

Fig. 2. Adult *F. fimetaria* female and male, left, and *F. candida* female and male, right.

Agency (DK-EPA) (Petersen and Gjelstrup, 1995), however it was used even earlier in studies to test for DDT effects (Van de Bund, 1965; Scopes and Lichtenstein, 1967). Scopes and Lichtenstein even published a filter paper method on how to use *F. fimetaria* for general insecticide residue testing (Scopes and Lichtenstein, 1967). Adults of *F. fimetaria* are 0.8-1.4 mm long (Folker-Hansen *et al.*, 1996), e.g. males 0.9 mm and females 1.3. mm, with a dry weight of 10-40 μg per individual at 20° C. Female *F. candida* can become 2.0-2.5 mm long (Crouau and Moia, 2006; Widarto *et al.*, 2007), and has a dry weight of 140 μg for adults at the asymptotic maximum size. Adult *F. candida* males although rarely found are about 1.25 mm long. *F. fimetaria* reproduces only sexually, and sexual dimorphism is not detectable at low magnification before an age of 20 days after hatching. Males have a more slender body, and they are only half as big as the females (Fig. 2).

Both species are widely distributed (Fig. 1), but maps created particularly from older records cannot be fully trusted due to confusion of the two species (Hopkin, 2008a). *F. fimetaria* is common in a range of habitats including agricultural soil, and its preference for high organic matter hot spots seems similar to *F. candida* (Fjellberg, 1980). It occurs less frequently in meadows and in the soils of urban settlements (Chernova *et al.*, 2003). The easiest way to get *F. fimetaria* is to collect soil samples from agricultural fields, meadows or grassland and make a heat/dry extraction of the soil. In buried lumps of organic hotspots like manure or sludge *F. fimetaria* can be found in huge numbers (Krogh *et al.*, 1997), and the collection of the lumps is a good source for starting a *F. fimetaria* culture.

F. candida is a cosmopolitan species found almost all over the globe (Fig. 1) and is considered a tramp species (Hopkin, 1997). However, only few outdoor records exists for *F. candida* who prefers high organic matter like in compost, green-houses, flower pots or manure (Fjellberg, 1980; Chernova *et al.*, 2003; Fjellberg, 2007a), hence the records used to generate the maps of Fig. 1 refers mainly to these domestic habitats. In line with this it is rare in Australian soils (Greenslade and Vaughan, 2003). However, it should be noted that the lack of presence of a standard test species in certain parts of the world may not at all invalidate its

general use; it may well have a similar response as other collembolans under the simplified artificial conditions offered in a standard test (see section 2.5).

Discrimination of *F. candida* and *F. fimetaria* from species of the same genus is not problematic with the unique position of manubrial setae and other characteristics (Fjellberg, 1980; Potapov, 2000; Potapov and Babenko, 2000; Fjellberg, 2007b).
However, when establishing cultures from field populations, care should be taken to avoid confusion between white and eyeless relatives from the *F. fimetaria* group such as *Folsomia lawrencei, Folsomia kerni* and *Folsomia litsteri*. Using recent keys, e.g. Fjellberg (2007b), should prevent such mistakes. Small *F. litsteri* was considered to be juvenile *F. candida* and bigger *F. litsteri* to be *F. lawrencei* (Josef Rusek pers. comm.), but later Steve Hopkin considered *F. litsteri* to be a true species (Hopkin, 2008b) and this is maintained by Fjellberg (2007b).

2.2 Comparison of the two species

While the size difference is very obvious for the two species behavioural differences have also been observed, but have rarely been explored scientifically (Chernova *et al.*, 2003). The collembolan family, Sminthuridae, has long been known to display relatively complex mating behaviour (Schaller, 1952), and similarly, the podurids have a sperm transfer requiring male-female interactions as otherwise believed to be non-interactive for the arthropleone collembolans (Schliwa and Schaller, 1963). Although not yet reported for *Folsomia* the observations by Goloschapova *et al.* (2006) indicate that isotomids may have more complex mating behaviour than usually assumed.

When being disturbed *F. fimetaria* will respond by bending down the head and retracting the antenna downwards and inwards to the head, in contrast *F. candida* will start scattering and jumping. Only few studies have made direct comparisons between the basic biological properties of these two species, however aspects such as fecundity and preference responses to a range of fungi have been demonstrated to be significantly different (Larsen *et al.*, 2008).

At 20^{o} C, the average duration of the five juvenile instars are 3 days for *F. candida* (Snider, 1973) and maximum 4 days for *F. fimetaria* (Jensen *et al.*, 2001). Sexual maturity is attained in the 6th instar occurring around age 15-16 days for *F. candida* and a few days later for *F. fimetaria*[2] (Snider, 1973; Holmstrup and Krogh, 1996; Widarto *et al.*, 2007).

It is generally assumed that sexually reproducing collembolans need fertilisation for every reproductive instar (Hopkin, 1997). To substantiate this hypothesis specifically reported for only a few non-isotomid species, 24 couples of 25-28 days old, 8th instar, *F. fimetaria* males and females, and 24 single females were isolated and the oviposition pattern of reproduction was followed for 3 weeks at 20^{o} C (Krogh, 2006). None of the single females produced any eggs and the couples produced averages of 10 and 30 eggs in instars 8 and 10, respectively, with a maximum clutch size of 60 eggs. The same figures for *F. candida* were 48 and 71 eggs with a maximum clutch of eggs of 114 (Snider, 1973). Egg development for *F. fimetaria* took 9.5 days, hence similar to 9-11 days observed for *F. candida* (Snider, 1973). The time between the 8th and 10th reproductive instars were 7 days, with 9 days between the 10th to 12th instars; 1-2 days shorter then the same instars for *F. candida*. The infertility of isolated females stresses that even if females are

[2] Life history data on *F. fimetaria* are not yet precise enough to give accurate figures.

coming from a mixed male-female population, as is the case for the reproductive test, this does not enable a female to produce fertile eggs, so the uptake of spermatophores is crucial just at oviposition time shortly after shedding the cuticle.

One of the most interesting differences between the two collembolans is the intracellularly presence of *Wolbachia* bacteria in *F. candida* and the absence of it in *F. fimetaria*[3]. *F. candida* has always been reported to reproduce parthenogenetically in laboratory cultures and the presence of males in laboratory cultures has never been reported in the literature, since early studies by Goto (1960), Milne (1960), Marshall & Kevan (1962) and Green (1964). Presence of intracellular bacteria in *F. candida* ovaries has been known since the study by Palévody (1972), and Vandekerckhove *et al.* (1999) demonstrated the presence of *Wolbachia* in *F. candida* ovary cells, fat bodies and institial cells. However, the exact mechanism by which *Wolbachia* operates in *F. candida* has not yet been resolved and neither is it yet established if *Wolbachia* indeed is the reason for parthenogenesis in *F. candida* (Riparbelli *et al.*, 2006), although it seems plausible (Koivisto and Braig, 2003). When males and females have been found in field populations the population are supposed to reproduce sexually, however as sex rarely has been determined in specimens from field samples, it has never been realized whether naturally occurring *F. candida* populations reproduce sexually or could have a very low rate of male production. Elin Jørgensen, environmental technician at NERI, discovered few *F. candida* males in our laboratory cultures in 1993. At that time it was not clear if these males actively took part in sexual reproduction and if a sexually reproducing *F. candida* population could emerge with these males. A second question arising from the presence of *Wolbachia* in *F. candida* was whether the rate of males would change during the life-time of female *F. candida*. We now know that the males, when reared with females in 10:10 proportion, do not seem to enable establishment of a sexual population with a normal ratio of males and females. Our observations indicate that only about 1 male is produced per 10,000 female offspring during the 8^{th} and 10^{th} reproductive instars, however for older *F. candida* females, it increases to one for every thousand juveniles.

2.3 Genetic variability

When investigating genetic differences, low variability was found in laboratory populations of *F. candida* compared to *F. fimetaria* (Simonsen and Christensen, 2001). Low genetic variability is considered a benefit for a standard test species because it may decrease variability of survival and reproduction between individuals as well as response to toxicants. The variability between clones has been demonstrated to convey minor differences in responses to chemicals and for some chemicals, no differences in sensitivity could be detected at all (Crommentuijn *et al.*, 1995; Chenon *et al.*, 2000). Genetic variability of *F. fimetaria* has not yet been investigated. To ensure that the species used for testing is well characterised, species cultures would have to be delivered by laboratories with a quality assurance system, such as GLP, who can certify the genetic strain and clone variability.

[3] We have made an analysis of *Wolbachia* in *F. candida* and *F. fimetaria* (Krogh et al in prep.)

2.4 Alternative Collembolan test species

Several authors have suggested alternative collembolan species to be used for testing standards because *F. candida* has limited ecological relevance due to its absence from many natural or agricultural habitats. This has led to suggestions of such species as *Paronychiurus kimi* (Son *et al.*, 2007), *Sinella communis* and *Proisotoma minuta* (Greenslade and Vaughan, 2003) as appropriate test species. Other collembolan species could be selected for testing such as e.g. *Isotoma viridis* (Wiles and Krogh, 1998), *Isotoma anglicana*, *Orchesella cincta*, *Sinella curviseta*, *Orthonychiurus folsomi* (Environment-Canada, 2007), and *Mesaphorura macrochaeta*. The result of a bibliographic search of papers referring to single collembolan species is presented in Annex 3 to give an indication of the present level of scientific knowledge. A number of prerequisites must be fulfilled in advance before using alternative species:

- an unequivocal identification
- a sound rationale for the selection of the species
- ensuring that the reproductive biology is included in the testing phase so it will be a potential target during the exposure
- life-history must be known: age at maturation, duration of egg development and instars subject to exposure
- optimal growth and reproduction conditions are provided with the test substrate and food supply
- variability is sufficiently low for precise and accurate toxicity estimation.

The choice of *F. fimetaria* as a test species was supported in an evaluation based on practical arguments, acceptability of tests and ecological significance (Van Gestel, 1998).

2.5 Differences in susceptibility of the two species

While Krogh (1995) reported no crucial differences between *F. fimetaria* and *F. candida*, Diao *et al*. (2007) found a difference which proved to be significant for mortality. Pedersen *et al.* (2000) found that male *F. fimetaria* differed from females in their copper body burden but reported no statistically significant differences between the growth and reproduction endpoints for the two species.

2.6 Variability in Reproduction Rates

Variability of *F. candida* reproduction is obvious from different scientific publications. Van Amelsvoort and Usher (1989) observed probably the lowest reproduction rate of *F. candida* fed Baker's yeast, with the population already declining after the first clutch appeared; this was in remarkable contrast to the classical findings by Snider (1973)[4] where *F. candida* produced eggs throughout its lifetime. According to her findings, 10 *F. candida* females would on the average produce 628 juveniles on plaster-charcoal during the first two reproductive instars, instar 6 and 8; this is probably possible in soil as well. However, if the eggs of the third clutch produced by instar 10, hatched before the 4 week test duration of the *F. candida* test, a mean of 1342 juveniles would be produced per replicate. This would require that the duration of instars and egg development are faster than the

[4] This observation led to the conclusion that yeast would affect the life history tactics by *F. candida*.

average. For *F. fimetaria*, which would produce 400 juveniles during the 3 week test suggested here (section 2.2), the variability may be due to similar changes in timing and instar duration. Attempts to clarify the sources of variability was done by Axelsen et al. (1998) in a modelling exercise. They found that a precise sexual differentiation, when individuals for testing are selected from a synchronous culture of *F. fimetaria*, was important for variability. Crouau and Cazes (2003) demonstrated that the individual age and test duration was important for *F. candida* testing when performed according to ISO 11267 (ISO, 1999).

3 Testing results obtained at NERI, 1994 to 1999

3.1 Introduction

Since 1992, plenty of toxicity tests and experiments have been conducted with *F. fimetaria* at Department of Terrestrial Ecology, Danish National Environmental Research Institute (NERI). This section describes a compilation of a subset of these tests to illustrate the intra-laboratory variability of an experienced testing facility. The data has previously been reported to Environment Canada (Krogh, 2004).

The database included the control reproduction observed in 57 tests with *F. fimetaria* (Annex 4). The procedure followed was a standard test guideline in effect at the laboratory since 1994 (Krogh, 1995; Wiles and Krogh, 1998). The tests were performed during a period of 6 years, potentially representing variability of culture health and performance properties. Different soil types ranging from sandy soils to clay soils were used in the tests.

3.2 Performance

The mean survival of initially 20 adult *F. fimetaria,* 10 females and 10 males, and their reproduction in the 3 week standard tests were: 17.7 [17.2-18.2] and 430.8 [405-457], respectively; for frequency distributions see Fig. 3; the reproduction was normally distributed, P>15% (Kolmogorov's D statistic). 5% of the tests would have a reproduction ≤233 according to the normally distributed reproduction. The average CV was 18.1 [15.4-21.2][5] None of the tests had a mean reproduction less than 100, but 7% had a coefficient of variation CV>30% (Annex 4), which is the validity criteria of the ISO *F. candida* test (ISO, 1999). Two of the CV's qualified as outliers, according to the validity criterion and 14% of the tests had an average adult mortality >20%, the validity criteria, and 2% a mortality >30%.

F. fimetaria performed generally well in all soils tested, so when reduced performance was observed this might be the result of other factors, such as health condition, seasonality or feeding condition.

3.3 Influence of soil type

Linking soil properties to collembolan performance, i.e. survival and reproduction, must be done with caution, due to the fact that the NERI data does not originate from experimentally designed studies with soil factors applied as treatments, but from independently assessments of the performance. Thus, the level of performance in the tests may have been caused by the actual condition of the test

[5] Confidence intervals of the log-normal distribution: $\overline{Y} + \frac{S^2}{2} \pm t\sqrt{\frac{S^2}{n} + \frac{S^4}{2(n-1)}}$ (Olsson, 2005) obtained by back-transformation.

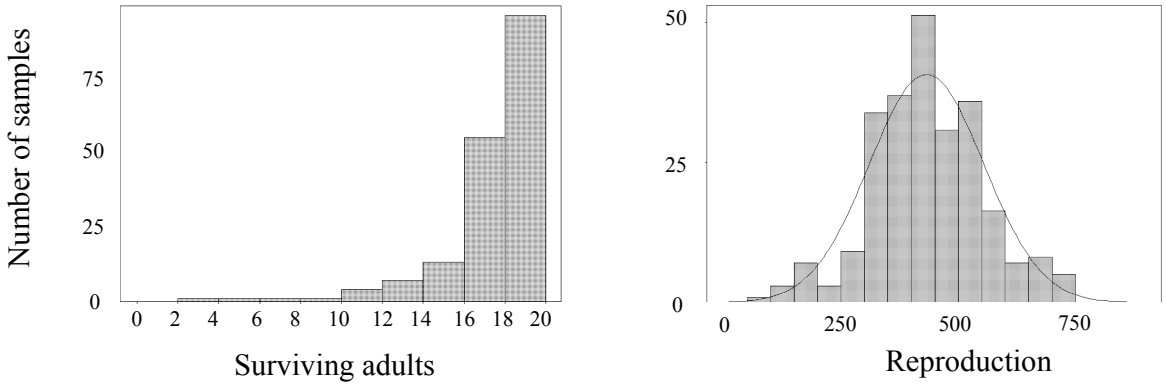

Fig. 3. Frequency distributions of surviving *F. fimetaria* adults of the initial 20 males and females and their reproduction for each replicate sample analysed, n=243, the normal distribution with mean 433.7 and variance 14,924 is included on the reproduction graph.

animals. Uncontrolled microbial factors differing from test to test may exert an influence on performance too.

To explore the relationships between the two performance measurements and soil characteristics, the correlations are given in Table 1. Adult survival was not correlated with soil constituents but reproductive output, in terms of number of juveniles, was significantly positively correlated with clay and silt but was negatively correlated with sand content of the soil.

3.4 Conclusion

The soil particle fractions clay and silt was positively correlated with the reproduction, while sand was negatively correlated with the reproduction. The performance of *F. fimetaria* was generally good for all soil types tested, with only less than an average of 200 juveniles per replicate in a control series observed in 2 tests. Survival was on the average 88.5% and was not affected by the soil types. Test performance was equivalent to the requirements for the *F. candida* test and therefore supports the same validity criteria as stated in ISO 11267: a mean maximum adult mortality of 20%, a mean minimum reproductive output of 100 juveniles in the controls with a maximum coefficient of variation (CV) of 30%.

The tests with *F. fimetaria* met the validity criteria as defined in the ISO 11267 standard (ISO, 1999) of at least 100 juveniles in the controls and in 91% of the tests the CV<30%

Table 1 Pearson correlation coefficients and the significance of the correlations between the soil characteristics and performance data of *F. fimetaria* from standard tests. Number of observations, n=243.

		Correlation, r	Correlation strength, r^2	Significance
Clay	Organic matter	7.5%	0.6%	24%
Silt	Organic matter	14%	1.9%	3%
Silt	Clay	91%	83%	<0.01%
Sand	Organic matter	-22%	5.0%	0%
Sand	Clay	-95%	90%	<0.01%
Sand	Silt	-99%	97%	<0.01%
Adult	Organic matter	1.5%	0.0%	92%
Adult	Clay	4.2%	0.2%	66%
Adult	Silt	4.0%	0.2%	65%
Adult	Sand	-3.5%	0.1%	71%
Juveniles	Organic matter	-1.0%	0.0%	87%
Juveniles	Clay	17%	2.9%	0.75%
Juveniles	Silt	22%	4.6%	0.07%
Juveniles	Sand	-21%	4.3%	0.1%
Juveniles	Adult	34%	11%	<0.01%

4 Ringtest results

4.1 Test guideline

A draft OECD test guideline was developed in the prevalidation phase of this project (OECD, 2006b) by Scott-Fordsmand and Krogh (2005) and was changed according to input from ringtest participants and further refined during the final reporting phase (Annex 6). Existing OECD guidelines were used as templates to ensure consistency and to ensure that the content was sufficient to perform the test.

F. candida and *F. fimetaria* is reared in lab cultures in closed containers with a bottom layer of a mixture of plaster of Paris and activated charcoal in a ratio of 9:1 by weight. The charcoal absorbs waste products that may be harmful to the optimal productivity of the cultures. The black colour of the bottom layer eases the visibility of the white collembolans. Wholes and furrows in the plaster may help stimulating oviposition (Fountain and Hopkin, 2005), although this was not needed for our cultures to thrive. The substrate is kept moist but not waterlogged to ensure saturated air humidity. The collembolans are watered and fed granulated dry Baker's yeast weekly; during this operation they are aerated.

Breeding of synchronous cultures is induced by transferring adults to fresh containers and collecting the eggs after three days, recommendable over a week-end. Alternatively the adults may be removed from the substrate and the eggs left behind. In the first case eggs are collected, in the second case adults are removed. After approx. 10 days the eggs hatch and at the age of 9-12 days the juveniles of *F. candida* or the 23-26 days old adults of *F. fimetaria* are ready for testing. Allowing for an age range span of 3 days in the test has important practical consequences, as it now provides for a working schedule that does not involve working with the test during the weekend spanning over 3 days.

The test exposes the collembolans to chemicals through the test soil, which is the artificial OECD soil based on a recipe originating from the earthworm acute test (OECD, 1984). On the day of preparing the mixture of moist soil and chemical collembolans, 10 *F. candida* or 10 male and 10 female *F. fimetaria* are added.

Test and breeding conditions are 20 ^{o}C and a light:dark cycle of 12:12 hours and light intensity of 400–800 lux.

At test termination after 3 weeks for *F. fimetaria* and 4 weeks for *F. candida* the collembolans are removed from the soil by flotation or heat extraction. While flotation immediately terminates the test, heat extraction runs for 2 days where the collembolans actively have to move out of the soil.

The practicability of performing the tests with the two species is identical with the exception of the need to discriminate the *F. fimetaria* males from females.

4.2 Participants

Participants spanned a broad range of laboratories from highly experienced professional contract laboratories to research laboratories at universities. This has aided in exposing the guideline procedure to diverse situations exposing weak or

yet unresolved issues even for the existing ISO standard test for *F. candida*. A list of the 14 participating laboratories is presented in Annex 1; they have been given a code to enable linking the data to a certain laboratory in Annex 2. A total of 51 tests were performed in the ringtest exercise (Table 4).

4.3 Model chemicals

The three model compounds chosen for the ringtest are evaluated for use as positive controls and reference chemicals for the guideline. Boric acid is the preferred candidate because it is easily accessible, while dimethoate is less accessible and the commercial production may cease, and $CuCl_2$ is more difficult to handle in the test due to the need to compensate for a pH effect changing with the $CuCl_2$ concentration. While boric acid and copper chloride are generally available, dimethoate was kindly delivered to the participants from Cheminova.

The model chemicals boric (H_3BO_4), copper chloride ($CuCl_2$), and the insecticide dimethoate, were chosen to cover 3 different modes of action, i.e. effects caused by: acidity, heavy metal inhibition of fecundity and inhibition of choline esterase. The benefit of boric acid is its accessibility and it has been suggested as a positive control for tests with plants, mites and collembolans (Environment-Canada, 2005b, 2007; OECD, 2007). Boric acid was applied in the concentrations corresponding to 0, 25, 50, 100, 200, 400, 800 mg kg^{-1}; anhydrous copper chloride in the concentrations: 0, 200, 400, 800, 1200, 1600, 2000; and dimethoate in the concentrations 0, 0.25, 0.5, 1, 2, 3, 4 mg kg^{-1}.

4.4 Range finding

In many cases range-finding tests were not performed or did not contribute to an appropriate final concentration series. A general problem of range-finding is that it is usually performed as a lethal test, but is used to guide the selection of concentrations for reproduction tests. Obviously this would give faulty guidance for chemicals with sublethal effects.

4.5 Statistical analysis

Statistical analyses for the estimation of control mortality and reproduction and concentrations causing a decrease of 10% and 50% in reproduction or survival (i.e., LC_{10}, LC_{50}, EC_{50} and EC_{50}) and their 95% confidence limits were performed using SAS/STAT® version 9.1.3 procedures NLIN and NLMIXED (SAS-Institute-Inc., 2004b). Non-linear modelling was used to estimate concentration-response relationships by fitting the binomially distributed mortality data to the mortality rate (m) formula (probit analysis):

$$m = c + (1 - c)\,\Phi(a+bd)$$

where c is control mortality rate, Φ (phi) is the cumulative normal probability function, a slides the curve along the x-axis, b determines the slope, and d is the mg kg^{-1} concentration of the testing compound in soil. Other models were employed when it was more appropriate to fit the actual mortality data:
asymptotic growth, $c+(1-c)(1-e^{ad})$, and exponential growth, $c \cdot e^{ad}$. The reproduction data was fit to the sigmoid model:

$$\frac{k}{1+\left(\frac{d}{EC_{50}}\right)^{a}}$$

and to exponential decay, $c \cdot e^{-ad}$, and a convex decrease, $(k/(1-b)) \cdot (1-b \cdot e^{(ad)})$.

Often a concentration-response curve does not contain sufficient information to estimate parameters for a non-linear curve such as the logistic or exponential because the curve is simply linear, the variability is too high[6], or the fitting procedure cannot attain reasonable parameters, i.e., it cannot converge. In such cases, there still may be a clear and significant decrease of the response with increasing concentration, and therefore, a linear section of the data can be selected by choosing a lower and an upper concentration limit within the decreasing section. Responses outside and on these borders were then added together and a new linear dataset created containing the sum of data for the upper and lower limit and the original data between these concentrations.

95% confidence limits are written in brackets [ ] throughout. Tests for normality were performed with the distribution analysis tool of SAS/INSIGHT (SAS-Institute-Inc., 2004a). The Coefficient of Variation (CV) is calculated as $\%\frac{\text{STD}}{\text{Mean}}$.

4.6 Experimental design

A spacing factor of 1.8 has been recommended for other tests such as the *H. aculeifer* and the enchytraeid test (OECD, 2004a, 2007), while the guideline on plant growth states that "the number and spacing of the concentrations or rates should be sufficient to generate a reliable concentration-response relationship and regression equation and give an estimate of the ECx or ERx." (OECD, 2006c). The ringtest does not *per se* support the spacing factor approach as an inspection of the concentration-response figures reveal (Fig. 4 to Fig. 7). As the purpose of using the spacing factor is to evenly cover the whole response curve, the actual result of the factor is to lump together many low concentrations at the expense of covering the higher concentrations.

4.7 Test conditions

The draft guideline (Annex 6), prescribes a soil humidity content of approximately 50% of the soil's WHC, but it should be ensured that the soil will maintain a crumbled structure. Hence, the water content is not regulated according to the usual 50% of the WHC. Generally the loss of water is controlled during the test and should not impose any stress on the collembolans.

4.8 Control mortality

The highest mortality was observed in tests with *F. fimetaria* (Table 2).For *F. candida* control mortality was less than 20% for 79% of the tests and *F. fimetaria* had a mortality of less than 20% for 44% of the tests (Table 2). These proportions were significantly different. The failure of some tests to meet the mortality validity criterion is indeed expected to happen even for highly experienced laboratories but at a much lower rate as observed here for *F. fimetaria*.

[6] Presently no OECD guideline has validity criteria for the power of a test and the confidence limits of EC_X-estimates, and high variability will lead to lower power and undesirable wide confidence intervals. A maximum of 50% width of the confidence interval would be a reasonable validity criterion. To implement such a criterion guidance should be followed concerning modelling as provided by Environment Canada and OECD (Environment-Canada, 2005a; OECD, 2006a).

Table 2 Summary of control performance evaluation criteria for the two collembolan tests for all tests, including tests not fulfilling the validity criteria, and detection of number of outliers for the boric acid tests. Percentages are the % of tests fulfilling the criteria. CV: Coefficient of Variation for the reproduction. Juv.: Reproductive output of the test in number of juveniles. Raw data presented in Annex 4.

	F. fimetaria	*F. candida*
Mean reproduction	132 [67-197]	399 [310-488]
Mean mortality	35% [22-48]	14% [8.7-20]
Mean control mortality <20%	44%	79%
Mean control reproduction > 100 juv.	50%	97%
CV < 30%	44%	76%
Mean CV	59.5	25.5
Mean CV when reproduction>100 juv.	28.8	24.8
Outliers: Inter-laboratory variability of LC_{50}, *h* (P<1%)	0	1
Outliers: Inter-laboratory variability of EC_{50}, *h* (P<1%)	0	0

4.9 Control reproduction

The validity criterion for the *F. candida*, *F. fimetaria* and *O. folsomi* control reproduction is an average minimum of 100 juveniles (ISO, 1999; Environment-Canada, 2007). The coefficient of variability (CV) of the reproduction has been set to a maximum of 30% (ISO, 1999), identical to the earthworm and draft mite reproduction tests (ISO, 1998; OECD, 2004b, 2007). For the ringtest, it was suggested to adopt the validity criteria of 100 juveniles and a CV of <30% for both species; therefore, these values are used for the evaluation of the ringtest results (Table 2). For comparison it should be noted that experience from the ringtest paving the way for the *F. candida* ISO 11267 standard has shown that variability in terms of the CV was greater than 30% for 30% of the tests (BBA, 1995) and 10% of the tests had a mean number of juveniles in the controls less than 100. Intrinsically *F. candida* has a reproduction rate twice the reproduction rate of *F. fimetaria*.

One of the *F. candida* tests had a reproductive output below the suggested validity criteria of 100 juveniles and 24% produced less than 200 juveniles. *F. fimetaria* produced less than 100 juveniles in 43% of the tests. The mean reproductive CV for *F. fimetaria* was significantly larger than the CV for *F. candida* (ANOVA F-test P<0.1%) (Table 2). But when excluding the data sets not meeting the mean minimum 100 juvenile reproduction criterion, the mean CV of the *F. candida* control reproduction was 23.6 [19-28] (n=33) and 28.7 [17-40] (n=8) for *F. fimetaria*, which did not differ significantly from each other (one-way ANOVA, P>5%) for the mean or the variance. Thus, this demonstrates that if a sufficient reproduction is obtained, a *F. fimetaria* test would have a normally accepted CV. In other words it can be concluded that the precision of the control reproduction is

Table 3 Mean LC_{50} and EC_{50} for the two species and the three model compounds. Numbers in brackets: 95% confidence limits.

Species	Compound	N	LC_{50}	EC_{50}
F. candida	Boric acid	16	259 [154-364]	90.8 [61.8-120]
	Copper	11	1541 [442-2639]	1251 [423-2080]
	Dimethoate	8	2.1 [0.74-3.4]	1.65 [0.4-2.9]
F. fimetaria	Boric acid	9	560 [271-849]	107 [67.9-146]
	Copper	2		1260 [-3551-6070]
	Dimethoate	3	1.0 [-1.7-3.7]	0.81 [-1.9-3.5]

potentially identical for the two species. The reproduction was particularly high in three *F. candida* tests (ref. no. 4, 48, 49), and this may be explained by the appearance of a third clutch (see section 2.6).

4.10 Variability of testing results

The inter-laboratory variability is evaluated by calculating *h*, the standardized difference of a toxicity test result observed for one laboratory from the mean toxicity values as given in Table 2 (Weyers *et al.*, 2002). The test statistic $(x-\mu)/STD$ is t-distributed and if x, the individual toxicity estimate from one laboratory, deviates considerably from the mean, it is considered an outlier. The criterion for outliers consists of toxicity estimates that differ from the mean at the 1% level of significance (Weyers *et al.*, 2002). For mortality, only the LC_{50} of 815 mg kg^{-1} for boric acid (ref. no. 43) qualified as an outlier, which was the outcome of an otherwise fully valid *F. candida* test. For the boric acid reproduction tests, none of the EC_{50} values were detected as outliers.

Graphical presentations of the EC_{50}'s and the LC_{50}'s are presented in Fig. 8 and Fig. 9 for all three testing compounds. However, as boric acid testing results were most numerous only those have been used for evaluation of the endpoint variability.

Boric acid has a pronounced sublethal effect for both species (Table 3). The variances of the two identical mean EC_{50}'s for *F. candida* and *F. fimetaria* with boric acid were not significantly different (P>10%) and both proved to conform to a normal distribution (P>15% for Kolmogorov's D). The precision of the EC_{50} estimates in terms of width of the 95% confidence limits (Table 4), were roughly spread up to ±50% around the EC_{50} for both species, and they were statistically identical. This precision depends on a proper model choice and it would not reflect the true precision if the model has a poor fit.

The LC_{50} for *F. fimetaria* were significantly higher than the LC_{50} of *F. candida* (ANOVA, F-test) (Table 3), but the apparent higher variability of *F. fimetaria* LC_{50}'ies is related to the mean and vanishes by transformation to obtain variance-homogeneity. The precision in terms of the width of the 95% confidence limits of the LC_{50}-estimates were seemingly better for *F. candida* ranging up to ±50% of the LC_{50}, but the wider range of *F. fimetaria*, ±100%, did not differ significantly (ANOVA, P>5%) (Table 4). When excluding the two *F. fimetaria* boric acid tests with a control mortality >50%, the LC_{50}'ies of *F. fimetaria* tests still varied within a factor of 2.6 (n=7, CV=38%) and the *F. candida* tests varied within a factor of 3.1 (n=16, CV=70%) in relation to the mean LC_{50}. This alternative to the outlier analysis way of describing variability (Table 2) gives the same result, and it is concluded that variability of the LC_{50} and EC_{50} toxicity outcome does not differ for the two species.

4.11 Conclusion

The reliability and performance of the test with the new standard species *F. fimetaria* were assessed by comparing its performance with the *F. candida* test, which then acted as a reference test method currently accepted by regulatory agencies, while still being a candidate species of the new draft guideline. The range of criteria used for this assessment was largely fulfilled, but the control reproduction and survival performed badly in some tests. In spite of this the toxicity was accurately and precisely estimated, in particular when invalid test results were omitted.

Table 4 Control mortality and reproduction and toxicity endpoints of the ringtest in terms of LC10, LC50, EC10 and EC50 estimated from the complete concentration-response data of each test. Missing cells is due to no effects detected or 50% effect levels outside the concentration range. C.V.: Coefficicient of variability of the control reproduction.

Ref. no.			Control mortality %	LC10	LC50	Control reproduction	C.V.	EC10	EC50
1	*F. candida*	Boric acid	17 [12-22]	101 [62.4-139]	121 [-12.5-254.1]	181 [152-211]	43.6	38.5 [8.8-68.1]	71.1 [45.6-96.7]
3	*F. candida*	Boric acid	10 [4-16]	101 [48.9-154]	195 [167-224]	1246 [1191-1301]		6.9 [6.1-7.8]	45.7 [40.1-51.2]
7	*F. candida*	Boric acid	14 [3-25]	55.8 [8.6-103]	108 [82-133]	413 [347-480]	15.6	38.8 [-24.0-102]	61.0 [45.9-76.1]
9	*F. candida*	Boric acid	0.0[7] [-7.8-7.5]	186 [87.1-285]	316 [275-357]	329 [303-355]	12.8	159 [129.0-189]	216 [201-231]
12	*F. candida*	Boric acid	47 [38-57]			288 [229-348]	41.1	76.0 [30.0-122]	120 [87-153]
16	*F. candida*	Boric acid	34 [25-43]	30.5 [17.0-44.0]	200 [112-289]	392 [342-443]	29.1	42.3 [17.7-66.8]	78.4 [57.9-98.9]
21	*F. candida*	Boric acid	6.5 [2.8-10.1]	172 [117-227]	306 [271-341]	413 [390-436]	14.8	30.8 [23.6-38.0]	59.3 [52.9-65.7]
26	*F. candida*	Boric acid	45 [36-54]	21.6 [13.4-29.9]	142 [88.2-196]	140 [110-171]	61.1	2.7 [2.3-3.1]	13.5 [11.7-15.4]
31	*F. candida*	Boric acid	6.3 [1.5-11.1]	22.9 [17.3-28.5]	151 [114-188]	338 [303-373]	24.6	20.0 [10.6-29.4]	42.7 [34.0-51.4]
32	*F. candida*	Boric acid	5.3 [1.6-9.1]	610 [-349-1568]		319 [291-347]	19.0	79.1 [48.4-110]	149 [124-175]
37	*F. candida*	Boric acid	4.1 [0.5-7.6]	227 [224-229]	333 [322-344]	207 [192-222]	5.5	92.3 [70.3-114]	137 [115-160]
38	*F. candida*	Boric acid	0.8 [-1.6-3.2]	195 [131-258]	378 [334-423]	360 [326-394]	31.6	112.7 [48.4-177]	169 [132-205]
43	*F. candida*	Boric acid	19 [10-28]	243 [178-308]	815 [492-1138]	356 [297-414]	22.3	23.1 [-67.6-114]	89.9 [15.4-164]
46	*F. candida*	Boric acid	14 [2-27]	71.4 [-35.3-178]	232 [176-287]	314 [261-368]	48.2	13.6 [3.3-23.9]	40.2 [27.1-53.3]
49	*F. candida*	Boric acid	12 [7-16]	136 [98-173]	201 [182-220]	988 [905-1072]	15.9	25.6 N.E.	99.8 [88.1-112]
53	*F. candida*	Boric acid	11 [0.1-0.2]	94.8 [74.2-115]	124 [90.8-157]	508 [462-554]	12.8	29.7 [17.8-41.6]	59.8 [49.0-70.7]
2	*F. candida*	Copper	10 [6-14]	980 [-12045-14006]	1096 [-7472-9664]	198 [174-222]	25.0	96.4 [38.9-154]	256 [188-324]
10	*F. candida*	Copper	2.2 [-1.9-6.4]	560 [359-760]	2799 [1797-3802]	260 [240-280]	27.6	885 [876-895]	1227 [1178-1276]
15	*F. candida*	Copper	25 [19-31]	1667 [1644-1690]	1935 [1821-2048]	528 [428-628]	32.6	268 [99-437]	1811 [1534-2088]
22	*F. candida*	Copper	4.2 [0.8-7.5]	1464 [532-2396]		453 [427-479]	12.3	285 [226-345]	516 [460-573]
25	*F. candida*	Copper	47 [38-56]	261 [259-263]	507 [497-517]	140 [109-171]		13.1 [-147.8-173.9]	58.3 [-297.3-414.0]
30	*F. candida*	Copper	7.3 [1.0-13.7]	194 [95.5-293]	346 [298-394]	339 [304-375]		129 [70-189]	212 [181-242]
35	*F. candida*	Copper	5.5 [1.9-9.1]	1134 [891-1377]	2563 [1860-3265]	129 [106-152]	24.3	245 [20-470]	741 [455-1028]
36	*F. candida*	Copper	3.2 [0.2-6.1]			663 [609-716]	16.4	447 [325-570]	2236 [1623-2849]
41	*F. candida*	Copper	10 [4-15]	917 [347-1488]		359 [321-398]	23.7	214 [207-222]	472 [433-510]
44	*F. candida*	Copper	27 [17-37]			341 [267-416]	38.0	827 [-2730-4383]	4133 [-13651-21916]
48	*F. candida*	Copper	11 [6-16]			756 [689-822]	8.8	320 [64.5-575.0]	2104 [424.1-3782.9]

[7] The probit model actually resulted in a negative mortality.

Ref. no.		Control mortality %	LC10	LC50	Control reproduction	C.V.	EC10	EC50
4	*F. candida* Dimethoate	5.5 [2.2-8.8]	1.2 [0.8-1.7]	1.7 [1.5-1.8]	1241 [1192-1290]	8.8	1.1 [0.9-1.2]	1.4 [1.3-1.5]
11	*F. candida* Dimethoate	3.5 [0.4-6.7]	0.3 [0.3-0.3]	0.7 [0.6-0.7]	130 [112-148]	29.5	0.2 [0.2-0.3]	0.3 [0.2-0.4]
17	*F. candida* Dimethoate	19 [12-26]	0.5 [0.4-0.5]	1.5 [1.4-1.7]	590 [491-689]	14.5	0.37 [0.0077-0.72]	1.3 [0.89-1.78]
23	*F. candida* Dimethoate	4.7 [1.8-7.6]	1.7 [1.3-2.1]	2.6 [2.3-2.8]	150 [137-163]	25.4	0.9 [0.6-1.1]	1.3 [1.0-1.6]
24	*F. candida* Dimethoate	78 [73-84]	0.6 [0.5-0.6]	0.8 [0.7-0.8]	24.2 [17.0-31.4]	88.9	0.6 [0.5-0.6]	0.8 [0.7-0.8]
42	*F. candida* Dimethoate	29 [21-36]	2.1 [2.1-2.1]	2.6 [2.5-2.6]	263 [201-325]	24.3	1.2 [1.2-1.3]	2.0 [1.8-2.3]
45	*F. candida* Dimethoate	6.7 [-3-16]	1.9 [1.7-2.1]	5.5 [4.7-6.3]	488 [434-543]	17.1	1.0 [0.8-1.2]	5.0 [4.1-5.9]
56	*F. candida* Dimethoate	33 [0.2-0.4]	1.0 [0.7-1.4]	1.2 [-3.6-6.1]	403 [359-447]		0.8 [0.3-1.3]	1.1 [0.8-1.3]
5	*F. fimetaria* Boric acid	39 [35-43]	132 [128-136]	258 [238-278]	59.6 [55.5-63.8]	17.3	21.8 [21.6-22.0]	37.0 [36.0-38.0]
13	*F. fimetaria* Boric acid	30 [25-35]			6.2 [5.1-7.3]	28.3		
19	*F. fimetaria* Boric acid	42 [37-48]	157 [28-286]	785 [140-1429]	70.9 [52.3-89.6]	63.3	62.4 [-14.0-138.8]	112.7 [52.5-173]
28	*F. fimetaria* Boric acid	75 [70-80]	55.2 [55.0-55.4]	76.0 [74.8-77.2]	26.6 [18.6-34.6]		42.2 [38.1-46.3]	111.0 [90.7-131]
33	*F. fimetaria* Boric acid	16 [12-21]	140 [72-208]	699 [359-1039]	137.8 [128.2-147.4]	18.3	27.9 [21.7-34.1]	183.5 [143-224]
40	*F. fimetaria* Boric acid	8.9 [5.6-12]	190 [139-242]	951 [693-1209]	266 [246-286]	12.2	36.6 [17.9-55.2]	142.5 [111-174]
47	*F. fimetaria* Boric acid	58 [50-65]	100 [60-139]	440 [264-617]	54.2 [24.1-84.4]	98.9	22.4 [-91.7-137]	78.0 [-98.9-255]
50	*F. fimetaria* Boric acid	37 [32-43]	142 [-4-289]	712 [-22-1445]	292 [253-332]	15.7	56.2 [20.6-91.8]	130.1 [96.5-164]
52	*F. fimetaria* Boric acid	19 [7.6-31]			195 [137-254]	38.0	22.0 [-7.2-51.3]	62.3 [27.4-97.3]
14	*F. fimetaria* Copper	21 [16-26]			7.2 [6.4-8.0]	20.5		
18	*F. fimetaria* Copper	32 [26-39]			166 [123-209]	44.1		
34	*F. fimetaria* Copper	3.9 [1.0-6.9]	1892 [667-3117]		242 [189-295]	41.7	85.2 [-98.3-269]	881.0 [245.5-1516]
39	*F. fimetaria* Copper	4.1 [1.3-6.9]	2537 [485-4589]		266 [239-293]	18.8	0.0 [.-.]	1638.2 [1458-1819]
6	*F. fimetaria* Dimethoate	40 [33-47]	0.09 [0.07-0.11]	0.59 [0.45-0.73]	13.0 [6.7-19.3]	49.6	0.00 [-0.02-0.02]	0.12 [-0.30-0.53]
20	*F. fimetaria* Dimethoate	11 [7-15]	0.86 [0.84-0.87]	2.28 [2.22-2.34]	291 [243-339]	41.0	1.16 [0.28-2.04]	2.07 [1.46-2.68]
29	*F. fimetaria* Dimethoate	73 [68-77]	0.04 [0.02-0.05]	0.25 [0.15-0.34]	3.3 [0.7-5.9]		0.05 [0.01-0.09]	0.25 [0.07-0.44]

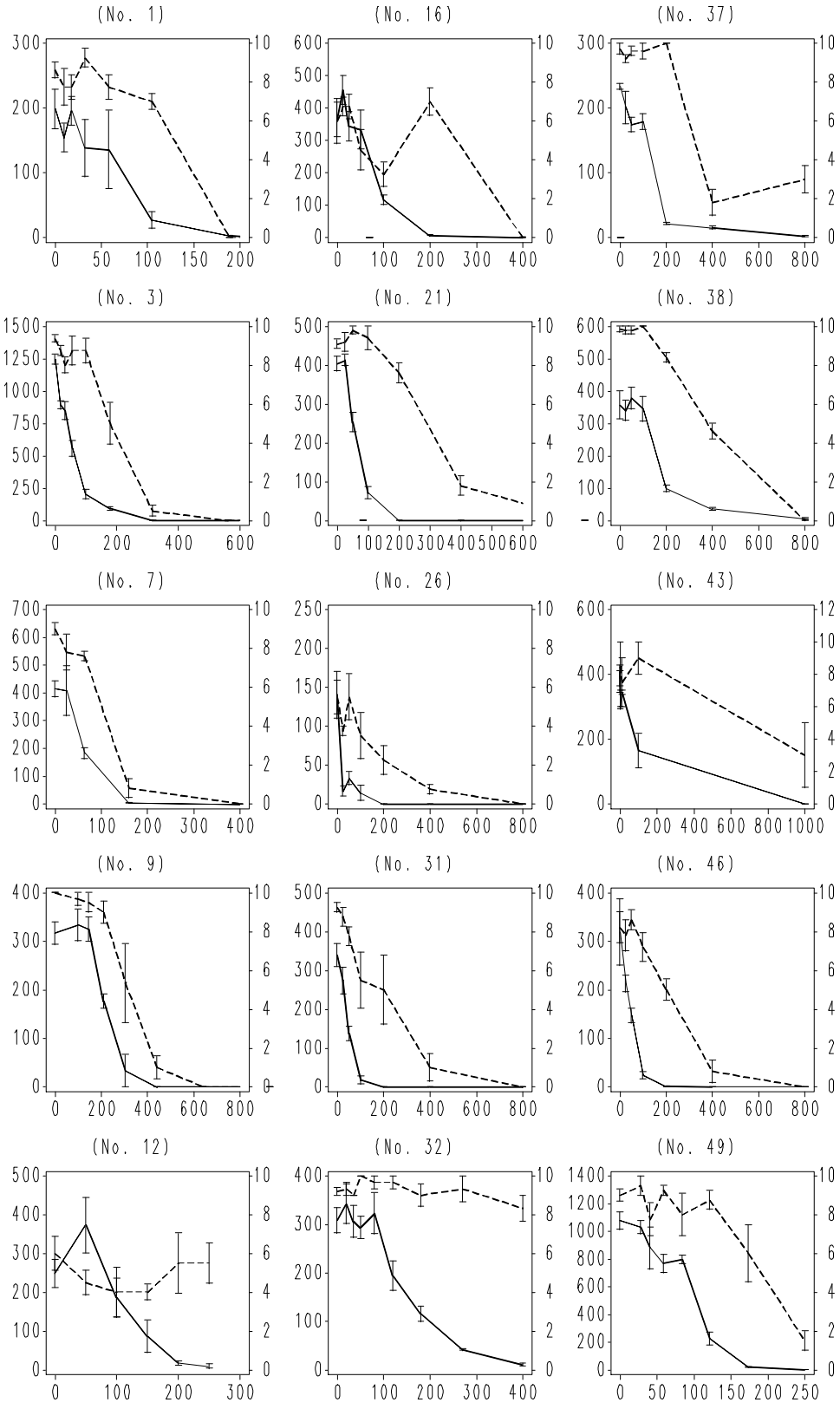

Fig. 4. *F. candida* testing results with boric acid. Horizontal axis: Nominal concentration of boric acid, mg kg^{-1} soil; Left vertical axis: number of juveniles produced per replicate; Right vertical axis surviving adults per replicate. Vertical bars: standard error of the mean. Numbers in brackets: ringtest ref. no. used to anonymize the laboratory. Broken line: adult survival per replicate; unbroken line reproduction, number of juveniles per replicate produced by initially 10 adults.

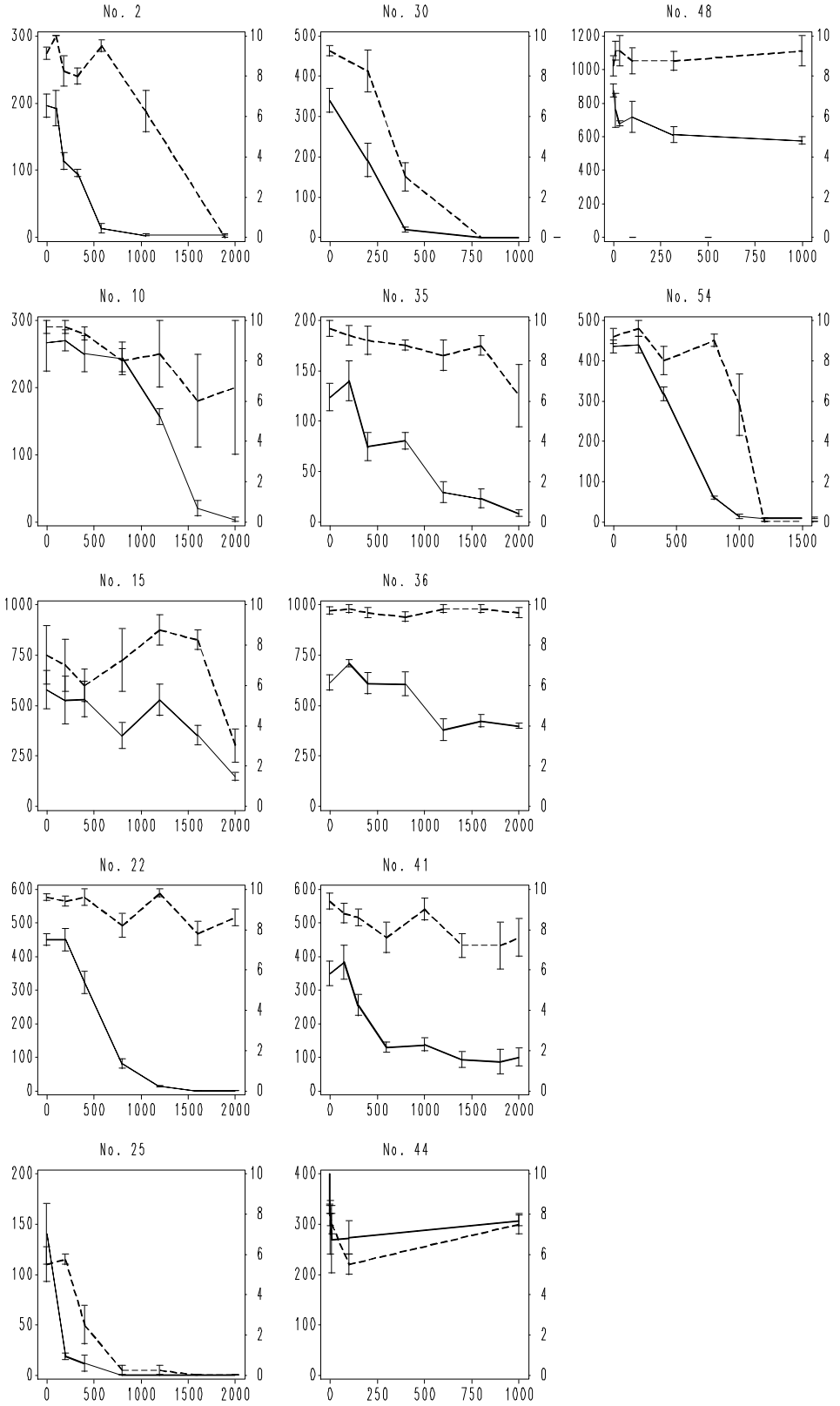

Fig. 5. *F. candida* testing results with nominal $CuCl_2$ concentration. Legend as Fig. 4.

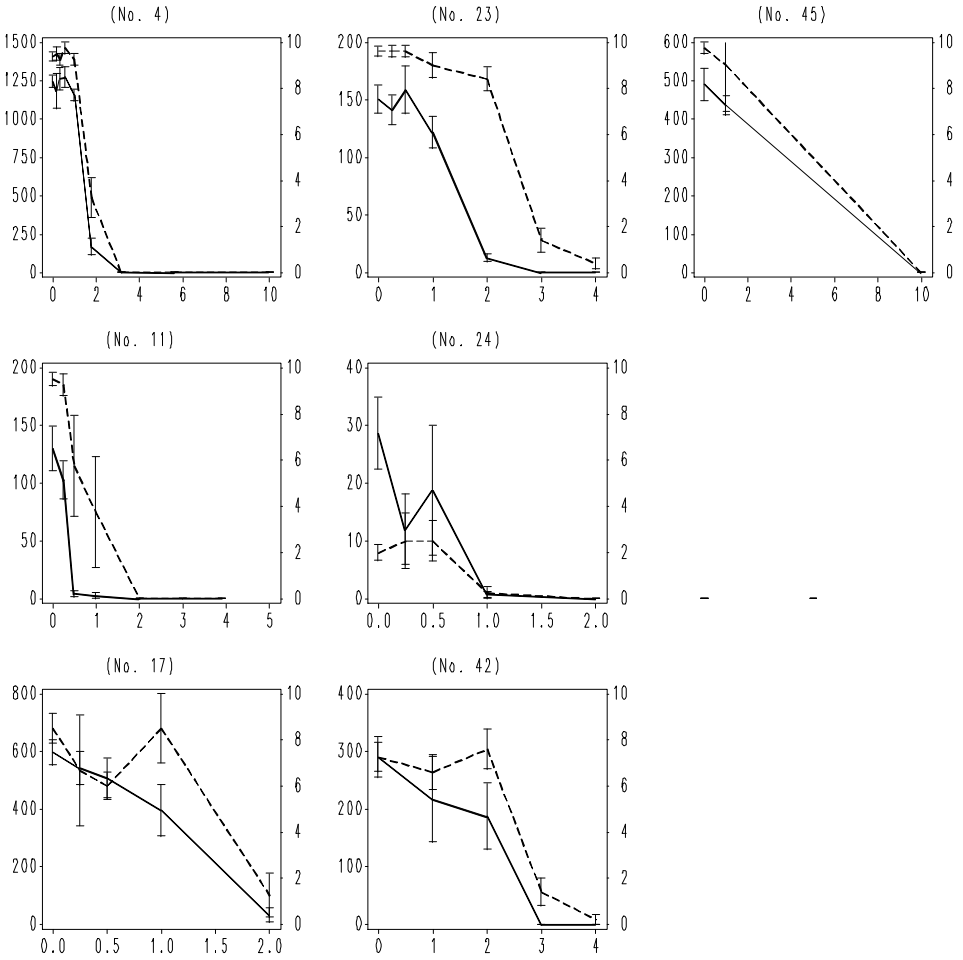

Fig. 6. *F. candida* testing results with nominal dimethoate concentration. Legend as Fig. 4

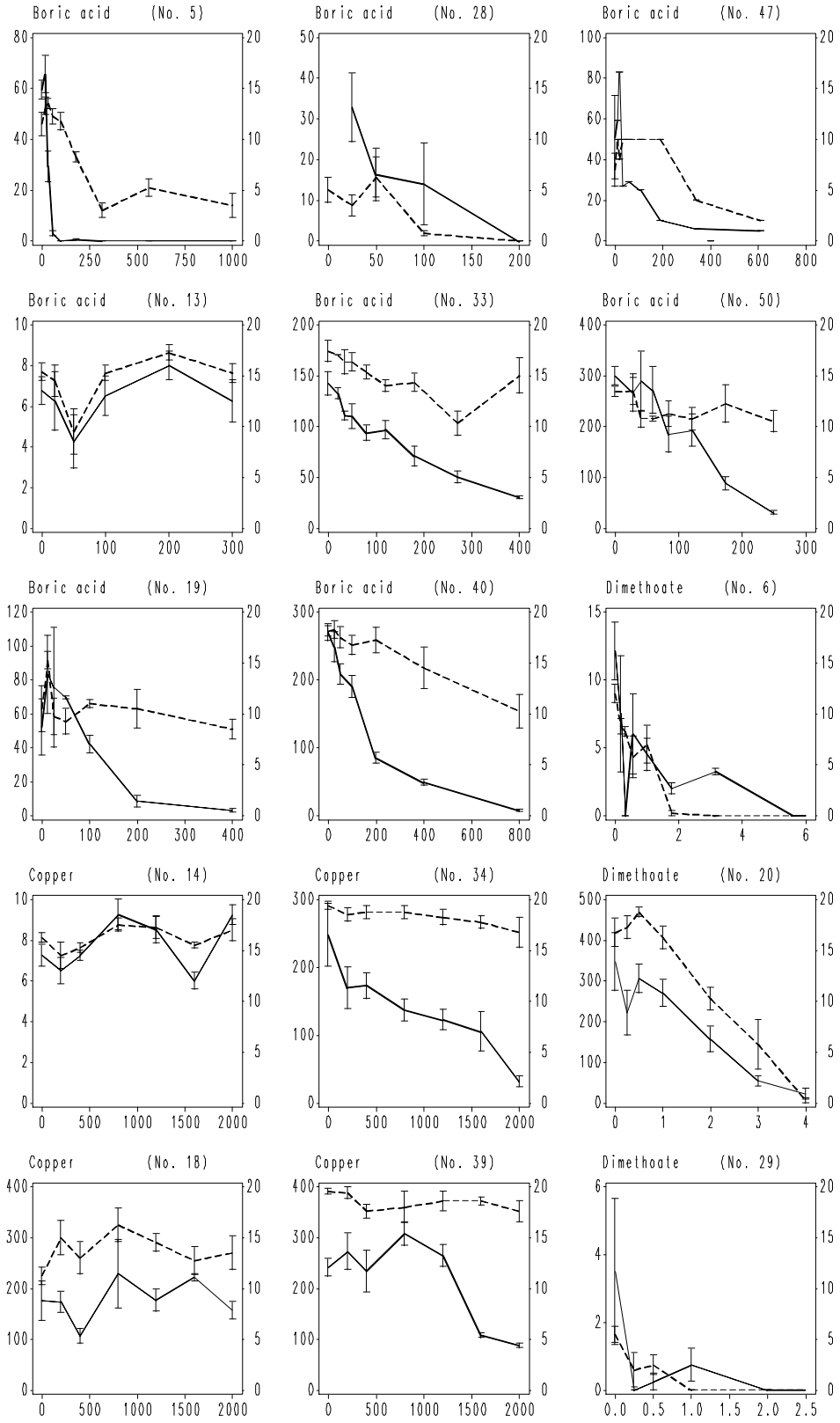

Fig. 7. *F. fimetaria* testing results with the three model compounds. Legend as Fig. 4, except unbroken line is the reproduction of juveniles per replicate produced by initially 20 adults.

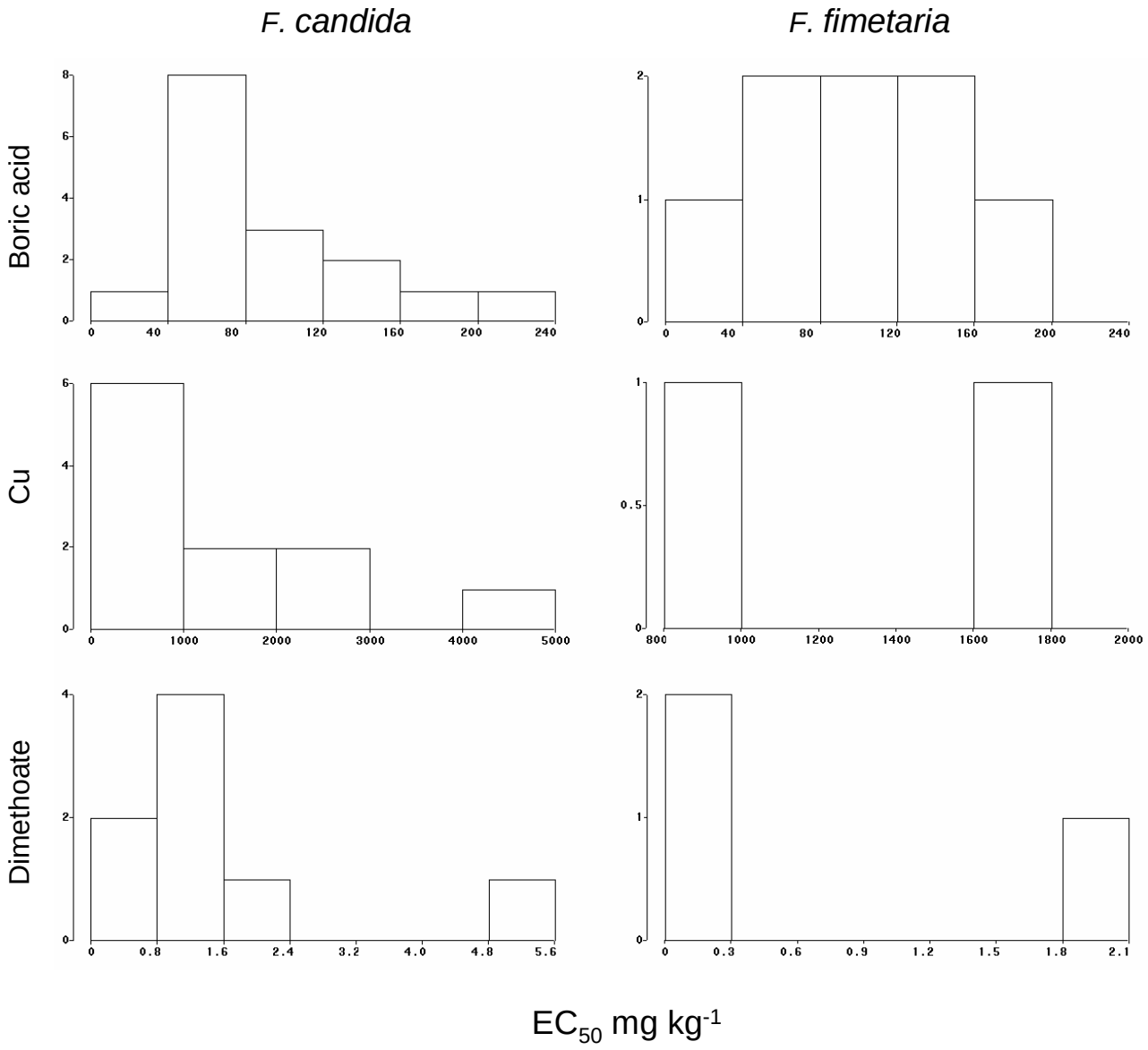

Fig. 8. Frequency distribution of chronic reproduction EC_{50}'ies from the ringtest. Y-axis number of occurrences of EC_{50}.

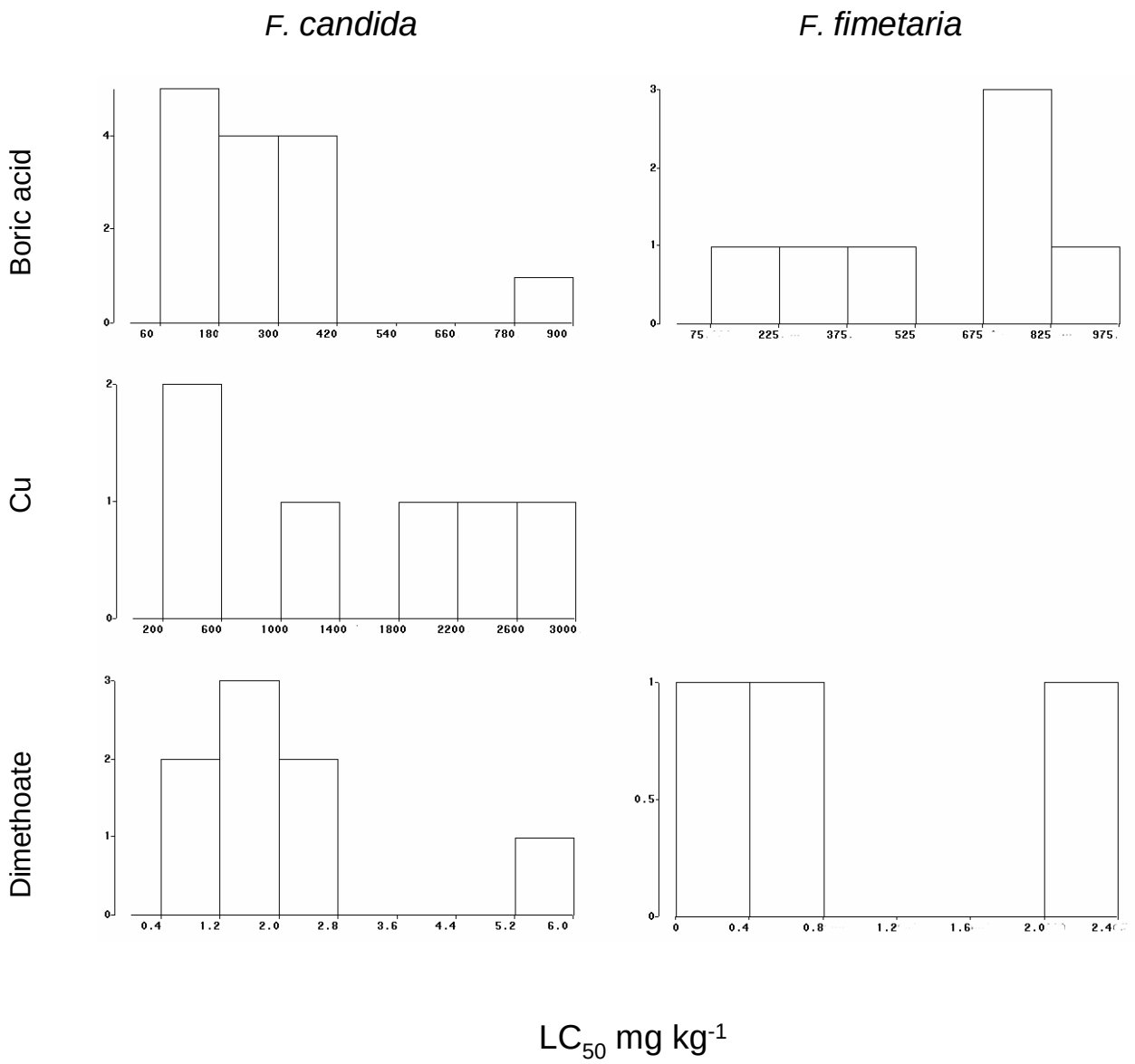

Fig. 9. Frequency distribution of chronic LC_{50}'ies from the ringtest. Y-axis number of occurrences of LC_{50}.

5 Summary and conclusions

The collembolans *Folsomia fimetaria* L. and *F. candida* Willem are proposed for inclusion in the *OECD guidelines for testing of chemicals* programme. The present ISO guideline 11267 for the collembolan *F. candida* has been applied successfully for toxicity testing in soil since its release, but due to the restricted parthenogenetic reproductive biology of *F. candida*, a collembolan species with a sexual mode of reproduction is needed. The relative *F. fimetaria* reproduces sexually and fulfils basic requirements for performance and feasibility of laboratory test animals. Each female reproductive instar of *F. fimetaria* requires the presence of males, of which three instars are usually completed during the standard reproductive tests with *F. candida* and *F. fimetaria*.

Extensive experience of the ringtest coordinating laboratory with the *F. fimetaria* test demonstrates intra-laboratory repeatability during the years 1994 to 1999. Fifty-seven control reproduction data sets tests with *F. fimetaria* all fulfilled the validity criterion for reproduction while the survival and the CV criteria were not met in 14% and 7% of the tests, respectively.

To provide sufficient information for the adoption of these collembolans for the OECD test guideline programme an international ringtest was initiated in 2005. For both species, test validity is achieved with a mean maximum adult mortality of 20%, a mean minimum reproductive output of 100 juveniles in the controls with a maximum coefficient of variation (CV) of 30%. Due to less experience of the participants in performing the *F. fimetaria* test, the control reproduction validity criteria were not met in half of the test, while the *F. candida* test generally was successful.

As the proposed test for *F. fimetaria* is mechanistically and functionally similar to the previously validated ISO *F. candida* test method with established performance criteria, the reliabilities of the test methods were compared and the overall test performance was evaluated against a range of criteria: control survival and reproduction and their variability, variability of toxicity endpoints for the model chemicals, the precision of the LC_{50} and EC_{50} and intra- and interlaboratory variability. Most data was produced for boric acid, so it was selected for this analysis. Survival was successful for 44% of the *F. fimetaria* tests and 79% of the *F. candida* tests. *F. fimetaria* tests with a valid reproduction had a CV similar to *F. candida*. A mean control reproduction of 130 and 400 for *F. fimetaria* and *F. candida*, respectively, resulted in 43% *F. fimetaria* tests not meeting the validity criteria, while the *F. candida* tests were practically all valid. The precision of the EC_{50} and LC_{50} estimates were identical for the two species. Stable EC_{50} and LC_{50} estimates with low inter-laboratory differences were produced by both tests with no outliers, except for one *F. candida* LC_{50} figure, and identical mean and variance for EC_{50} values with boric acid. Thus, based on the overall inter- and intralaboratory validation results the following validity criteria are proposed for both species in the draft test guideline and should be met in the untreated controls for a test to be considered valid:

- Adult mortality should not exceed a mean of 20 % at the end of the tests
- An average minimum of 100 juveniles per vessel should be produced during the test

- The coefficient of variation of the number of the juveniles per vessel should be less than 30%
- The reference compound, boric acid, should cause a 50% decrease in reproduction at 100 mg kg^{-1} in an OECD artificial soil substrate with 5% organic matter.

The proposed draft guideline includes comprehensive information and details to successfully perform toxicity testing with either of the two collembolan species. It is especially recommended to employ *F. fimetaria* for chemicals that are suspected to interfere with any parts of the reproductive biology of sexually reproducing species, while *F. candida* may be used more generally to assess less specific toxicity.

6 Acknowledgements

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Annex 2 Laboratory code

All the datasets were coded by a reference no. in Table 4, and in Fig. 4 to Fig. 7 to conceal the identity of the participating laboratories. However, the tests performed by each coded laboratory is presented below. B: Boric acid; C: copper chloride; D: Dimethoate.

Laboratory no.	Testing data set reference no. (Ref. no.)	*F. candida*	*F. fimetaria*
1	21, 22, 23	BCD	
2	1, 2	BC	
3	52, 53	B	B
4	12, 13, 14, 15, 16, 17, 18, 19, 20	BCD	BCD
5	55, 56	BD	
6	47, 48	CB	
7	36, 37, 38, 39, 40	BC	BC
8	9, 10, 11	BCD	
9	3, 4, 5, 6	BD	
10	49, 50	B	B
11	24, 25, 26, 27, 28, 29, 30, 31	BCD	BCD
12	7, 8	B	
13	32, 33, 34, 35	BC	BC
14	41, 42, 43, 44, 45, 46	BCD	

Annex 3 Bibliometric statistics

An impression of the accumulated knowledge available on collembolans was obtained by searching for common collembolan species names in Science Citation Index (ISI Web of Knowledge/Web of Science accessed Jan 2008). Thus, whenever a species name occurred in a title, abstract or keyword of papers it was counted as a citation. To indicate the attention papers on a certain species have received, the average number of citations was included as a "citation rate" (auto-citations excluded).

Species	ISI indexed papers	Citations	Citation rate
F. candida	398	2082	5
Orchesella cincta	109	1025	9
Onychiurus armatus/Protaphorura armata[8]	85	919	11
F. fimetaria	63	401	6
Parisotoma notabilis and *Isotoma notabilis*	42	467	11
Proisotoma minuta	38	155	4
Onychiurus arcticus/Megaphorura arctica	37	381	10
Isotoma viridis/anglicana	36	250	7
Sminthurus viridis	36	115	3
Folsomia quadrioculata	27	225	8
Isotomiella minor	20	138	7
Isotomurus palustris / *I. prasinus*	18	134	7
Sinella curviseta	13	50	4
Tullbergia macrochaeta, *Mesaphorura macrochaeta*, or *T. krausbaueri*	14	91	7
Paronychiurus kimi[9]	5	5	1
Onychiurus folsomi / *Orthonychiurus folsomi*[9]	2	9	5

Some species is present under more than one name either because the genus name has been changed or because a species complex has been illuminated and a new species name has been given to a member of a species complex.

[8] The similar onychiurid pair *Onychiurus fimatus*/*Protaphorura fimata* was mentioned 26 times, and cited 219 times, so the species group are similarly "popular" than *O. cincta*.
[9] Due to its association with Onychiurinae it should be considered together with the other members of this subfamily, which will make the subfamily Onychiurinae the second most commonly published species group.

Annex 4 Intralaboratory variability

Survival and reproduction of control replicate results from 57 *F. fimetaria* standard tests performed over a period of 6 years (n=243) at NERI. Mean and 95% C.L., are given for adult survival and reproduction. Adults: Average number of surviving adults of the initial 10 males and 10 females at test termination; Reproduction: Average number of juvenile *F. fimetaria* at test termination; 95% C.L.: Confidence Limits.

Soil origin	Adults 95% C.L.	Reproduction 95% C.L.	C.V.	Organic matter	Clay	Silt	Fine sand	Coarse sand	Code	Reference
Finnish	17.6[15.7-19.5]	498[335-662]	17.0	8.6	8.6	31.5	22.8	28.5	44	(Martikainen and Krogh, 1999)
OECD 10%	17.8[18.6-20.4]	451[321-580]	18.0	8.6	9.4	16.9	62.7	2.5	46	-
OECD 5%	17.6[16.5-18.7]	396[338-453]	11.8	4.4	8.9	13.6	67.6	5.5	50	-
OECD 2%	18.2[16.6-19.8]	382[304-460]	16.5	1.8	9.1	15.3	70	3.9	48	-
LUFA 2.2	19.4[18.7-20.1]	522[387-658]	20.9	3.9	5.1	5.6	34.8	54.6	74	
LUFA 2.2	15.2[12.8-17.6]	356[301-412]	12.6	3.9	5.1	5.6	34.8	54.6	75	
LUFA 2.2	18.0[15.7-20.3]	386[264-509]	19.9	3.9	5.1	5.6	34.8	54.6	76	
LUFA 2.2	20.0[-]	473[301-646]	29.3	3.9	5.1	5.6	34.8	54.6	73	(Martikainen and Krogh, 1999)
LUFA 2.2	19.0[17.7-20.3]	174[156-191]	6.3	3.9	5.1	5.6	34.8	54.6	54	-
LUFA 2.2	17.5[15.4-19.6]	158[116-199]	16.6	3.9	5.1	5.6	34.8	54.6	55	-
Flakkebjerg	18.0[-]	565[494-635]	7.8	1.8	15.1	34.2	25.5	23.4	4	
Askov	20.0[13.6-25.4]	506[349-663]	19.5	2.3	11.2	23.8	28.1	34.6	1	
Askov	19.0[16.7-21.3]	587[399-775]	20.2	2.3	11.2	23.8	28.1	34.6	2	
Askov	19.8[17.7-21.8]	498[363-632]	26.4	2.3	11.2	23.8	28.1	34.6	3	
Jyndevad	19.5[-]	506[336-676]	21.1	2.5	4.1	3.3	22.9	68	5	
Jyndevad	19.8[18.2-21.3]	420[85-754]	19.6	2.5	4.1	3.3	22.9	68	6	
Jyndevad	15.8[6.0-25.5]	405[198-612]	32.1	2.5	4.1	3.3	22.9	68	7	
Lundgård	9.5[-3.1-22.1]	287[-39-612]	71.4	2.7	6.2	8.6	15.8	66.9	8	(Holmstrup and Krogh, 2001)
Askov	15.8[11.6-19.9]	368[248-488]	20.5	2.8	13	22.3	23.6	38.4	9	(Holmstrup *et al.*, 2001)
Flakkebjerg	15.3[9.2-21.3]	296[147-444]	31.6	1.8	15.1	34.2	25.5	23.4	10	
Askov	18.5[16.4-20.6]	392[239-545]	24.5	2.7	13	22.3	23.6	38.4	11	(Sverdrup *et al.*, 2001)
Askov	18.0[17.6-19.4]	457[367-546]	12.3	2.7	13	22.3	23.6	38.4	12	-
Askov	18.3[16.7-19.8]	548[474-621]	8.5	2.7	13	22.3	23.6	38.4	13	-
Askov	19.0[17.2-20.8]	660[551-770]	10.4	2.7	13	22.3	23.6	38.4	14	-
Askov	19.3[16.9-21.6]	642[500-784]	13.9	2.7	13	22.3	23.6	38.4	15	-
Askov	18.5[15.7-21.3]	307[238-375]	14.1	2.7	13	22.3	23.6	38.4	16	-
Askov	18.3[14.5-22.0]	352[289-415]	11.3	2.7	13	22.3	23.6	38.4	17	-
Askov	19.5[17.9-21.1]	408[311-505]	15.0	2.7	13	22.3	23.6	38.4	18	-
Askov	19.0[15.8-22.2]	423[291-555]	19.7	2.7	13	22.3	23.6	38.4	19	
Askov	17.3[11.8-22.7]	483[356-609]	16.5	2.7	13	22.3	23.6	38.4	20	
Askov	19.5[15.8-19.8]	451[310-591]	25.1	2.7	13	22.3	23.6	38.4	21	
Askov	14.5[12.4-16.6]	443[303-582]	19.8	2.7	13	22.3	23.6	38.4	22	
Askov	18.8[16.4-21.1]	536[416-656]	14.1	2.7	13	22.3	23.6	38.4	23	
Askov	17.0[12.5-21.5]	572[397-748]	19.3	2.7	13	22.3	23.6	38.4	24	
Askov	18.3[16.7-19.8]	523[455-590]	7.0	2.7	13	22.3	23.6	38.4	25	

Soil origin	Adults	95% C.L.	Reproduction	95% C.L.	C.V.	Organic matter	Clay	Silt	Fine sand	Coarse sand	Code	Reference
Askov	18.3	[16.6-19.9]	495	[426-564]	16.6	2.7	13	22.3	23.6	38.4	26	
Askov	19.5	[17.9-21.1]	480	[426-534]	7.0	2.7	13	22.3	23.6	38.4	56	
Askov	14.0	[8.2-19.8]	340	[299-380]	7.5	2.7	13	22.3	23.6	38.4	57	(Sverdrup *et al.*, 2002)
Askov	16.3	[13.0-19.5]	292	[111-474]	39.1	2.7	13	22.3	23.6	38.4	58	-
Askov	17.0	[15.2-18.8]	320	[272-368]	9.4	2.7	13	22.3	23.6	38.4	59	-
Askov	18.0	[16.7-19.3]	308	[188-429]	24.6	2.7	13	22.3	23.6	38.4	60	-
Norway	18.8	[16.4-21.1]	464	[322-606]	19.2	1.4	5.8	11.3		82.9	36	(Amundsen *et al.*, 1999)
Norway	18.8	[16.4-21.1]	371	[285-457]	14.5	1.4	5.8	11.3		82.9	37	-
Norway	19.3	[16.9-21.6]	387	[207-568]	29.3	1.4	5.8	11.3		82.9	38	-
Norway	17.5	[15.4-19.6]	348	[310-386]	6.9	1.4	5.8	11.3		82.9	39	-
Norway	17.8	[15.0-20.5]	400	[346-455]	8.6	1.4	5.8	11.3		82.9	40	-
Norway	19.3	[16.9-21.6]	456	[333-578]	16.9	1.4	5.8	11.3		82.9	41	-
Norway	17.0	[-]	510	[434-586]	9.4	1.4	5.8	11.3		82.9	42	-
Norway	18.5	[16.7-19.3]	457	[358-557]	13.7	1.4	5.8	11.3		82.9	27	-
Norway	16.3	[11.7-20.8]	425	[407-444]	2.7	1.4	5.8	11.3		82.9	28	-
Norway	16.3	[11.5-21.0]	415	[350-480]	9.9	1.4	5.8	11.3		82.9	29	-
Norway	16.0	[10.6-21.4]	453	[363-543]	12.5	1.4	5.8	11.3		82.9	35	-
Norway	15.0	[7.4-22.6]	444	[309-578]	19.1	1.4	5.8	11.3		82.9	30	-
Norway	19.8	[19.0-20.5]	523	[465-581]	15.4	1.4	5.8	11.3		82.9	31	-
Norway	18.0	[14.8-21.2]	420	[290-551]	50.0	1.4	5.8	11.3		82.9	32	-
Norway	18.5	[16.4-20.6]	434	[320-547]	16.5	1.4	5.8	11.3		82.9	33	-
Norway	16.8	[13.7-19.8]	389	[340-437]	7.8	1.4	5.8	11.3		82.9	34	-

Annex 5 Control mortality and reproduction

Control values estimated from the control data only, in contrast to values in Table 4, that were based on the complete concentration-response dataset.

Ref.no.	Species	Compound	n	Adults	CV	Mortality, %	Reproduction	CV
1	*F. candida*	Boric acid	8	8.6[7.6-9.6]	14	13.8[3.8-23.7]	199[126-271]	44
3[10]	*F. candida*	Boric acid	8	9.4[8.9-9.8]	5.5	6.3[1.9-10.6]	1244[1152-1336]	8.8
7	*F. candida*	Boric acid	5	9.0[8.1-9.9]	7.9	10.0[1.2-18.8]	414[334-494]	16
9	*F. candida*	Boric acid	3	10.0	0.0	0.0	317[216-417]	13
12	*F. candida*	Boric acid	8	6.0[3.9-8.1]	42	40.0[19.0-61.0]	249[163-334]	41
16	*F. candida*	Boric acid	4	6.0[2.3-9.7]	38	40.0[3.3-76.7]	365[196-534]	29
21	*F. candida*	Boric acid	10	9.1[8.6-9.6]	8.1	9.0[3.7-14.3]	405[362-447]	15
26[10]	*F. candida*	Boric acid	8	5.5[3.5-7.5]	45	45.0[24.5-65.5]	140[69-212]	61
31[10]	*F. candida*	Boric acid	8	9.3[8.7-9.8]	7.6	7.5[1.6-13.4]	339[269-409]	25
32	*F. candida*	Boric acid	5	9.2[8.6-9.8]	4.9	8.0[2.4-13.6]	308[236-381]	19
37	*F. candida*	Boric acid	7	9.7[9.0-10.4]	7.8	2.9[-4.1-9.8]	233[221-245]	5.5
38	*F. candida*	Boric acid	7	9.9[9.5-10.2]	3.8	1.4[-2.1-4.9]	358[253-462]	32
43	*F. candida*	Boric acid	4	7.8[6.2-9.3]	12	22.5[7.3-37.7]	386[249-523]	22
46	*F. candida*	Boric acid	5	8.2[6.0-10.4]	22	18.0[-4.2-40.2]	319[128-510]	48
49	*F. candida*	Boric acid	7	9.0[8.2-9.8]	9.1	10.0[2.4-17.6]	1076[918-1234]	16
51	*F. candida*	Boric acid	6	9.0[8.3-9.7]	7.0	10.0[3.4-16.6]	349[277-421]	20
53	*F. candida*	Boric acid	6	9.3[8.5-10.2]	8.7	6.7[-1.9-15.2]	537[465-610]	13
55	*F. candida*	Boric acid	6	8.0[7.1-8.9]	10	20.0[11.2-28.8]	345[251-440]	26
2	*F. candida*	Copper	8	9.1[8.4-9.8]	9.1	8.8[1.8-15.7]	196[155-237]	25
10	*F. candida*	Copper	3	9.7[8.2-11.1]	6.0	3.3[-11.0-17.7]	267[84-450]	28
15	*F. candida*	Copper	4	7.5[2.9-12.1]	38	25.0[-20.9-70.9]	578[278-878]	33
22	*F. candida*	Copper	10	9.6[9.2-10.0]	5.4	4.0[0.3-7.7]	449[410-489]	12
25[10]	*F. candida*	Copper	8	5.5[3.5-7.5]	45	45.0[24.5-65.5]	140[69-212]	61
30[10]	*F. candida*	Copper	8	9.3[8.7-9.8]	7.6	7.5[1.6-13.4]	339[269-409]	25
35	*F. candida*	Copper	5	9.6[8.5-10.7]	9.3	4.0[-7.1-15.1]	124[86-161]	24
36	*F. candida*	Copper	7	9.7[9.3-10.2]	5.0	2.9[-1.7-7.4]	614[521-707]	16
41	*F. candida*	Copper	5	9.4[8.3-10.5]	10	6.0[-5.1-17.1]	349[246-452]	24
44	*F. candida*	Copper	4	8.3[7.5-9.0]	6.1	17.5[9.5-25.5]	415[164-666]	38
48	*F. candida*	Copper	4	8.5[6.9-10.1]	12	15.0[-0.9-30.9]	875[753-997]	8.8
54	*F. candida*	Copper	5	9.2[8.2-10.2]	9.1	8.0[-2.4-18.4]	435[390-480]	8.3
4[10]	*F. candida*	Dimethoate	8	9.4[8.9-9.8]	5.5	6.3[1.9-10.6]	1244[1152-1336]	8.8
11	*F. candida*	Dimethoate	4	9.5[8.6-10.4]	6.1	5.0[-4.2-14.2]	130[69-191]	29
17	*F. candida*	Dimethoate	4	8.5[6.4-10.6]	15	15.0[-5.5-35.5]	596[458-734]	15

[10] These control data are duplicated as two tests shared the same controls, so for summaries and statistics only one figure is used.

Ref.no.	Species	Compound	n	Adults	CV	Mortality, %	Reproduction	CV
23	*F. candida*	Dimethoate	10	9.6[9.1-10.1]	7.3	4.0[-1.0-9.0]	150[123-178]	25
24	*F. candida*	Dimethoate	16	2.0[1.2-2.8]	71	80.0[72.5-87.5]	29[15-42]	86
42	*F. candida*	Dimethoate	4	7.3[5.2-9.3]	17	27.5[7.5-47.5]	290[178-402]	24
45	*F. candida*	Dimethoate	4	9.8[9.0-10.5]	5.1	2.5[-5.5-10.5]	490[357-623]	17
5	*F. fimetaria*	Boric acid	8	11.5[8.8-14.2]	28	42.5[28.9-56.1]	59[51-68]	17
13	*F. fimetaria*	Boric acid	8	15.4[13.4-17.4]	16	23.1[13.1-33.1]	6.8[5.2-8.3]	28
19	*F. fimetaria*	Boric acid	4	10.5[3.3-17.7]	43	47.5[11.6-83.4]	52.3[-0.4-105]	63
28	*F. fimetaria*	Boric acid	8	5.0[2.1-7.9]	70	75.0[60.3-89.7]		
33	*F. fimetaria*	Boric acid	5	17.4[14.5-20.3]	13	13.0[-1.3-27.3]	142[110-174]	18
40	*F. fimetaria*	Boric acid	7	18.1[16.9-19.4]	7.4	9.3[3.1-15.5]	270[240-301]	12
47	*F. fimetaria*	Boric acid	6	7.0[2.8-11.2]	57	65.0[44.0-86.0]	50.8[-1.9-104]	99
50	*F. fimetaria*	Boric acid	7	13.4[12.3-14.6]	9.5	32.9[27.0-38.7]	300[256-343]	16
52	*F. fimetaria*	Boric acid	2	17.5[11.1-23.9]	4.0	12.5[-19.3-44.3]	197[-476-870]	38
14	*F. fimetaria*	Copper	8	16.3[15.1-17.4]	8.5	18.8[12.9-24.6]	7.3[6.0-8.5]	21
18	*F. fimetaria*	Copper	4	11.3[8.5-14.0]	15	43.8[30.2-57.3]	176[53-299]	44
27	*F. fimetaria*	Copper	8	5.0[2.1-7.9]	70	75.0[60.3-89.7]	0.4[-0.2-1.0]	198
34	*F. fimetaria*	Copper	5	19.4[18.3-20.5]	4.6	3.0[-2.6-8.6]	248[119-376]	42
39	*F. fimetaria*	Copper	7	19.6[18.8-20.3]	4.0	2.1[-1.5-5.8]	242[200-284]	19
6	*F. fimetaria*	Dimethoate	8	12.0[9.8-14.2]	22	40.0[29.1-50.9]	12.1[7.1-17.2]	50
20	*F. fimetaria*	Dimethoate	4	16.8[12.4-21.1]	16	16.3[-5.7-38.2]	347[121-573]	41
29	*F. fimetaria*	Dimethoate	16	5.5[3.8-7.2]	58	72.5[64.0-81.0]	3.5[-1.1-8.1]	246

Annex 6 Draft test guideline