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OECD GUIDELINE FOR THE TESTING OF CHEMICALS

Enchytraeid Reproduction Test

INTRODUCTION

1. This Test Guideline is designed to be used for assessing the effects of test chemicals on the reproductive output of the enchytraeid worm, *Enchytraeus albidus* Henle 1873, in soil. It is based principally on a method developed by the Umweltbundesamt, Germany (1) that has been ring-tested (2). Other methods for testing the toxicity of test chemicals to Enchytraeidae and earthworms have also been considered (3)(4)(5)(6)(7)(8).

INITIAL CONSIDERATIONS

2. Soil-dwelling annelids of the genus *Enchytraeus* are ecologically relevant species for ecotoxicological testing. Whilst enchytraeids are often found in soils containing earthworms it is also true that they are often abundant in many soils where earthworms are absent. Enchytraeids can be used in laboratory tests as well as in semi-field and field studies. From a practical point of view, many *Enchytraeus* species are easy to handle and breed, and their generation time is significantly shorter than that of earthworms. The duration for a reproduction test with enchytraeids is therefore only 4-6 weeks while for earthworms (*Eisenia fetida*) it is 8 weeks.

3. Basic information on the ecology and ecotoxicology of enchytraeids in the terrestrial environment can be found in (9)(10)(11)(12).

PRINCIPLE OF THE TEST

4. Adult enchytraeid worms are exposed to a range of concentrations of the test chemical mixed into an artificial soil. The test can be divided into two steps: (a) a range-finding test, in case no sufficient information is available, in which mortality is the main endpoint assessed after two weeks exposure and (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 the definitive test is six weeks. After the first three weeks, the adult worms are removed and morphological changes are recorded. After an additional three weeks, the number of offspring, hatched from the cocoons produced by the adults, is counted. The reproductive output of the animals exposed to the test chemical is compared to that of the control(s) in order to determine (i) the no observed effect concentration (NOEC) and/or (ii) EC_x (e.g. EC_{10}, EC_{50}) by using a regression model to

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estimate the concentration that would cause a x % reduction in reproductive output. The test concentrations should bracket the EC_x (e.g. EC_{10}, EC_{50}) so that the EC_x then comes from interpolation rather than extrapolation.

INFORMATION ON THE TEST CHEMICAL

5. The water solubility, the log K_{ow}, the soil water partition coefficient (e.g. OECD Guideline 106 or 121) and the vapour pressure of the test chemical should preferably be known. Additional information on the fate of the test chemical 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 chemical will differ accordingly. The Guideline may not be applicable to substances for which the air/soil partition coefficient is greater than one, or to substances with vapour pressure exceeding 300 Pa, at 25°C. Other factors such as high water solubility or high adsorption to soil limiting the volatilisation potential should be taken into account when deciding whether or not the test chemical can be tested. For volatile, unstable or readily degrading substances (e.g. using data generated from a TG 307 study), or where there is otherwise uncertainty in maintaining the nominal soil concentration, analytical measurements of the exposure concentrations at the beginning, during and at the end of the test should be considered.

7. Before use of the Test Guideline for the testing of a mixture intended for a regulatory purpose, it should be considered whether, and if so why, it may provide adequate results for that purpose. Such considerations are not needed, when there is a regulatory requirement for testing of the mixture.

VALIDITY OF THE TEST

7. For the test to be valid, the following performance criteria should be met in the controls:

- adult mortality should not exceed 20% at the end of the range-finding test (if performed) and after the first three weeks of the reproduction test.
- assuming that 10 adults per vessel were used in setting up the test, an average of at least 25 juveniles per vessel should have been produced at the end of the test.
- the coefficient of variation around the mean number of juveniles should not be higher than 50% 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 should be tested 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. A suitable reference substance is carbendazim, which has been shown to affect survival and reproduction of enchytraeids (13)(14) or other chemicals whose toxicity data are well known could be also used. A formulation of carbendazim known by the trade name of Derosal ™ supplied by AgrEvo Company (Frankfurt, Germany) and containing 360 g/l (32.18%) active ingredient was used in a ring-test (2). The EC_{50} for reproduction determined in the ring test was in the range of 1.2 ± 0.8 mg active ingredient (a.i) /kg dry mass (2). If a positive toxic standard is included in the test series, one concentration is used and the number of replicates should be the same as that in

the controls. For carbendazim, the testing of 1.2 mg a.i./kg dry weight (tested as a liquid formation) is recommended.

DESCRIPTION OF THE TEST

Test vessels and equipment

9. The test vessels should be made of glass or other chemically inert material. Glass jars (e.g. volume: 0.20 - 0.25 litre; diameter: ≈ 6 cm) are suitable. 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 lids should be 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;
- photo basin.

Preparation of the artificial soil

11. An artificial soil is used in this test (5)(7) with the following composition (based on dry weights, dried to a constant weight at 105 ^{o}C):

- 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 culturing the worms before it is used in a test;
- 20% kaolin clay (kaolinite content preferably above 30%);
- approximately 0.3 to 1.0% calcium carbonate ($CaCO_3$, pulverised, analytical grade) to obtain a pH of 6.0 ± 0.5; the amount of calcium carbonate to be added may depend principally on the quality/nature of the peat;
- approximately 70% air-dried quartz 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 an artificial soil for culturing the worms and for achieving the test validity criteria before using the soil in a definitive test. It is especially recommended to make such a check to ensure that the performance of the test is not compromised if the organic carbon content of the artificial soil is reduced, e.g. by lowering the peat content to 4-5% and increasing the sand content accordingly. By such a reduction in organic carbon content, the possibilities of adsorption of test chemical to the soil (organic carbon) may be decreased and the availability of the test chemical to the worms may increase. It has been demonstrated that *Enchytraeus albidus* can comply with the validity criteria on reproduction when tested in field soils with lower organic carbon content than mentioned above (e.g. 2.7%) (15), and there is experience – though limited – that this can also be achieved in artificial soil with 5% peat.

Note: When using natural soil in additional (e.g. higher tier) testing, the suitability of the soil and achieving the test validity criteria should also be demonstrated.

12. 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 days 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 (see (16) and Annex 3). If the soil is more acidic than the required range (see paragraph 11), 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, referred to in paragraph 11, but excluding the $CaCO_3$

13. The maximum water holding capacity (WHC) of the artificial soil is determined in accordance with procedures described in Annex 2. One or two days before starting the test, the dry artificial soil is pre-moistened by adding enough de-ionised water to obtain approximately half of the final water content, that being 40 to 60% of the maximum water holding capacity. 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 40-60 % of the maximum WHC by the addition of the test chemicalsolution and/or by adding distilled or de-ionised water (see paragraphs 19-21). The moisture content is determined at the beginning and at the end of the test (by drying to constant weight at 105 °C) and should be within the optimal range for the survival of the worms. A rough check of the soil moisture content can be obtained by gently squeezing the soil in the hand, if the moisture content is correct small drops of water should appear between the fingers.

Selection and preparation of test animals

14. The recommended test species is *Enchytraeus albidus* Henle 1837 (white potworm), a member of the family *Enchytraeidae* (order *Oligochaeta*, phylum *Annelida*). *E. albidus* is one of the largest species of enchytraeids, with specimens of up to 35 mm in length being recorded (17)(18). *E. albidus* has a world-wide distribution and is found in marine, freshwater and terrestrial habitats, mainly in decaying organic matter (seaweed, compost) and rarely in meadows (9). Its broad ecological tolerance and some morphological variations might indicate that different races exist.

15. *E. albidus* is commercially available, as a fish food. It should be checked whether the culture is contaminated by other, usually smaller, species (1) (19). If contamination occurs, all worms should be washed with water in a petri dish. Large adult specimens of *E. albidus* should then be selected (using a stereomicroscope) to start a new culture and all other worms are discarded. *E. albidus* can be bred easily in a wide range of organic materials (see Annex 4). The life-cycle of *E. albidus* is short since maturity is reached between 33 days (at 18 °C) and 74 days (at 12 °C) (1). Only cultures that have been kept without problems in the laboratory for at least 5 weeks (one generation) will be used for the test.

16. Other species of the *Enchytraeus* genus are also suitable, e.g. *E. buchholzi* Vejdovsky 1879 or *E. crypticus* Westheide & Graefe 1992 (see Annex 5). If other species of *Enchytraeus* are used, they must be clearly identified and the rationale for the selection of the species should be reported.

17. The animals used in the tests are adult worms. They should have eggs (white spots) in the clitellum region, and they should be approximately the same size (about 1 cm long). Synchronisation of the breeding culture is not necessary.

18. If the enchytraeids are not bred in the same soil type and under the conditions (including feeding) used for the final test they must be acclimatised for at least 24 hours and up to three days. A larger number of

adults than that needed for performing the test should initially be acclimatised to allow scope for rejection of damaged or otherwise unsuitable specimens. At the end of the acclimatisation period, only worms containing eggs and exhibiting no behavioural abnormalities (e.g. trying to escape from the soil) are selected for the test. The worms are carefully removed using jeweller´s tweezers, hooks or loops and placed in a petri dish containing a small amount of fresh water. Reconstituted fresh water as proposed in OECD Test Guideline 211 (*Daphnia magna*, Reproduction Test) is preferred for this purpose since de-ionised, de-mineralised or tap water could be harmful to the worms. The worms are inspected under a stereomicroscope and any that do not contain eggs are discarded. Care is taken to remove and discard any mites or springtails that might have infected the cultures. Healthy worms not used for the test are returned to the stock culture.

Preparation of test concentrations

Test chemical soluble in water

19. A solution of the test chemical is prepared in deionised water in a quantity sufficient for all replicates of one test concentration. It is recommended to use an appropriate quantity of water to reach the required moisture content, i.e. 40 to 60% of the maximum WHC (see paragraph 13). Each solution of test chemical is mixed thoroughly with one batch of pre-moistened soil before being introduced into the test vessel.

Test substance insoluble in water

20. For test chemicals insoluble in water but soluble in organic solvents, the test chemical can be dissolved in the smallest possible volume of a suitable vehicle (e.g. acetone). Only volatile solvents should be used. 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 chemical 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.

21. For test chemicals 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 chemical to obtain the desired test concentration. This mixture of quartz sand and test chemical 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.

22. Test chemicals should not normally be tested at concentrations higher than 1000 mg/kg dry mass of soil. Testing at higher concentrations may however be required in accordance with the objectives of a specific test.

PROCEDURE

Test groups and controls

23. For each test concentration, an amount of test soil corresponding to 20 g dry weight is placed into the test vessel (see paragraphs 19-21). Controls, without the test chemical, are also prepared. Food is added to each vessel in accordance with procedures described in paragraph 29. Ten worms are randomly allocated to each test vessel. The worms are carefully transferred into each test vessel and placed on the surface of the soil using, for example, jeweller's tweezers, hooks or loops. The number of replicates for test concentrations and

for controls depends on the test design used (see paragraph 34). The test vessels are positioned randomly in the test incubator and these positions are re-randomised weekly.

24. If a vehicle is used for application of the test chemical, 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 chemical. A control series containing additional quartz sand (2.5 g per vessel) should be run for test chemicals requiring administration in accordance with the procedures described in paragraph 21.

Test conditions

25. The test temperature is 20 ± 2 °C. To discourage worms 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.

26. 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.

27. 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 but not containing worms. Food should only be added to these soil samples at the start of the test to facilitate microbial activity. The amount of food added should be the same as that added to the test cultures. It is not necessary to add further food to these vessels during the test.

Feeding

28. A food capable of maintaining the enchytraeid population can be used. Rolled oats, preferably autoclaved before use to avoid microbial contamination (heating is also appropriate), have been found to be a suitable feeding material.

29. Food is first provided by mixing 50 mg of ground rolled oats with the soil in each vessel before introducing the worms. Thereafter, food is supplied weekly up to Day 21. Feeding is not carried out on Day 28 since the adults have been removed at this stage and the juvenile worms need relatively little additional food beyond this point. Feeding during the test comprises 25 mg of ground rolled oats per vessel placed carefully on the surface of the soil so as to avoid injuring the worms. In order to reduce fungal growth, the oats flakes should be buried in the soil by covering with small amounts of soil. If food remains uneaten the ration should be reduced.

Design for the range-finding test

30. When necessary, a range-finding test is conducted with, for example, five test chemical concentrations of 0.1, 1.0, 10, 100, and 1000 mg/kg (dry weight of soil). One replicate for each treatment and control is sufficient.

31. The duration of the range-finding test is two weeks. At the end of the test, mortality of the worms is assessed. A worm is recorded as dead if it has no reaction to a mechanical stimulus at the anterior end. Additional information to mortality may also be useful in deciding on the range of concentrations to be used in the definitive test. Changes in adult behaviour (e.g. the inability to dig into the soil; lying motionless against the glass wall of the test vessel) and morphology (e.g. the presence of open wounds) should therefore also be recorded along with the presence of any juveniles. The latter can be determined using the staining method described in Annex 6.

32. The LC_{50} can be approximately determined by calculating the geometrical mean of mortality data. In setting the concentration range for the definitive test, effects on the reproduction are assumed to be lower than the LC_{50} by a factor of up to 10. However, this is an empirical relation ship and in any specific case it might be different. Additional observations made in the range-finding test such as the occurrence of juveniles can help refine the test chemical concentration range to be used for the definitive test.

33. In order for an accurate determination of the LC_{50} performing the test using at least four replicates each of the test chemical concentration and an adequate number of concentrations to cause at least four statistically significantly different mean responses at these concentrations) is recommended. A similar number of the concentrations and replicates for the controls are used when they are applicable.

Design for the definitive reproduction test

34. Three designs are proposed based on recommendations arising from a ring test (2):

- For determination of the NOEC, at least five concentrations in a geometric series should be tested. Four replicates for each test concentration plus eight controls are recommended. The concentrations should be spaced by a factor not exceeding 1.8.
- For determination of the EC_x (e.g. EC_{10}, EC_{50}), at least five concentrations should be tested and the concentrations should bracket EC_x in order to enable EC_x interpolation and not extrapolation. At least four replicates for each test concentration and four control replicates are recommended. The spacing factor may vary, i.e. less than or equal to 1.8 in the expected effect range and above 1.8 at the higher and lower concentrations.
- A combined approach allows for determination of both the NOEC and EC_x. Eight treatment concentrations in a geometric series should be used. Four replicates for each treatment plus eight controls are recommended. The concentrations should be spaced by a factor not exceeding 1.8.

35. Ten adult worms per test vessel should be used (see paragraph 23). Food is added to the test vessels at the beginning of the test and then once a week (see paragraph 29) up to and including Day 21. On Day 21 the soil samples are carefully hand searched and living adult worms are observed and counted and changes in behaviour (e.g. inability to dig into the soil; lying motionless against the glass wall of the test vessel) and in morphology (e.g. open wounds) are recorded. All adult worms are then removed from the test vessels and the test soil. Adults are then humanely euthanized, preferably by rapid freezing at -80°C or cryopreservation. The test soil containing any cocoons that had been produced are incubated for three additional weeks under the same test conditions except that feeding takes place only on Day 35 (i.e. 25 mg ground rolled oats per vessel).

36. After six weeks, the newly hatched worms are counted. The method based on Bengal red staining (see Annex 6) is recommended although other wet (but not heat) extraction and floatation techniques (see Annex 6) have also proved suitable (4)(10)(11)(20). Bengal red staining is recommended because wet extraction from a soil substrate can be hampered by turbidity caused by suspended clay particles. At termination of the test, newly hatched worms are counted and humanely euthanized, preferably by rapid freezing at -80°C or cryopreservation.

Limit test

37. If no effects are observed at the highest concentration in the range-finding test (i.e. 1000 mg/kg), the reproduction test can be performed as a limit test, using 1000 mg/kg in order to demonstrate that the NOEC for reproduction is greater than this value.

Summary and timetable for the test

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

Time	Range-finding test	Definitive test
Day –7 or earlier	- Prepare artificial soil (mixing of dry constituents)	- Prepare artificial soil (mixing of dry constituents)
Day –5	- Check pH of artificial soil - Measure max WHC of soil	- Check pH of artificial soil - Measure max WHC of soil
Day –5 to –3	- Sort worms for acclimatisation	- Sort worms for acclimatisation
Day – 3 to 0	- Acclimatise worms for at least 24 hours	- Acclimatise worms for at least 24 hours
Day –1	- Pre-moisten artificial soil and distribute into batches	- Pre-moisten artificial soil and distribute into batches
Day 0	- Prepare stock solutions - Apply test chemical - Weigh test substrate into test vessels - Mix in food - Introduce worms - Measure soil pH and moisture content	- Prepare stock solutions - Apply test chemical - Weigh test substrate into test vessels - Mix in food - Introduce worms - Measure soil pH and moisture content
Day 7	- Check soil moisture content	- Check soil moisture content - Feed
Day 14	- Determine adult mortality - Estimate number of juveniles - Measure soil pH and moisture content	- Check soil moisture content - Feed
Day 21		- Observe adult behaviour - Remove adults - Determine adult mortality - Check soil moisture content - Feed
Day 28		- Check soil moisture content - No feeding
Day 35		- Check soil moisture content - Feed
Day 42		- Count juvenile worms - Measure soil pH and moisture content

DATA AND REPORTING

Treatment of results

39. Although an overview is given in Annex 7, no definitive statistical guidance for analysing test results is given in this guideline.

40. In the range finding test, the main endpoint is mortality. Changes in behaviour (e.g. inability to dig into the soil; lying motionless against the glass wall of the test vessel) and morphology (e.g. open wounds) of the adult worms should however also be recorded along with the presence of any juveniles. Probit analysis (21) or logistic regression should normally be applied to determine the LC_{50}. However, in cases where this method of analysis is unsuitable (e.g., if less then three concentrations with partial kills

are available), alternative methods can be used. These methods could include moving averages (22), the trimmed Spearman-Karber method (23) 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}).

41. In the definitive test, test endpoint is fecundity (i.e. number of juveniles produced). However, as in the range-finding test, all other harmful signs should be recorded in the final report. The statistical analysis requires the arithmetic mean and the standard deviation per treatment and per control for reproduction to be calculated.

42. 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 of 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 appropriate tests see paragraph 45 and Annex 7).

43. Further statistical testing and inference depends on whether the replicate values are normally distributed and are homogeneous with regard to their variance.

NOEC Estimation

44. The application of powerful tests should be preferred. One should use information e.g. from previous experience with ring-testing or other historic data on whether data are approximately normally distributed. Variance homogeneity (homoscedascity) is more critical. Experience tells that the variance often increases with increasing mean. In these cases, a data transformation could lead to homoscedascity. However, such a transformation should be based on experience with historic data rather than on data under investigation. With homogeneous data, multiple t-tests such as Williams' test (α = 0.05, one-sided) (24)(25) or in certain cases Dunnett´s test (26)(27) should be performed. It should be noted that, in the case of unequal replication, the table 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 of strong deviation from monotonicity the Dunnett's test is more appropriate. If there are deviations from homoscedascity, it may be reasonable to investigate possible effects on variances more closely to decide whether the t tests can be applied without loosing much power (28). Alternatively, a multiple U-test, e.g. the Bonferroni-U-test according to Holm (29), or when these data exhibit heteroscedasticity but are otherwise consistent with a underlying monotone dose-response, an other non-parametric test [e.g. Jonckheere-Terpstra (30) (31) or Shirley (32) (33)] can be applied and would generally be preferred to unequal-variance t-tests. (see also the scheme in Annex 7).

45. 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 (29).

EC_x Estimation

46. To compute any EC_x value, the per-treatment means are used for regression analysis (linear or non-linear), after an appropriate dose-response function has been obtained. For the growth of worms as a continuous response, EC_{x-} -values can be estimated by using suitable regression analysis (35). Among suitable functions for quantal data (mortality/survival and number of offspring produced) 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 EC_x 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 Fieller (cited in Finney (21)) or other modern appropriate methods.

47. Alternatively, the response is modelled as a percent or proportion of model parameter which is interpreted as the control mean response.In these cases, the normal (logistic, Weibull) sigmoid curve can often be easily fitted to the results using the probit regression procedure (21). In these cases the weighting function has to be adjusted for metric responses as given by Christensen (36). However, if hormesis has been observed, probit analysis should be replaced by a four-parameter logistic or Weibull function, fitted by a non-linear regression procedure (37). If a suitable dose-response function cannot be fitted to the data, one may use alternative methods to estimate the EC_x, and its confidence limits, such as Moving Averages after Thompson (22) and the Trimmed Spearman-Karber procedure (23).

Test report

48. The test report must include the following information:

Test chemical:

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Mono-constituent substance:

- physical appearance, water solubility, and additional relevant physicochemical and environmental fate properties, measured or estimated (e.g. hydrolysis, vapour pressure, log Kow, log Koc, log Kd (soil), log Koa, air/soil partitioning coefficient, biodegradability in soil or other biodegradability information).
- chemical identification, such as IUPAC or Chemical Abstract (CA) Index name, CAS Registry Number, SMILES or InChI code, structural formula, purity, chemical identity of impurities as appropriate and practically feasible, etc. (including the organic carbon content, if appropriate).

Multi-constituent substance, UVCB and mixture:

- characterised as far as possible by chemical identity (see above), quantitative occurrence and relevant physicochemical properties of the constituents.

Test species:

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

Test conditions:
- ingredients and preparation of the artificial soil;
- method of application of the test chemical;
- description of the test conditions, including temperature, moisture content, pH, etc.;
- full description of the experimental design and procedures.

Test results:

- mortality of adult worms after two weeks and the number of juveniles at the end of the range-finding test;
- mortality of adult worms 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 EC_x (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 all information and observations helpful for the interpretation of the results.

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

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ANNEX 1

DEFINITIONS

For the purpose of this Guideline the following definitions are applicable:

EC_x (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. In this test the effect concentrations are expressed as a mass of test chemical per dry mass of the test soil.

LC_0 (No lethal concentration) is the concentration of a test chemical that does not kill any of exposed test organisms within a given time period. In this test the LC_0 is expressed as a mass of test chemical per dry mass of the test soil.

LC_{50} (Median lethal concentration) is the concentration of a test chemical kills 50% of exposed test organisms within a given time period. In this test the LC_{50} is expressed as a mass of test chemical per dry mass of the test soil.

LC_{100} (Totally lethal concentration) is the concentration of a test chemical kills 100% of exposed test organisms within a given time period. In this test the LC_{100} is expressed as a mass of test chemical per dry mass of the test soil.

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

NOEC (No Observed Effect Concentration) is the highest test chemical 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.

Reproduction rate is the mean number of juvenile worms produced per a number of adults over the test period.

ANNEX 2

DETERMINATION OF THE MAXIMUM WATER HOLDING CAPACITY

Determination of the water holding capacity of the artificial soil

The following method has been found appropriate. It is described in Annex C of the ISO DIS 11268-2.

Collect a defined quantity (e.g. 5 g) of the test soil substrate using a suitable device (auger tube etc.). Cover the bottom of the tube with a piece of filter paper and, after filling with water, 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 closed 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

References:

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ANNEX 3

DETERMINATION OF SOIL pH

The following method for determining the pH of a soil sample is based on the description in ISO 10390 (Soil Quality - Determination of pH).

A defined quantity of soil is dried at room temperature for at least 12 hours. A suspension of the soil (containing at least 5 grams of soil) is then made up in five times its volume of either 1 M 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. After shaking, the suspension is 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).

References:

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ANNEX 4

CULTURING CONDITIONS OF *ENCHYTRAEUS sp.*

Enchytraeids of the species *Enchytraeus albidus* (as well as other *Enchytraeus* species) can be cultured in large plastic boxes (e.g. 30 x 60 x 10 cm) filled with a 1:1 mixture of artificial soil and natural, uncontaminated garden soil. Compost material must be avoided since it could contain toxic chemicals such as heavy metals. Fauna should be removed from the soil before use (e.g. by deep-freezing). A substrate comprising only of artificial soil can also be used but the reproduction rate may be lower than that obtained with a mixed soil substrate. The substrate used for culturing should have a pH of 6.0 ± 0.5.

The culture is kept in the dark at a temperature of 15 to 20 °C ± 2 °C. Temperatures higher than 23 °C must be avoided. The soil must be kept moist but not wet. The correct soil moisture content is indicated when small drops of water appear between the fingers when the soil is gently squeezed. The production of anoxic conditions must be avoided by ensuring that covers to culture containers allow adequate gaseous exchange with the atmosphere. The soil should be carefully broken up each week to facilitate aeration.

The worms can be fed on rolled oats. The oats should be stored in sealed vessels and autoclaved or heated before use in order to avoid infestation with flour mites (e.g. *Glyzyphagus sp.*, *Astigmata*, *Acarina*) or predacious mites [e.g. *Hypoaspis* (*Cosmolaelaps) miles*, *Gamasida*, *Acarina*]. After a heat treatment, the food should be ground so that it can easily be strewn on the soil surface. From time to time, the rolled oats can be supplemented by the addition of vitamins, milk and cod-liver oil. Other suitable food sources are baker's yeast and the fish food "Tetramin".

Feeding takes place approximately twice a week. An appropriate quantity of rolled oats is strewn on the soil surface or carefully mixed into the substrate when breaking up the soil to facilitate aeration. The absolute amount of food provided depends on the number of worms present in the substrate. As a guide, the amount of food should be increased if it is all consumed within one day of being provided. Conversely, if food still remains on the surface at the time of the second feeding (one-week later) it should be reduced. Food contaminated with fungal growth should be removed and replaced. After three months, the worms should be transferred into a freshly prepared substrate.

Culturing conditions are deemed satisfactory if the worms: (a) do not try to leave the soil substrate, (b) move quickly through the soil, (c) exhibit a shiny outer surface without adhering soil particles, (d) are more or less whitish in colour, (e) exhibit a variety of age ranges in the cultures and (f) reproduce continuously.

ANNEX 5 (Informative)

TEST PERFORMANCE WITH OTHER *ENCHYTRAEUS* SPECIES

Selection of species

Species other than *E. albidus* may be used but the test procedure and the validity criteria should be adapted accordingly. Since many *Enchytraeus*-species are readily available and can be satisfactorily maintained in the laboratory, the most important criterion for selecting a species other than *E. albidus* is ecological relevance and, additionally, comparable sensitivity. There may also be formal reasons for a change of species. For example, in countries where *E. albidus* does not occur and cannot be imported (e.g. due to quarantine restrictions), it will be necessary to use another *Enchytraeus* species.

Examples of suitable alternative species

- *Enchytraeus crypticus* (Westheide & Graefe 1992): In recent years, this species has often been used in ecotoxicological studies because of the simplicity of its breeding and testing. However, it is small and this makes handling more difficult compared with *E. albidus* (especially at stages prior to use of the staining method). *E. crypticus* has not been found to exist with certainty in the field, having only been described from earthworm cultures. Its ecological requirements are therefore not known.

- *Enchytraeus buchholzi* (Vejdovsky 1879): This name probably covers a group of closely related species that are morphologically difficult to distinguish. Its use for testing is not recommended until the individuals used in a test can be identified to species. *E. buchholzi* is usually found in meadows and disturbed sites such as roadsides.

- *Enchytraeus luxuriosus*: This species was originally known as *E. "minutus",* but has been recently described (1). It was first found by U. Graefe (Hamburg) in a meadow close to St. Peter-Ording (Schleswig-Holstein, Germany). *E. luxuriosus* is approximately half the size of E. albidus but larger than the other species discussed here; this could make it a good alternative to *E. albidus*.

- *Enchytraeus bulbosus* (Nielsen & Christensen 1963): This species has hitherto been reported from German and Spanish mineral soils, where it is common but not usually very abundant. In comparison to other small species of this genus, it is relatively easy to identify. Nothing is known about its behaviour in laboratory tests or its sensitivity to chemicals. It has, however, been found to be easy to culture (E. Belotti, personal communication).

Breeding conditions

All the *Enchytraeus*-species mentioned above can be cultured in the same substrates used for *E. albidus*. Their smaller size means that the culture vessels can be smaller and that, while the same food can be used, the ration size must be adjusted. The life-cycle of these species is shorter than for *E. albidus* and feeding should be carried out more frequently.

Test conditions

The test conditions are generally the same as those applying to *E. albidus,* except that:

- the size of the test vessel can (but need not) be smaller;
- the duration of the reproduction test can (but need not) be shorter, i.e. four instead of six weeks however, the duration of the Range-Finding Test should not be changed;
- in view of the small size of the juvenile worms the use of the staining method is strongly recommended for counting;
- the validity criterion relating to "number of juveniles per test vessel in the control" should be changed to "50".

References

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ANNEX 6

DETAILED DESCRIPTION OF EXTRACTION TECHNIQUES

Staining with Bengal red

This method, originally developed in limnic ecology (1) was first proposed for the counting of juvenile enchytraeids in the Enchytraeidae reproduction test by W. de Coen (University of Ghent, Belgium). Independently, a modified version (Bengalred mixed with formaldehyde instead of ethanol) was developed by RIVM Bilthoven (2)(3).

At the end of the Definitive Test (i.e. after six weeks), the soil in the test vessels is transferred to a shallow container. A Bellaplast vessel or a photo basin with ribbed bottom is useful for this purpose, the latter because the "ribs" restrict movement of the worms within the field of observation. The juveniles are fixed with ethanol (approx. 5 mL per replicate). The vessels are then filled with water up to a layer of 1 to 2 cm. A few drops (200 to 300 µL) of Bengalred (1% solution in ethanol) are added (0.5% eosin is an alternative) and the two components are mixed carefully. After 12 hours, the worms should be stained a reddish colour and should be easy to count because they will be lying on the substrate surface. Alternatively, the substrate/alcohol mixture can be washed through a sieve (mesh size: 0.250 mm) before counting the worms. Using this procedure, the kaolinite, peat, and some of the sand will be washed out and the reddish coloured worms will be easier to see and count. The use of illuminated lenses (lens size at least 100 x 75 mm with a magnification factor 2 to 3x) will also facilitates counting.

The staining technique reduces counting time to a few minutes per vessel and as a guide it should be possible for one person to assess all the vessels from one test in a maximum of two days.

Wet extraction

The wet extraction should be started immediately the test finishes. The soil from each test vessel is placed into plastic sieves with a mesh size of approximately 1 mm. The sieves are then suspended in plastic bowls without touching the bottom. The bowls are carefully filled up with water until the samples in the sieves are completely under the water surface. To ensure a recovery rate of more than 90% of the worms present, an extraction period of 3 days at 20 ± 2 °C should be used. At the end of the extraction period the sieves are removed and the water (except for a small amount) is slowly decanted, taking care not to disturb the sediment at the bottom of the bowls. The plastic bowls are then shaken slightly to suspend the sediment in the overlying water. The water is transferred to a petri dish and, after the soil particles have settled), the enchytraeids can be identified, removed and counted using a stereomicroscope and soft steel forceps.

Flotation

A method based on flotation has been described in a note by R. Kuperman (4). After fixing the contents of a test vessel with ethanol, the soil is flooded with Ludox (AM-30 colloidal silica, 30 wt. % suspension in water) up to 10 to 15 mm above the soil surface. After thoroughly mixing the soil with the flotation agent for 2 – 3 minutes, the juvenile worms floating on the surface can easily be counted.

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ANNEX 7

OVERVIEW OF THE STATISTICAL ASSESSMENT OF DATA (NOEC DETERMINATION)