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OECD GUIDELINE FOR THE TESTING OF CHEMICALS

Earthworm Reproduction Test (*Eisenia fetida/ Eisenia andrei*)

INTRODUCTION

1. This Test Guideline is designed to be used for assessing the effects of test chemicals in soil on the reproductive output (and other sub-lethal end points) of the earthworm species *Eisenia fetida* (Savigny 1826) or *Eisenia andrei* (Andre 1963) (1)(2). The test has been ring-tested (3). A guideline for the earthworm acute toxicity test has been published by OECD (4). A number of other international and national guidelines for earthworm acute and chronic tests have been published (5)(6)(7)(8).

2. *Eisenia fetida /Eisenia andrei* are considered to be a one of representatives of soil fauna and earthworms in particular. Background information on the ecology of earthworms and their use in ecotoxicological testing is available (7)(9)(10)(11)(12).

PRINCIPLE OF THE TEST

3. Adult worms are exposed to a range of concentrations of the test chemical either mixed into the soil or, in case of pesticides, applied into or onto the soil using procedures consistent with the use pattern of the test chemical. The method of application is specific to the purpose of the test. The range of test concentrations is selected to encompass those likely to cause both sub-lethal and lethal effects over a period of eight weeks. Mortality and growth effects on the adult worms are determined after 4 weeks of exposure. The adults are then removed from the soil and effects on reproduction assessed after a further 4 weeks by counting the number of offspring present in the soil. The reproductive output of the worms exposed to the test chemical is compared to that of the control(s) in order to determine the (i) no observed effect concentration (NOEC) and/or (ii) EC_x (e.g. EC_{10}, EC_{50}) by using a regression model to 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 (see Annex 1 for definitions).

INFORMATION ON THE TEST CHEMICAL

This Guideline was adopted by the OECD Council by written procedure on 29 July 2016 [C(2016)103].

4. The following information relating to the test chemical should be available to assist in the design of appropriate test procedures:

- water solubility;
- log Kow;
- vapour pressure;
- and information on fate and behaviour in the environment, where possible (e.g. rate of photolysis and rate of hydrolysis where relevant to application patterns).

5. This Guideline is applicable to all test chemicals irrespective of their water solubility. The method 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. data generated from a TG 307 study may be considered), 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.

6. 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.

REFERENCE SUBSTANCE

7. The NOEC and/or the EC_x of a reference substance must be determined to provide assurance that the laboratory test conditions are adequate and to verify that the response of the test organisms does not change statistically over time. It is advisable to test a reference substance at least once a year or, when testing is carried out at a lower frequency, in parallel to the determination of the toxicity of a test chemical. Carbendazim or benomyl are suitable reference substances that have been shown to affect reproduction (3). Significant effects should be observed between (a) 1 and 5 mg active ingredient (a.i.)/kg dry mass or (b) 250-500 g/ha or 25-50 mg/m^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.

VALIDITY OF THE TEST

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

- each replicate (containing 10 adults) to have produced ≥ 30 juveniles by the end of the test;
- the coefficient of variation of reproduction to be ≤ 30 %;
- adult mortality over the initial 4 weeks of the test to be ≤10 %.

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 report.

DESCRIPTION OF THE TEST

Test vessels and equipment

9. Test containers made of glass or other chemically inert material of about one to two litres capacity should be used. The containers should have a cross-sectional area of approximately 200 cm² so that a moist substrate depth of about 5-6 cm is achieved when 500 to 600 g dry mass of substrate is added. The design of the container cover should permit gaseous exchange between the substrate and the atmosphere and access to light (e.g. by means of a perforated transparent cover) whilst preventing the worms from escaping. If the amount of test substrate used is substantially more than 500 to 600 g per test container the number of worms should be increased proportionately.

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;
- water bath.

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 C):

- 10 per cent sphagnum peat (as close to pH 5.5 to 6.0 as possible, no visible plant remains, finely ground, dried to measured moisture content);
- 20 per cent kaolin clay (kaolinite content preferably above 30 per cent);
- 0.3 to 1.0% calcium carbonate ($CaCO_3$, pulverized, analysis grade) to obtain an initial pH of 6.0 ± 0.5.
- 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.

Note 1: The amount of $CaCO_3$ required will depend on the components of the soil substrate including food, and should be determined by measurements of soil sub-samples immediately before the test. pH is measured in a mixed sample in a 1 M solution of potassium chloride (KCl) or a 0.01 M solution of calcium chloride ($CaCl_2$) (13).

Note 2: The organic carbon content of the artificial soil may be 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 *Eisenia fetida* can comply with the validity criteria on reproduction when tested in field soils with lower organic carbon content (e.g. 2.7%) (14), and there is experience that this can also be achieved in artificial soil with 5% peat. Therefore, it is not necessary before using such a soil in a definitive test to demonstrate the suitability of the artificial soil for allowing the test to comply with the validity criteria unless the peat content is lowered more than specified above.

Note 3: 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) in a well ventilated area. Before starting the test, the dry artificial soil is 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 (corresponding to $50 \pm 10\%$ moisture dry mass). This will produce a substrate that has no standing or free water when it is compressed in the hand. The maximum water holding capacity (WHC) of the artificial soil is determined in accordance with procedures described in Annex 2 or ISO 11274 (15).

13. If the test chemical is applied on the soil surface or mixed into soil without water, the final amount of water can be mixed into the artificial soil during preparation of the soil. If the test substance is mixed into the soil together with some water, the additional water can be added together with the test substance (see paragraph 19).

14. Soil moisture content is determined at the beginning and at the end of the test in accordance with ISO 11465 (16) and soil pH in accordance with Annex 3 or ISO 10390 (13). These determinations should be carried out in a sample of control soil and a sample of each test concentration soil. The soil pH should not be adjusted when acidic or basic substances are tested. The moisture content should be monitored throughout the test by weighing the containers periodically (see paragraph 26 and 30).

Selection and preparation of test animals

15. The species used in the test is *Eisenia fetida* or *Eisenia andrei* (1)(2). Adult worms between two months and one year old and with a clitellum are required to start the test. The worms should be selected from a synchronised culture with a relatively homogeneous age structure (Annex 4). Individuals in a test group should not differ in age by more than 4 weeks.

16. The selected worms should be acclimatised for at least one day with the type of artificial soil substrate to be used for the test. During this period the worms should be fed on the same food to be used in the test (see paragraphs 31 to 33).

17. Groups of 10 worms should be weighed individually randomly assigning the groups to the test containers at the start of the test. The worms are washed prior to weighing (with deionised water) and the excess water removed by placing the worms briefly on filter paper. The wet mass of individual worms should be between 250 and 600 mg for *E. andrei* and between 300 and 600 mg for *E. fetida*.

Preparation of test concentrations

18. Two methods of application of the test chemical can be used: mixing the test chemical into the soil (see paragraphs 19-21) or application to the soil surface (see paragraphs 22-24). The selection of the appropriate method depends on the purpose of the test. In general, mixing of the test chemical into the soil is recommended. However application procedures that are consistent with normal agricultural practice may be required (e.g. spraying of liquid formulation or use of special pesticide formulations such as granules or seed dressings). Solvents used to aid treatment of the soil with the test chemical should be selected on the basis of their low toxicity to earthworm and appropriate solvent control must be included in the test design (see paragraph 27).

Mixing the test chemical into the soil

Test chemical soluble in water

19. A solution of the test chemical in de-ionised water is prepared immediately before starting the test in a quantity sufficient for all replicates of one concentration. A co-solvent may be required to facilitate for the preparation of the test solution. It is convenient to prepare an amount of solution necessary to reach the final moisture content (40 to 60% of maximum water holding capacity). The solution is mixed thoroughly with the soil substrate before introducing it into a test container.

Test chemical insoluble in water

20. The test chemical is dissolved in a small volume of a suitable organic solvent (e.g. acetone) and then sprayed onto, or mixed into, a small quantity of fine quartz sand. The solvent is then removed by evaporation in a fume hood for at least a few minutes. The treated sand is then mixed thoroughly with the pre-moistened artificial soil. De-ionised water is then added (an amount required) to achieve a final moisture content of 40 to 60 % of the maximum water holding capacity and mixed in. The soil is then ready for placing in test containers vessels. Care should be taken that some solvents may be toxic to earthworms.

Test chemical insoluble in water and organic solvents

21. A mixture comprised of 10 g of finely ground industrial quartz sand with a quantity of the test chemical necessary to achieve the test concentration in the soil is prepared. The mixture is then mixed thoroughly with the pre-moistened artificial soil. De-ionised water is then added to an amount required to achieve a final moisture content of 40 to 60% of the maximum water holding capacity is then added and mixed in. The soil is then ready for placing to the test containers.

Application of the test chemical to the soil surface

22. The soil is treated after the worms are added. The test containers are first filled with the moistened soil substrate and the weighed worms are placed on the surface. Healthy worms normally burrow immediately into substrate and consequently any remaining on the surface after 15 minutes are defined as damaged and must be replaced. If worms are replaced, the new ones and those substituted should be weighed so that total live weight of the exposure group of worms and the total weight of the container with worms at the start is known.

23. The test chemical is applied. It should not be added to the soil within half an hour of introducing the worms (or if worms are present on the soil surface) so as to avoid any direct exposure to the test chemical by skin contact. When the test chemical is a pesticide it may be appropriate to apply it to the soil surface by spraying. The test chemical should be applied to the surface of the soil as evenly as possible using a suitable laboratory-scale spraying device to simulate spray application in the field. Before application the cover of the test container should be removed and replaced by a liner which protects the side walls of the container from spray. The liner can be made from a test container with the base removed. The application should take place at a temperature within 20 ± 2 °C of variation and for aqueous solutions, emulsions or dispersions at a water application rate of between 600 and 800 µl/m^2. The rate should be verified using an appropriate calibration technique. Special formulations like granules or seed dressings should be applied in a manner consistent with agricultural use.

24. Test containers should be left uncovered for a period of one hour to allow any volatile solvent associated with the application of the test substance to evaporate**.** Care should be taken that no worm will escape from the test vessels within this time.

PROCEDURE

Test groups and controls

25. A loading of 10 earthworms in 500 - 600 g dry mass of artificial soil (i.e. 50-60 g of soil per worm) is recommended. If larger quantities of soil are used, as might be the case if testing pesticides with

special modes of application such as seed dressings, the loading of 50-60 g of soil per worm should be maintained by increasing the number of worms. Ten worms are prepared for each control and treatment container. The worms are washed with water and wiped and then placed on absorbent paper for a short period to allow excess water to drain.

26. To avoid systematic errors in distributing the worms to the test containers the homogeneity of the test population should be determined by individually weighing 20 worms sampled randomly from the population from which the test worms are to be taken. Having ensured homogeneity, batches of worms are then be selected, weighed and assigned to test containers using a randomisation procedure. After the addition of the test worms, the weight of each test container should be measured to ensure that there is an initial weight that can be used as the basis for monitoring soil moisture content throughout the test as described in paragraph 30. The test containers are then covered as described in paragraph 9 and placed in the test chamber.

27. Appropriate controls are prepared for each of the methods of test substance application described in paragraphs 18 to 24. The relevant procedures described are followed for preparing the controls except that the test chemical is not added. Thus, where appropriate, organic solvents, quartz sand or other vehicles are applied to the controls in concentrations/amounts consistent with those used in the treatments. Where a solvent or other vehicle is used to add the test chemical an additional control without the vehicle or test chemical should also be prepared and tested to ensure that the vehicle has no bearing on the result.

Test conditions

28. The test temperature is 20 ± 2 °C. 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 containers.

29. The test containers are not aerated during the test but the design of the test vessel covers should provide opportunity for gaseous exchange whilst limiting evaporation of moisture (see paragraph 9).

30. The water content of the soil substrate in the test containers is maintained throughout the test by re-weighing the test containers (minus their covers) periodically. Losses are replenished as necessary with de-ionised water. The water content should not vary by more than 10 % from that at the start of the test.

Feeding

31. Any food of a quality shown to be suitable for at least maintaining worm weight during the test is considered acceptable. Experience has shown that oatmeal, cow or horse manure is a suitable food. Checks should be made to ensure that cows or horses from which manure is obtained are not subject to medication or treatment with substances, such as growth promoters, nematicides or similar veterinary products that could adversely affect the worms during the test. Self-collected cow manure is recommended, since experience has shown that commercially available cow manure used as garden fertiliser may have adverse effects on the worms. The manure should be air-dried, finely ground and pasteurised before use.

32. Each fresh batch of food should be fed to a non-test worm culture before use in a test to ensure that it is of suitable quality. Growth and cocoon production should not be reduced compared to worms kept in a substrate that does not contain the new batch of food (conditions as described in OECD 207(4)).

33. Food is first provided one day after adding the worms and applying the test chemical to the soil. Approximately 5 g of food is spread on the soil surface of each container and moistened with de-ionised

water (about 5 ml to 6 ml per container). Thereafter food is provided once a week during the 4-week test period. If food remains uneaten the ration should be reduced so as to avoid fungal growth or moulding. The adults are removed from the soil on day 28 of the test. A further 5 g of food is then administered to each test container. No further feeding takes place during the remaining 4 weeks of the test.

Range-finding test

34. Prior knowledge of the toxicity of the test chemical should help in selecting appropriate test concentrations, e.g. from an acute test (4) and/or from range-finding studies. When necessary, a range-finding test is conducted with, for example, five test concentrations of 0.1, 1.0, 10, 100, and 1000 mg/kg (dry mass of soil). One replicate for each treatment and control is sufficient. The duration of the range-finding test is two weeks and the mortality is assessed at the end of the test.

Experimental design

35. Since a single summary statistic cannot be prescribed for the test, this Guideline makes provision for the determination of the NOEC and the EC_x. A NOEC is likely to be required by regulatory authorities for the foreseeable future. More widespread use of the EC_x, resulting from statistical and ecological considerations, may be adopted in the near future. Therefore, three designs are proposed, based on recommendations arising from a ring test of an enchytraeid reproduction test method (17):

36. In setting the range of concentrations, the following should be borne in mind:

- For determination of the NOEC, at least five/twelve 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 2.0.

- For determination of the EC_x (e.g. EC_{10}, EC_{50}), an adequate number of concentrations to cause at least four statistically significantly different mean responses at these concentrations is recommended. At least two replicates for each test concentration and six 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.

Test duration and measurements

37. On Day 28 the living adult worms are observed and counted. Any unusual behaviour (e.g. inability to dig into the soil; lying motionless) and in morphology (e.g. open wounds) are also recorded. All adult worms are then removed from the test vessels and counted and weighed. Transfer of the soil containing the worms to a clean tray prior to the assessment may facilitate searching for the adults. The worms extracted from the soil should be washed prior to weighing (with de-ionised water) and the excess water removed by placing the worms briefly on filter paper. Any worms not found at this time are to be recorded as dead, since it is to be assumed that such worms have died and decomposed prior to the assessment. Adult worms are humanely euthanized, preferably by rapid freezing at -80°C or cryopreservation.

38. If the soil has been removed from the containers it is then returned (minus the adult worms but containing any cocoons that have been produced). The soil is then incubated for four additional weeks under the same test conditions except that feeding only takes place once at the start of this phase of the test (see paragraph 33).

39. At the end of the second 4-week period, the number of juveniles hatched from the cocoons in the test soil and cocoon numbers are determined using procedures described in Annex 5. All signs of harm or damage to the worm should also be recorded throughout the test period. At termination of the test, hatched juveniles are counted and humanely euthanized, preferably by rapid freezing at -80°C or cryopreservation.

Limit test

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

DATA AND REPORTING

Treatment of results

41. Although an overview is given in Annex 6, no definitive statistical guidance for analysing test results is given in this guideline.

42. One 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 (18) 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 (19), the trimmed Spearman-Karber method (20) 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}).

43. The other endpoint is fecundity (e.g. 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 $\overline{X}$ and the standard deviation per treatment and per control for reproduction to be calculated.

44. If an analysis of variance has been performed, the standard deviation, s, and the degrees of freedom (df) may be replaced by the pooled variance estimate obtained from the ANOVA and by its degrees of freedom, respectively – provided variance does not depend on the concentration. In this case, use the single variances of control and treatments. Those values are usually calculated by commercial statistical software using the per-vessel results as replicates. If pooling data for the negative and solvent controls appears reasonable rather than testing against one of those, they should be tested to see that they are not significantly different (for the appropriate test, consider paragraph 47 and Annex 6).

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

NOEC Estimation

46. 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 transform 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) (21)(22) or certain cases Dunnett´s test (23)(24) 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 (25). Alternatively, a multiple U-test, e.g. the Bonferroni-U-test according to Holm (26), 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 (27)(28) or Shirley (29) (30)) can be applied and would generally be preferred to unequal-variance t-tests. (see also the scheme in Annex 6).

47. 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 (31).

EC_x Estimation

48. 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 (32). 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 (18)) or other modern appropriate methods.

49. Alternatively, the response is modeled 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 (18). In these cases the weighting function has to be adjusted for metric responses as given by Christensen (33). 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 (34). 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 (19) and the Trimmed Spearman-Karber procedure (20).

Test report

50. The test report must include the following information:

Test chemical:

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, UVCBs and mixtures:

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

Test organisms:
- test animals used: species, scientific name, source of organisms and breeding conditions;
- age, size (mass) range of test organisms.

Test conditions

- preparation details for the test soil;
- the maximum water holding capacity of the soil;
- a description of the technique used to apply the test chemical to the soil;
- details of auxiliary substances used for administering the test chemical;
- calibration details for spraying equipment if appropriate;
- description of the experimental design and procedure;
- size of test containers and volume of test soil;
- test conditions: light intensity, duration of light-dark cycles, temperature;
- a description of the feeding regime, the type and amount of food used in the test, feeding dates;
- pH and water content of the soil at the start and end of the test.

Test results:

- adult mortality (%) in each test container at the end of the first 4 weeks of the test;
- the total mass of adults at the beginning of the test in each test container;
- changes in body weight of live adults (% of initial weight) in each test container after the first four weeks of the test;
- the number of juveniles produced in each test container at the end of the test;
- a description of obvious or pathological symptoms or distinct changes in behaviour;
- the results obtained with the reference test chemical;
- 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;
- a plot of the dose-response-relationship;
- the results applicable to each test container;

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

LITERATURE

(1) Jaenicke, J. (1982). '*Eisenia foetida'* is two biological species. Megadrilogica 4, 6-8.

(2) Oien, N. and J. Stenerson (1984). Esterases of earthworm - III. Electrophoresis reveals that *Eisenia foetida* (Savigny) is two species. Comp. Biochem. Physiol. 78c (2), 277 - 282.

(3) Kula, C. (1996). Development of a test method on sublethal effects of pesticides on the earthworm species *Eisenia fetida/Eisenia andrei* – comparison of two ringtests. In: Riepert, F., Kula, C. (1996): Development of laboratory methods for testing effects of chemicals and pesticides on collembola and earthworms. Mitt. Biol. Bundesamst. f. Land- Forstwirtsch. Berlin-Dahlem, 320, p. 50-82.

(4) OECD Guideline for testing chemicals 207. Earthworm acute toxicity test. Adopted: 4 April 1984.

(5) ISO (International Organization for Standardization) (2012). Soil Quality – Effects of pollutants on earthworms. Part 2: Determination of effects on reproduction of *Eisenia fetida/Eisenia andrei*, No.11268-2. ISO, Geneve.

(6) ISO (International Organization for Standardization) (2012). Soil Quality – Effects of pollutants on earthworms (*Eisenia fetida*). Part 1: Determination of acute toxicity to *Eisenia fetida/Eisenia Andrei* using artificial soil substrate, No.11268-1. ISO, Geneve.

(7) SETAC (1998). Advances in Earthworm Ecotoxicology. Sheppard, S.C., Bembridge, J.D., Holmstrup, M., and L. Posthuma, (eds). SETAC Press, 456 pp.

(8) EPA (1996). Ecological effects test guidelines. Earthworm Subchronic Toxicity Test (850.62.00). United States Environmental Protection Agency. Office of Prevention, Pesticides and Toxic Substances. EPA712-C-96-167, April 1996.

(9) Bouché, M.B. (1972). Lombriciens de France, Ecologie et systématique. Publication de l'Institut National de la Recherche Agronomique.

(10) Edwards, C.A. (1983). Development of a standardized laboratory method for assessing the toxicity of chemical substances to earthworms. Report EUR 8714 EN, Commission of European Communities.

(11) Greig-Smith, P.W., H. Becker, P.J. Edwards and F. Heimbach (eds.) (1992). Ecotoxicology of Earthworms. Intercept.

(12) Edwards, C.A. and J. P. Bohlen, (1996). Biology and ecology of Earthworms, 3rd Edition. Chapman and Hall, London.

(13) ISO (International Organization for Standardization) (1994). Soil Quality - Determination of pH, No. 10390. ISO, Geneve.

(14) Hund-Rinke, K, Römbke, J., Riepert, F. & Achazi R. (2000): Beurteilung der Lebensraumfunktion von Böden mit Hilfe von Regenwurmtests. In: Toxikologische Beurteilung von Böden. Heiden, S., Erb, R., Dott, W. & Eisentraeger, A. (eds.). Spektrum Verl., Heidelberg. 59-81.

(15) ISO (International Organization for Standardization) (1998). Soil Quality –Determination of water retention characteristics –Laboratory methods, No. 11274. ISO, Geneve.

(16) ISO (International Organization for Standardization) (1993). Soil Quality –Determination of dry matter and water content on a mass basis – Gravimetric method, No. 11465. ISO, Geneve.

(17) Römbke, J. and Th. Moser (1999). Organisation and Performance of an International Ringtest for the validation of the Enchytraeid Reproduction Test. UBA-Texte 4/99, 150+ 223 pp.

(18) Finney, D.J. (1971). Probit Analysis (3rd ed.), pp. 19-76. Cambridge Univ. Press.

(19) Finney, D.J. (1978). Statistical Method in Biological Assay. - Charles Griffin & Company Ltd, London.

(20) Hamilton, M.A., R.C. Russo and R.V. Thurston. (1977). Trimmed Spearman-Karber Method for estimating median lethal concentrations in toxicity bioassays. Environ. Sci. Technol. 11(7), 714-719; Correction Environ. Sci. Technol. 12(1998), 417.

(21) Williams, D.A., (1971). A test for differences between treatment means when several dose levels are compared with a zero dose control. Biometrics 27, 103-117.

(22) Williams, D.A., (1972). The comparison of several dose levels with a zero dose control. Biometrics 28, 519-531.

(23) Dunnett, C.W., (1955). A multiple comparison procedure for comparing several treatments with a control. Amer. Statist. Ass. J. 50, 1096-1121.

(24) Dunnett, C.W., (1964) New tables for multiple comparisons with a control. Biometrics 20, 482-491.

(25) Hoeven, N. van der, (1998). Power analysis for the NOEC: What is the probability of detecting small toxic effects on three different species using the appropriate standardized test protocols? Ecotoxicology 7: 355-361

(26) Holm, S., (1979): A simple sequentially rejective multiple test procedure. Scand. J. Statist. 6, 65-70.

(27) Jonckheere, A. R. (1954); A Distribution-free k-Sample Test Against Ordered Alternatives, Biometrika 41, 133-145.

(28) Terpstra, T. J. (1952); The Asymptotic Normality and Consistency of Kendall's Test Against Trend, When Ties are Present in One Ranking, Indagationes Math. 14, 327-333.

(29) Shirley, E. A. (1979); The comparison of treatment to control group means in toxicology studies, Applied Statistics 28, 144-151.

(30) Williams, D.A. (1986); A Note on Shirley's Nonparametric Test for Comparing Several Dose Levels with a Zero-Dose Control, Biometrics 42, 183-186.

(31) Sokal, R.R. and F.J. Rohlf. (1981). Biometry. The Principle and practice of statistics in biological research. 2nd edition. W.H. Freeman and Company. New York.

(32) Bruce R.D. and Versteeg D.J. (1992) A statistical procedure for modelling continuous toxicity data. Environmental Toxicology and Chemistry 11:1485-1494

(33) Christensen, E.R., (1984). Dose-response functions in aquatic toxicity testing and the Weibull model. Water Research 18, 213-221.

(34) Van Ewijk, P.H. and J.A. Hoekstra. (1993). Calculation of the EC50 and its confidence interval when sub-toxic stimulus is present. Ecotox, Environ. Safety. 25, 25-32.

ANNEX 1

DEFINITIONS

The following definitions are applicable to this Guideline:

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. For example, an EC_{50} is a concentration estimated to cause an effect on a test end point in 50% of an exposed population over a defined exposure period. In this test the effect concentrations are expressed as a mass of test substance per dry mass of the test soil or as a mass of the test substance per unit area of the soil.

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 that 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 or as a mass of test chemical per unit area of 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 or as a mass of test chemical per unit area of soil. All test concentrations above the LOEC should normally show an effect that is statistically different from the control. Any deviations from the above must be justified in the test report.

NOEC (No Observed Effect Concentration) is the highest test 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: Mean number of juvenile worms produced per a number of adults over the test period.

ANNEX 2

DETERMINATION OF THE MAXIMUM WATER HOLDING CAPACITY OF THE SOIL

The following method for determining the maximum water holding capacity of the soil has been found to be appropriate. It is described in Annex C of the ISO DIS 11268-2 (1).

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

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

Where:
S = water-saturated substrate + mass of tube + mass of filter paper
T = tare (mass of tube + mass of filter paper)
D = dry mass of substrate

References:

(1) ISO (International Organization for Standardisation) (2012). Soil Quality – Effects of pollutants on earthworms . Part 2: Determination of effects on reproduction of *Eisenia fetida/Eisenia andrei*, No.11268-2. ISO, Geneve.

ANNEX 3

DETERMINATION OF SOIL pH

The following method for determining the pH of a soil is based on the description given in ISO 10390: Soil Quality – Determination of pH (1).

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

References:

(1) ISO (International Organization for Standardization) (2005). Soil Quality - Determination of pH, No. 10390. ISO, Geneve.

ANNEX 4

CULTURING OF *EISENIA FETIDA /EISENIA ANDREI*

Breeding should preferably be carried out in a climatic chamber at 20 °C ± 2 °C. At this temperature and with the provision of sufficient food, the worms become mature after about 2 to 3 months.

Both species can be cultured in a wide range of animal wastes. The recommended breeding medium is a 50:50 mixture of horse or cattle manure and peat. Checks should be made to ensure that cows or horses from which manure is obtained are not subject to medication or treatment with chemicals, such as growth promoters, nematicides or similar veternary products that could adversely affect the worms during the test. Self-collected manure obtained from an "organic" source is recommended, since experience has shown that commercially available manure used as garden fertiliser may have adverse effects on the worms. The medium should have a pH value of approximately 6 to 7 (adjusted with calcium carbonate), a low ionic conductivity (less than 6 mg or 0.5 % salt concentration) and should not be contaminated excessively with ammonia or animal urine. The substrate should be moist but not too wet. Breeding boxes of 10 to 50-litre capacity are suitable.

To obtain worms of standard age and size (mass), it is best to start the culture with cocoons. Once the culture has been established it is maintained by placing adult worms in a breeding box with fresh substrate for 14 days to 28 days to allow further cocoons to be produced. The adults are then removed and the juveniles produced from the cocoons used as the basis for the next culture. The worms are fed continuously with animal waste and transferred into fresh substrate from time to time. Experience has shown that air-dried finely ground cow or horse manure or oatmeal is a suitable food. It should be ensured that cows or horses from which manure is obtained are not subject to medication treatment with chemicals, such as growth promoters, that could adversly affect the worms during long term culture. The worms hatched from the cocoons are used for testing when they are between 2 and 12 months old and considered to be adults.

Worms can be considered to be healthy if they move through the substrate, do not try to leave the substrate and reproduce continuously. Substrate exhaustion is indicated by worms moving very slowly and having a yellow posterior end. In this case the provision of fresh substrate and/or a reduction in stocking density is recommended.

ANNEX 5

TECHNIQUES FOR COUNTING JUVENILE WORMS HATCHED FROM COCOONS

Hand sorting of worms from the soil substrate is very time-consuming. Two alternative methods are therefore recommended:

(a) The test containers are placed in a water bath initially at a temperature of 40°C but rising to 60°C. After a period of about 20 minutes the juvenile worms should appear at the soil surface from which they can be easily removed and counted.

(b) The test soil may be washed through a sieve using the method developed by van Gestel et al. (1) providing the peat and the manure or oatmeal added to the soil were ground to a fine powder. Two 0.5 mm mesh size sieves (diameter 30 cm) are placed on top of each other. The contents of a test container are washed through the sieves with a powerful stream of tap water, leaving the young worms and cocoons mainly on the upper sieve. It is important to note that the whole surface of the upper sieve should be kept wet during this operation so that the juvenile worms float on a film of water, thereby preventing them from creeping through the sieve pores. Best results are obtained when a showerhead is used.

Once all the soil substrate has been washed through the sieve, juveniles and cocoons can be rinsed from the upper sieve into a bowl. The contents of the bowl are then left to stand allowing empty cocoons to float on the water surface and full cocoons and young worms to sink to the bottom. The standing water can then be poured off and the young worms and cocoons transferred to a petri dish containing a little water. The worms can be removed for counting using a needle or a pair of tweezers.

Experience has shown that method (a) is better suited to extraction of juvenile worms that might be washed through even a 0.5 mm sieve.

The efficiency of the method used to remove the worms (and cocoons if appropriate) from the soil substrate should always be determined. If juveniles are collected using the hand sorting technique it is advisable to carry out the operation twice on all samples.

References:

(1) Van Gestel, C.A.M., W.A. van Dis, E.M. van Breemen, P.M. Sparenburg (1988). Comparison of two methods determining the viability of cocoons produced in earthworm toxicity experiments. Pedobiologia 32:367-371.

ANNEX 6

OVERVIEW OF THE STATISTICAL ASSESSMENT OF DATA (NOEC DETERMINATION)