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OECD GUIDELINES FOR THE TESTING OF CHEMICALS

Bioaccumulation in Terrestrial Oligochaetes

INTRODUCTION

1. Among the OECD Guidelines for the Testing of Chemicals relating to environmental fate, the Bioconcentration: Flow-through Fish Test (TG 305) and the Bioaccumulation in Sediment-dwelling Benthic Oligochates (TG 315) were published in 1996 and 2008 respectively. The extrapolation of aquatic bioaccumulation data to terrestrial organisms like earthworms is difficult, if possible at all. Model calculations based on a compound's lipophilicity, e.g. (14) (37), are currently used for the assessment of bioaccumulation of chemicals in soil, as e.g. in the EU Technical Guidance Document (19). The need for a compartment-specific test method has already been addressed, e.g. (55). Such a method is especially important for the evaluation of secondary poisoning in terrestrial food chains (4). Several national test methods address the issue of bioaccumulation in organisms other than fish e. g. (2) and (72). A method on the measurement of bioaccumulation from contaminated soils in earthworms (*Eisenia fetida*, Savigny) and potworms has been developed by the American Society for Testing and Materials (3). An internationally accepted, test method for the determination of bioaccumulation in spiked soil will improve the risk assessment of chemicals in terrestrial ecosystems e.g. (25) (29).

2. Soil-ingesting invertebrates are exposed to soil bound substances. Among these animals, terrestrial oligochaetes play an important role in the structure and function of soils (15) (20). Terrestrial oligochaetes live in soil and partly, at the soil surface (especially the litter layer); they frequently represent the most abundant species in terms of biomass (54). By bioturbation of the soil and by serving as prey these animals can have a strong influence on the bioavailability of substances to other organisms like invertebrates (e.g. predatory mites and beetles; e.g. (64)) or vertebrate (e.g. foxes and gulls) predators (18) (62). Some species of terrestrial oligochaetes currently used in ecotoxicological testing are described in Annex 5.

3. The ASTM Standard Guide for Conducting Laboratory Soil Toxicity or Bioaccumulation Tests with the Lumbricid Earthworm *Eisenia fetida* and the Enchytraeid Potworm *Enchytraeus albidus* (3) provides many essential and useful details for the performance of the present soil bioaccumulation test method. Further documents that are referred to in this guideline are the OECD Test Guideline 305: Bioconcentration: Flow-through Fish Test (49) and the OECD Test Guideline 315: Bioaccumulation in Sediment-dwelling Benthic Oligochates (53). Practical experience with soil bioaccumulation studies and publications from literature e.g. (1) (5) (11) (12) (28) (40) (43) (45) (57) (59) (76) (78) (79) are also major sources of information for this Test Guideline.

4. This test method is mostly applicable to stable, neutral organic chemicals, which tend to adsorb to soils. Testing for bioaccumulation of soil-associating, stable metallo-organic compounds may be possible with this method. It is also applicable to metals and other trace elements.

PRE-REQUISITE

5. Tests for measuring the bioaccumulation of a substance in terrestrial oligochaetes have been performed with heavy metals (see e.g. (63)) and persistent, organic substances having log K_{ow} values between 3.0 and 6.0 e.g. (40). Such tests also apply to:

- Substances that show a log K_{ow} of more than 6.0 (super-hydrophobic substances);
- Substances which belong to a class of organic substances known to have the potential to bioaccumulate in living organisms, e.g. surface active or highly adsorptive substances;
- Substances that indicate the potential for bioaccumulation from structural features, e.g. analogues of substances with known bioaccumulation potential; and
- Metals.

6. Information on the test substance such as common name, chemical name (preferably IUPAC name), structural formula, CAS registry number, purity, safety precautions, proper storage conditions and analytical methods should be obtained before beginning the study. In addition, the following information should be known:

(a) solubility in water;
(b) octanol-water partition coefficient, $K_{ow;}$
(c) soil-water partition coefficient, expressed as $K_{oc;}$
(d) vapour pressure;
(e) degradability (e.g. in soil, water);
(f) known metabolites.

7. Radiolabelled or non-radiolabelled test substance can be used. However, to facilitate analysis it is recommended to use a radiolabelled test substance. The decision will be made based on the detection limits or a requirement to measure parent compound and metabolites. If a radiolabelled chemical is used and total radioactive residues are measured, it is important that the radiolabelled residues in both the soil and the test organisms are characterised for percentages of parent compound and labelled non-parent, e.g. in samples taken at steady state or at the end of the uptake phase, to allow a bioaccumulation factor (BAF) calculation for the parent compound and for the soil metabolites of concern (see paragraph 50). The method described here may have to be modified, e.g., to provide sufficient biomass, for measuring non-radiolabelled organic test substance or metals. When total radioactive residues are measured (by liquid scintillation counting following extraction, combustion or tissue solubilisation), the bioaccumulation factor is based on the parent compound and metabolites. The BAF calculation should preferably be based on the concentration of the parent compound in the organisms and total radioactive residues. Subsequently, the biota-soil accumulation factor (BSAF), normalized to the lipid content of worm and organic carbon content (OC) of soil should be calculated from the BAF for reasons of comparability between results from different bioaccumulation tests.

8. Toxicity of the test substance to the species used in the test should be known, e.g. an effect concentration (EC_x) or lethal concentration (LC_x) for the time of the uptake phase (e.g. (19)). The selected concentration of the test substance should preferably be about 1% of its acute asymptotic LC_{50}, and at least ten-fold higher than its detection limit in soil by the analytical method used. If available, preference should be given to toxicity values derived from long-term studies on sublethal endpoints (51) (52). If such data are not available, an acute toxicity test will provide useful information (see e.g. (23)).

9. An appropriate analytical method of known accuracy, precision, and sensitivity for the quantification of the substance in the test solutions, in the soil, and in the biological material should be available, together with details of sample preparation and storage as well as material safety data sheets. Analytical detection limits of the test item in soil and worm tissue should also be known. If a ^{14}C-labelled test substance is used, the specific radioactivity (i.e. Bq mol^{-1}) and the percentage of radioactivity associated with impurities should be known. The specific radioactivity of the test substance should be high enough to facilitate analysis, and the test concentrations used should not elicit toxic effects.

10. The test can be performed with an artificial soil or with natural soils. Information on characteristics of the natural soil used, e.g. origin of soil or its constituents, pH, organic carbon content, particle size distribution (percent sand, silt, and clay), and water holding capacity (WHC), should be known before the start of the test (3) (48).

PRINCIPLE OF THE TEST

11. The parameters which characterise the bioaccumulation of a substance include the bioaccumulation factor (BAF), the uptake rate constant (k_s) and the elimination rate constant (k_e). Definitions are provided in Annex 1.

12. The test consists of two phases: the uptake (exposure) phase and the elimination (post-exposure) phase. During the uptake phase, replicated groups of worms are exposed to soil which has been spiked with the test substance. In addition to the test animals, groups of control worms are held under identical conditions without the test substance. The dry weight and lipid content of the test organisms are measured. This can be done using worms of the control group. Analytical background values (blank) can be obtained by analysing samples of the control worms and soil. For the elimination phase, the worms are transferred to a soil free of the test substance. An elimination phase is always required unless uptake of the test substance during the exposure phase has been insignificant. An elimination phase provides information on the rate at which the test substance is excreted by the test organisms (e.g. (27)). If a steady state has not been reached during the uptake phase, the determination of the kinetic parameters – kinetic bioaccumulation factor BAF_k, uptake and elimination rate constant(s) – should preferably be based on simultaneous fitting of the results of the uptake and elimination phases. The concentration of the test substance in/on the worms is monitored throughout both phases of the test.

13. During the uptake phase, measurements are made at sampling times up to 14 days (enchytraeids) or 21 days (earthworms) until the steady-state is reached (11) (12) (67). The steady state occurs when a plot of the concentration in worms against time is parallel to the time axis, and three successive concentration analyses made on samples taken at intervals of at least two days do not vary more than ± 20% of each other based on statistical comparisons (e.g., analysis of variance, regression analysis).

14. The elimination phase consists of transferring the test organisms to vessels containing the same substrate without the test substance. During the elimination phase, measurements are made at sampling times during 14 days (enchytraeids) or 21 days (earthworms) unless earlier analytical determination showed 90% reduction of the test substance residues in worms. The concentration of the test substance in the worms at the end of the elimination phase is reported as non-eliminated residues. The steady state bioaccumulation factor (BAF_{ss}) is calculated preferably both as the ratio of the concentration in worms (C_a) and in the soil (C_s) at apparent steady state, and as a kinetic bioaccumulation factor, BAF_K, as the ratio of the rate constant of uptake from soil (k_s) and the elimination rate constant (k_e) (see Annex 1 for definitions) assuming first-order kinetics (see Annex 2 for calculations). If first-order kinetics is obviously not applicable, other models should be employed.

15. The uptake rate constant, the elimination rate constant (or constants, where other models are involved), the kinetic bioaccumulation factor (BAF_K), and where possible, the confidence limits of each of these parameters are calculated from computerised model equations (see Annex 2 for guidance). The goodness of fit of any model can be determined from e.g. the correlation coefficient or the coefficient of determination (coefficients close to one indicate a good fit) or chi-squared. Also the size of the standard error or confidence limit around the estimated parameters may be indicative of the goodness of fit of the model.

16. To reduce variability in test results for substances with high lipophilicity, bioaccumulation factors should be expressed in relation to lipid content and organic carbon content (kg soil organic carbon (OC) kg^{-1} worm lipid content). This approach is based on the fact that for some chemical classes, there is a clear relationship between the potential for bioaccumulation and lipophilicity; this has been well established for fish (47). There is a relationship between the lipid content of fish and the bioaccumulation of such substances. For benthic organisms, similar correlations have been found e.g. (30) (44). Likewise for terrestrial oligochaetes this correlation has been demonstrated e.g. (5) (6) (7) (14). If sufficient worm tissue is available, the lipid content of the test animals can be determined on the same biological material as the one used to determine the concentration of the test substance. Alternatively, control animals can be used to measure the lipid content.

VALIDITY OF THE TEST

17. For a test to be valid the following criteria should be fulfilled for both controls and treatments:

- At the end of the test, the overall mortality during uptake and elimination phase should not exceed 10% (earthworms) or 20% (enchytraeids) of the total number of the introduced worms.

- For *Eisenia fetida and Eisenia andrei*, the mean mass loss as measured at the end of the uptake and at the end of the elimination phase should not exceed 20% compared to the initial fresh weight (f.w.) at start of each phase.

DESCRIPTION OF THE METHOD

Test species

18. Several species of terrestrial oligochaetes are recommended for bioaccumulation testing. The most commonly used species *Eisenia fetida* or *Eisenia andrei* (Lumbricidae), or *Enchytraeus albidus, Enchytraeus crypticus*, or *Enchytraeus luxuriosus* (Enchytraeidae)) are described in Annex 5.

Apparatus

19. Care should be taken to avoid the use of materials, for all parts of the equipment, which can dissolve, adsorb the test substance or leach other compounds, and have an adverse effect on the test animals. Standard rectangular or cylindrical vessels, made of chemically inert material and of suitable capacity can be used in compliance with the loading rate, i.e. the number of test worms. Stainless steel, plastic or glass may be used for any equipment having contact with the test media. The test vessels should be appropriately covered to prevent escaping of the worms, while allowing sufficient air supply. For substances with high adsorption coefficients, such as synthetic pyrethroids, silanised glass may be required. In these situations the equipment will have to be discarded after use (49). Radiolabelled test items and volatile chemicals should be prevented from escaping. Traps (e.g. glass gas washing bottles) should be employed containing suitable absorbents to retain any residues evaporating from the test vessels.

Soil

20. The test soil should be of a quality that will allow the survival and preferably the reproduction of the test organisms for the duration of the acclimation and test periods without them showing any abnormal appearance or behaviour. The worms should burrow in the soil.

21. The artificial soil described in the OECD Test Guideline 207 (48) is recommended for use as the substrate in the tests. Preparation of the artificial soil for use in the bioaccumulation tests and

recommendations for the storage of artificial soil are given in Annex 4. Air-dried artificial soil may be stored at room temperature until use.

22. However, natural soils from unpolluted sites may serve as test and/or culture soil. Natural soils should be characterised at least by origin (collection site), pH, organic carbon content, particle size distribution (percent sand, silt, and clay), maximum water holding capacity (WHC_{max}), and percent water content (3). Analysis of the soil or its constituents for micro-pollutants prior to use should provide useful information. If field soil from agricultural land is used, it should not have been treated with crop protection products or with manure from treated animals as fertilizers for at least one year and with organic fertilizers for at least six months prior to sampling (50). Manipulation procedures for natural soils prior to use in ecotoxicological tests with oligochaetes in the laboratory are described in (3). For natural soils the storage time in the laboratory should be kept as short as possible.

Application of the test substance

23. The test substance is incorporated into the soil. The physicochemical properties of the test substance should be taken into consideration. A water-soluble test substance should be completely dissolved in water before it be mixed with the soil. The recommended spiking procedure for poorly water-soluble test substance involves coating of one or more of the (artificial) soil constituents with the test substance. For example, the quartz sand, or a portion thereof, can be soaked with a solution of the test substance in a suitable organic solvent, which is then slowly evaporated to dryness. The coated fraction can then be mixed into the wet soil. The major advantage of this procedure is that no solvent is introduced into the soil. When a natural soil is used, the test substance may be added by spiking an air-dried portion of the soil as described above for the artificial soil, or by stirring the test substance into the wet soil, with subsequent evaporating step if a solubilising agent is used. In general, the contact of wet soil with solvents should be avoided as far as possible. The following should be considered (3):

- If a solvent other than water is used, it should be one that is water-miscible and/or can be driven off (for example, evaporated), leaving only the test chemical on the soil.

- If a solvent control is used, there is no need for negative control. The solvent control should contain the highest concentration of solvent added to the soil and should use solvent from the same batch used to make the stock solution. Toxicity and volatility of the solvent, and solubility of the test substance in the chosen solvent should be the main criteria used for the selection of a suitable solubilising agent.

24. For substances that are poorly soluble in water and in organic solvents, 2.0 – 2.5 g of finely ground quartz sand per test vessel can be mixed with the quantity of test substance, e.g. using mortar and pestle, to obtain the desired test concentration. This mixture of quartz sand and test substance is added to the pre-moistened soil and thoroughly mixed with an appropriate amount of de-ionised water to obtain the required moisture content. The final mixture is distributed to the test vessels. The procedure is repeated for each test concentration, and an appropriate control with 2.0 – 2.5 g of finely ground quartz sand per test vessel is also prepared.

25. The concentration of the test substance in the soil should be determined after spiking. The homogenous distribution of the test substance into the soil should be verified before introducing the test organisms. The method used for spiking, and the reasons for choosing a specific spiking procedure should be reported (24).

26. Equilibrium between the soil and the pore-water phase should ideally be established before adding the organisms; a time period of four days at 20°C is recommended. For many poorly water-soluble

organic chemicals the time required to reach a true equilibrium between adsorbed and dissolved fractions can be counted in days or months. Depending on the purpose of the study, for example when the environmental conditions are to be mimicked, the spiked soil may be 'aged' for a longer period, e.g. for metals three weeks at 20°C (22).

Culturing of the test organisms

27. Worms should be preferably kept in permanent laboratory culture. Guidance on laboratory culture methods for *Eisenia fetida* and *Eisenia andrei*, and *Enchytraeid* species, is provided in Annex 5 (see also (48) (51) (52)).

28. The worms used in the tests should be free from observable diseases, abnormalities and parasites.

PERFORMANCE OF THE TEST

29. The test organisms are exposed to the test substance during the uptake phase. The uptake phase should be of 14 days (enchytraeids) or 21 days (earthworms) unless it is demonstrated that steady state has been reached.

30. For the elimination phase, the worms are transferred to a soil free of test substance. The first sample should be taken at 4 – 24 h after the start of elimination phase. Examples of sampling schedules for a 21-day uptake phase and a 21-day elimination phase are given in Annex 3.

Test organisms

31. For many species of terrestrial enchytraeids the individual weight is very low (e.g. 5-10 mg wet weight per individual for *Enchytraeus albidus* and less for *Enchytraeus crypticus* or *Enchytraeus luxuriosus*); in order to perform the weight measurements and chemical analysis, it may be necessary to pool the worms of the replicate test vessels (i.e. all the worms of a replicate vessel will be used for obtaining one analytical tissue result). 20 individual enchytraeids are added to each replicate, and at least three replicates should be used. If the analytical detection limit of the test substance is high, more worms may be necessary. For test species with higher individual weight (*Eisenia fetida* and *Eisenia andrei*), replicate vessels containing one individual can be used.

32. The earthworms used in a test should be of similar weight (e.g. *Eisenia fetida* and *Eisenia andrei* should have an individual weight of 250 – 600 mg). Enchytraeids (e.g. *Enchytraeus albidus*) should have a length of approximately 1 cm. All worms used in a particular test should come from the same source, and should be adult animals with clitellum (see Annex 5). Since the weight and age of an animal might have an effect on the BAF-values (e.g. due to varying lipid content and/or presence of eggs), these parameters should be recorded accurately and taken into account in the interpretation of results. In addition, cocoons can be deposited during the exposure period, which will also have an impact on the BAF values. It is recommended that a sub-sample of the test worms be weighed before the test in order to estimate the mean wet and dry weights.

33. A high soil-to-worm ratio should be used in order to minimise the decrease of the test substance concentration in the soil during the uptake phase. For *Eisenia fetida* and *Eisenia andrei* a minimum amount of 50 g dry weight (d.w.) of soil per worm, and for enchytraeids, a minimum of 10-20 g d.w. of soil per test vessel are recommended. The vessels should contain a soil layer of 2-3 cm (enchytraeids) or 4-5 cm (earthworms).

34. The worms used in a test are removed from the culture (e.g. enchytraeids by using jeweller's tweezers). Adult animals are transferred to non-treated test soil for acclimation, and fed (see paragraph 36). If the test conditions differ from the culture conditions, an acclimation phase of 24-72 h should be sufficient to adapt the worms to the test conditions. After acclimation, earthworms are rinsed by transfer to glass dishes (e.g. petri dishes) containing clean water, and subsequently weighed before they are added to the test soil. Prior to weighing, excess water should be removed from the worms by gently touching them against the edge of the dish or by blotting them cautiously dry by using a slightly moistened paper towel.

35. Burrowing behaviour of the test organisms should be observed and recorded. In tests with earthworms, the animals (control and treatments) normally burrow in the soil within a period of a few hours; this should be checked no later than 24 h after addition of the worms to the test vessels. If the earthworms fail to burrow in the soil (e.g. more than 10% over more than half of the uptake phase), this indicates that either the test conditions are not appropriate or the test organisms are not healthy. In such a case the test should be stopped and repeated. Enchytraeids mainly live in the interstitial pores of the soil, and frequently their integument may be only partly in contact with the surrounding substrate; exposure of burrowing and non-burrowing enchytraeids is assumed to be equivalent and non-burrowing of the enchytraeids does not necessarily require the repetition of the test.

Feeding

36. Feeding should be envisaged when a soil with low total organic carbon content is used. When an artificial soil is used, a weekly feeding rate (i.e. the worms should be fed once a week) of 7 mg of dried dung per g soil dry weight is recommended for earthworms, and a weekly rate of 2-2.5 mg of ground oat flakes per g soil dry weight is recommended for enchytraeids (11). The first food ration should be mixed with the soil immediately before the test organisms are added. Preferably the same type of food like in the cultures should be used (see Annex 5).

Light and temperature

37. The tests should be carried out under a controlled 16/8 hours light/dark cycle, preferably 400 to 800 lx in the area of the test vessels (3). The test temperature should be 20 ± 2°C throughout the test.

Test concentrations

38. A single concentration is used. Situations where additional concentration(s) is(are) required should be justified. If toxicity (EC_x) of the test substance is close to the analytical detection limit, the use of radiolabelled test substance with high specific radioactivity is recommended. For metals, the concentration should be above the background level in tissue and soil.

Replicates

39. For the kinetic measurements (uptake and elimination phase), the minimum number of treated replicate vessels should be three per sampling point. The total number of replicates prepared should be sufficient to cover all sampling times during the uptake and the elimination phase.

40. For the biological observations and measurements (e.g. dry-to-wet weight ratio, lipid content) and for the analysis of background concentrations in worms and soil, at least 12 replicate vessels of a negative control (four sampled at start, four at end of uptake, and four at end of elimination) should be provided if no solvent other than water is used. If any solubilising agent is used for application of the test substance, a solvent control (four replicate vessels should be sampled at start, four at the end of the uptake phase, and four at the end of the elimination phase) containing all constituents except for test item should

be run in addition to the treated replicates. In this case, four additional replicate vessels of a negative control (no solvent) may also be provided for optional sampling at the end of the uptake phase. These replicates can be compared biologically with the solvent control in order to gain information on a possible influence of the solvent on the test organisms. It is recommended establishing a sufficient number of additional reserve replicate vessels (e.g. eight) for treatment and control(s).

Frequency of soil quality measurements

41. Soil pH, soil moisture content and the temperature (continuously) in the test room should be measured at the start and end of the uptake and elimination phases. Once per week the soil moisture content should be controlled by weighing the test vessels and comparing actual weights with initial weights at test start. Water losses should be compensated by adding deionised water.

Sampling and analysis of worms and soil

42. An example of schedule for the uptake and elimination phases in earthworm and enchytraeid bioaccumulation tests is given in Annex 3.

43. The soil is sampled from the test vessels for the determination of test substance concentration before inserting the worms, and during the uptake and elimination phases. During the test the concentrations of test substance are determined in the worms and the soil. In general, total soil concentrations are measured. As an option, concentrations in pore water may be measured; in such case, rationale and appropriate methods should be provided prior to initiation of a study, and included in the report.

44. The worms and soil are sampled at least at six occasions during the uptake and the elimination phases. If the stability of a test substance is demonstrated, the number of soil analyses can be reduced. It is recommended analysing at least three replicates at the beginning and at the end of the uptake phase. If the concentration in soil measured at the end of the uptake phase deviates from the initial concentration by more than 30%, the soil samples taken at other dates should also be analysed.

45. Remove the worms of a given replicate from the soil at each sampling time (e.g. after spreading the soil of the replicate on a shallow tray and picking the worms using soft jewellers' tweezers), rinse them quickly with water in a shallow glass or steel tray. Remove excess water (see paragraph 34). Transfer the worms carefully to a pre-weighed vessel, weigh them instantly, including gut content.

46. The earthworms (*Eisenia* sp.) should then be allowed to purge their gut overnight e.g. on a moist filter paper in a covered petri dish (see paragraph 34). After purging, the weight of the worms should be determined in order to assess a possible decrease in biomass during the test (see validity criteria in paragraph 17). Weighing and tissue analysis of Enchytraeids is carried out without purging, as this is technically difficult due to the small size of these worms. After final weight determination, the worms should be killed immediately, using the most appropriate method (e.g. using liquid nitrogen, or freezing at temperatures below -18°C).

47. During the elimination phase, the worms replace contaminated gut contents with clean soil. This means, measurements in un-purged worms (enchytraeids in this context) sampled immediately before the elimination phase include contaminated gut soil. For aquatic oligochaetes it is assumed that after the initial 4 – 24 h of the elimination phase, most of the contaminated gut content has been replaced by clean sediment e.g. (46). Similar findings have been reported for earthworms in studies on the accumulation of radiolabelled cadmium and zinc (78). In the non-purged enchytraeids, the concentration of this first sample of the elimination phase may be considered as the tissue concentration after gut purge. To account for

dilution of the test item concentration by uncontaminated soil during the elimination phase, the weight of the gut content may be estimated from worm wet weight/worm ash weight or worm dry weight/worm ash weight ratios.

48. The soil and worm samples should be preferably analysed immediately after removal (i.e. within 1-2 days) in order to prevent degradation or other losses, and it is recommended calculating the approximate uptake and elimination rates as the test proceeds. If the analysis is delayed, the samples should be stored by an appropriate method, e.g. by deep-freezing (≤ -18 ºC).

49. It should be checked that the precision and reproducibility of the chemical analysis, as well as the recovery of the test substance from soil and worm samples are satisfactory for the given method; the extraction efficiency, the limit of detection (LOD) and the limit of quantification (LOQ) should be reported. Likewise it should be checked that the test substance is not detectable in the control vessels in concentrations higher than background. When the concentration of the test substance in the test organism C_a is > 0 in the control worms, this should be included in the calculation of the kinetic parameters (see Annex 2). All samples should be handled throughout the test to minimise contamination and loss (e.g. resulting from adsorption of the test substance on the sampling device).

50. When working with radiolabelled substances, it is possible to analyse parent and metabolites. Quantification of parent compound and metabolites at steady state or at the end of the uptake phase provides important information. The samples should then be 'cleaned up' so that the parent compound can be quantified separately. If single metabolites exceed 10% of total radioactivity in the analysed sample(s), the identification of these metabolites is recommended.

51. The overall recovery, and the recovery of test substance in worms, soil, and if used, in traps containing absorbents to retain evaporated test substance, should be recorded and reported.

52. Pooling of the individuals sampled from a given test vessel is acceptable for enchytraeid worms which are smaller than earthworms. If pooling involves the reduction of the number of replicates, this limits the statistical procedures which can be applied to the data. If a specific statistical procedure and power are required, then an adequate number of replicate test vessels should be included in the test to accommodate the desired pooling, procedure and power.

53. It is recommended that the BAF be expressed both as a function of total dry weight and, when required (i.e. for highly hydrophobic substances), as a function of the lipid content. Suitable methods should be used for determination of lipid content (some existing methods – e.g. (31) (58) – should be adapted for this purpose). These methods use a chloroform/methanol extraction technique. However, to avoid the use of chlorinated solvents, a modification of the Bligh and Dyer method (9) as described in (17) should be used. Since the various methods may not give identical values, it is important to give details of the method used. When possible, i.e. if sufficient worm tissue is available, the lipid analysis should ideally be made on the same sample or extract as the one used for analysis of the test substance, since the lipids often have to be removed from the extract before it can be analysed chromatographically (49). Alternatively, control animals may be used to measure the lipid content, which can then be used to normalise BAF values. This latter approach reduces the contamination of equipment with the test substance.

DATA AND REPORTING

Treatment of results

54. The uptake curve of the test substance is obtained by plotting its concentration in/on the worms during the uptake phase against time on arithmetic scales. When the curve has reached a plateau, or steady-state (see definitions in Annex 1), the steady state bioaccumulation factor BAF_{ss} is calculated from:

$$\frac{C_a \text{ at steady state or at end of uptake phase (mean)}}{C_s \text{ at steady state or at end of uptake phase (mean)}}$$

C_a is the concentration of test substance in the test organism

C_s is the concentration of test substance in the soil

55. When no steady state is reached, the BAF_K, based on the rate constants, should be determined instead of BAF_{ss}, as described below:

- Determine the accumulation factor (BAF_K) as the ratio k_s/k_e.
- Uptake and elimination rates are preferably calculated simultaneously (see Equation 11 in Annex 2)
- The elimination rate constant (k_e) is usually determined from the elimination curve (i.e. a plot of the concentration of the test item in the worms during the elimination phase). The uptake rate constant k_s is then calculated given k_e and a value of C_a which is derived from the uptake curve – See Annex 2 for a description of these methods. The preferred method for obtaining BAF_K and the rate constants, k_s, and k_e, is to use non-linear parameter estimation methods on a computer. If the elimination is obviously not first-order, then more complex models should be employed.

Test report

56. The test report should include the following information:

Test substance:

– Any available information on acute or long term toxicity (e.g. EC_x, $LC_{x,}$ NOEC) of the test substance towards soil-dwelling oligochaetes;

– purity, physical nature and, physicochemical properties e.g. log K_{ow}, water solubility;

– substance identification data; source of the test item, identity and concentration of any solvent used;

– if radiolabelled test substance is used, the precise position of the labelled atoms, the specific radioactivity, and the radiochemical purity.

Test species:

– scientific name, strain, source, any pre-treatment, acclimation, age, size-range, etc.;

Test conditions:

- test procedure used;
- type and characteristics of illumination used and photoperiod(s);
- test design (e.g. number and size of test vessels, soil mass and height of soil layer, number of replicates, number of worms per replicate, number of test concentrations, duration of uptake and elimination phases, sampling frequency);
- rationale for the choice of test vessel material;
- method of test item preparation and application as well as reasons for choosing a specific method;
- the nominal test concentrations, the means of the measured values and their standard deviations in the test vessels, and the method by which these values were obtained;
- source of the constituents of the artificial soil or – if natural media are used – origin of the soil, description of any pre-treatment, results of the controls (survival, biomass development, reproduction), soil characteristics (pH, total organic carbon content, particle size distribution (percent sand, silt, and clay), WHC_{max}, percent water content at start and at end of the test, and any other measurements made);
- detailed information on the treatment of soil and worm samples, including details of preparation, storage, spiking procedures, extraction, and analytical procedures (and precision) for the test item in worms and soil, and lipid content (if measured), and recoveries of the test item;

Results:

- mortality of the control worms and the worms in each test vessel and any observed abnormal behaviour (e.g., soil avoidance, lack of reproduction in a bioaccumulation test with enchytraeids);
- the dry weight to wet weight ratio of the soil and the test organisms (useful for normalisation);
- the wet weights of the worms at each sampling time; for earthworms, the wet weights at start of the test, and at each sampling time before and after gut purging;
- the lipid content of the test organisms (if determined);
- curves, showing the uptake and elimination kinetics of the test substance in the worms, and the time to steady state;
- C_a and C_s (with standard deviation and range, if appropriate) for all sampling times (C_a expressed in g kg^{-1} wet and dry weight of whole body, C_s expressed in g kg^{-1} wet and dry weight of soil). If a biota-soil accumulation factor (BSAF) is required (e.g. for comparison of results from two or more tests performed with animals of differing lipid content), C_a may additionally be expressed as g kg^{-1} lipid content of the organism, and C_s may be expressed as g kg^{-1} organic carbon (OC) of the soil;

- BAF (expressed in kg soil·kg^{-1} worm), soil uptake rate constant k_s (expressed in g soil kg^{-1} of worm day^{-1}), and elimination rate constant k_e (expressed in day^{-1}); BSAF (expressed in kg soil OC kg^{-1} worm lipid content) may be reported additionally;

- if measured: percentages of parent compound, metabolites, and bound residues (i.e. the percentage of test substance that cannot be extracted with common extraction methods) detected in soil and test animals;

- methods used for the statistical analyses of data;

Evaluation of results:

- compliance of the results with the validity criteria as listed in paragraph 17;

- unexpected or unusual results, e.g. incomplete elimination of the test substance from the test animals;

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ANNEX 1

DEFINITIONS AND UNITS

Bioaccumulation is the increase in concentration of the test substance in or on an organism relative to the concentration of the test substance in the surrounding medium. Bioaccumulation results from both bioconcentration and biomagnification processes (see below).

Bioconcentration is the increase in concentration of the test substance in or on an organism, resulting from the uptake of the substance exclusively from the surrounding medium (i.e. via the body surface and ingested soil), relative to the concentration of the test substance in the surrounding medium.

Biomagnification is the increase in concentration of the test substance in or on an organism, resulting mainly from uptake from contaminated food or prey, relative to the concentration of the test item in the food or prey. Biomagnification can lead to a transfer or accumulation of the test item within food webs.

The elimination of a test substance is the loss of this substance from the test organism tissue by active or passive processes that occurs independently of presence or absence of the test item in the surrounding medium.

The bioaccumulation factor (BAF) at any time during the uptake phase of this bioaccumulation test is the concentration of test substance in/on the test organism (C_a in $g \cdot kg^{-1}$ dry weight of worm) divided by the concentration of the substance in the surrounding medium (C_s as $g \cdot kg^{-1}$ of dry weight of soil); the BAF has the units of kg soil$\cdot kg^{-1}$ worm.

The steady state bioaccumulation factor (BAF_{ss}) is the BAF at steady state and does not change significantly over a prolonged period of time, the concentration of the test substance in the surrounding medium (C_s as g kg^{-1} of dry weight of soil) being constant during this period of time.

Bioaccumulation factors calculated directly from the ratio of the soil uptake rate constant and the elimination rate constant (k_s and k_e, see below) are termed kinetic bioaccumulation factor (BAF_K).

The biota-soil accumulation factor (BSAF) is the lipid-normalised concentration of the test substance in/on the test organism divided by the organic carbon-normalised concentration of the test substance in the soil at steady state. C_a is then expressed as $g \cdot kg^{-1}$ lipid content of the organism, and C_s as $g \cdot kg^{-1}$ organic content of the soil; the BSAF has the units of kg OC$\cdot kg^{-1}$ lipid.

A plateau or steady state is defined as the equilibrium between the uptake and elimination processes that occur simultaneously during the exposure phase. The steady state is reached in the plot of BAF against time when the curve becomes parallel to the time axis and three successive analyses of BAF made on samples taken at intervals of at least two days are within 20% of each other, and there are no statistically significant differences among the three sampling periods. For test substances which are taken up slowly, more appropriate intervals would be seven days (49).

The organic carbon-water partitioning coefficient (K_{oc}) is the ratio of a substance's concentration in/on the organic carbon fraction of a soil and the substance´s concentration in water at equilibrium.

The octanol-water partitioning coefficient (K_{ow}) is the ratio of a substance's solubility in n-octanol and water at equilibrium, also sometimes expressed as P_{ow}. The logarithm of K_{ow} (log K_{ow}) is used as an indication of a substance´s potential for bioaccumulation by aquatic organisms.

The uptake or exposure phase is the time during which the test organisms are exposed to the test substance.

The soil uptake rate constant (k_s) is the numerical value defining the rate of increase in the concentration of the test item in/on the test organism resulting from uptake from the soil phase. k_s is expressed in g soil kg^{-1} of worm d^{-1}.

The elimination phase is the time, following the transfer of the test organisms from a contaminated medium to a medium free of the test item, during which the elimination (or the net loss) of the substance from the test organisms is studied.

The elimination rate constant (k_e) is the numerical value defining the rate of reduction in the concentration of the test item in/on the test organism, following the transfer of the test organisms from a medium containing the test item to a substance-free medium; k_e is expressed in d^{-1}.

ANNEX 2

CALCULATION OF UPTAKE AND ELIMINATION PARAMETERS

The main endpoint of a bioaccumulation test is the bioaccumulation factor, BAF. The measured BAF can be calculated by dividing the concentration in the test organism, C_a, by the concentration in the soil, C_s, at steady state. If the steady state is not reached during the uptake phase, the BAF_K is calculated from the rate constants instead of BAFss. However, it should be noted if the BAF is based on steady state concentrations or not.

The usual means for obtaining the kinetic bioaccumulation factor (BAF_K), the soil uptake rate constant (k_s) and the elimination rate constant (k_e) is to use non-linear parameter estimation methods on a computer, e.g., based on the models described in (68). Given a set of sequential time concentration data and the model equations:

$$C_a = \frac{k_s}{k_e} \times C_s(1-e^{-k_e t}) \qquad 0 < t < t_c \qquad \text{[equation 1]}$$

or

$$C_a = \frac{k_s}{k_e} \times C_s(e^{-k_e(t-tc)} - e^{-k_e t}) \qquad t > t_c \qquad \text{[equation 2]}$$

where
C_a = concentration of substance in worms [g kg^{-1} wet or dry weight]
k_s = uptake rate constant in tissue [g soil kg^{-1} of worm d^{-1}]
C_s = concentration of substance in soil [g kg^{-1} of wet or dry weight]
k_e = elimination rate constant [d^{-1}]
t_c = time at the end of the uptake phase,

these computer programs calculate values for BAF_K, k_s and k_e.

When the background concentration in the non-exposed worms e.g. on day 0 differs significantly from zero (this may e.g. be the case for metals), this background concentration ($C_{a,0}$) should be included in these equations, to make them read:

$$C_a = C_{a,0} + \frac{k_s}{k_e} \times C_s(1-e^{-k_e t}) \qquad 0 < t < t_c \qquad \text{[equation 3]}$$

and

$$C_a = C_{a,0} + \frac{k_s}{k_e} \times C_s(e^{-k_e(t-tc)} - e^{-k_e t}) \qquad t > t_c \qquad \text{[equation 4]}$$

In cases where a significant decrease of the test substance concentration in the soil is observed over time during the uptake phase, the following models can be used e.g. (67) (79):

$$C_s = C_0(e^{-k_0 t}) \qquad \text{[equation 5]}$$

where C_s = concentration of substance in the soil [g kg^{-1} wet or dry weight]
k_0 = degradation rate constant in soil [d^{-1}]
C_0 = initial concentration of substance in soil [g kg^{-1} of wet or dry weight]

$$C_a = \frac{k_s}{k_e - k_0} \times (e^{-k_0 t} - e^{-k_e t}) \qquad 0 < t < t_c \qquad \text{[equation 6]}$$

$$C_a = \frac{k_s}{k_e - k_0} \times (e^{-k_0 tc} - e^{-k_e tc}) * e^{-k(t-tc)} \qquad t > t_c \qquad \text{[equation 7]}$$

where C_a = concentration of substance in worms [g kg^{-1} wet or dry weight]
k_s = uptake rate constant in tissue [g soil kg^{-1} of worm d^{-1}]
k_0 = degradation rate constant in soil [d^{-1}]
k_e = elimination rate constant [d^{-1}]
t_c = time at the end of the uptake phase.

When steady state is reached during the uptake phase (i.e. $t = \infty$), equation 1

$$C_a = \frac{k_s}{k_e} \times C_s(1-e^{-k_e t}) \qquad 0 < t < t_c \qquad \text{[equation 1]}$$

may be reduced to:

$$C_a = \frac{k_s}{k_e} \times C_s$$

or

$$C_a/C_s = k_s/k_e = BAF_K \qquad \text{[equation 8]}$$

Then $k_s/k_e \times C_s$ is an approach to the concentration of the test item in the worm tissue at steady state ($C_{a,ss}$).

The biota-soil accumulation factor (BSAF) can be calculated as follows:

$$BSAF = BAF_K * \frac{f_{oc}}{f_{lip}} \qquad \text{[equation 9]}$$

where f_{oc} is the fraction of soil organic carbon, and f_{lip} is the fraction of worm lipid, both preferably determined on samples taken from the test, and based either on dry weight or on wet weight, respectively.

The elimination kinetics can be modelled using the data from the elimination phase and applying the following model equation and a computer-based non-linear parameter estimation method. If the data points plotted against time indicate a constant exponential decline of the test item concentration in the animals, a one-compartment model (equation 9) can be used to describe the time course of elimination.

$$C_a(t) = C_{a,ss} \text{ x } e^{-k_e t} \qquad \text{[equation 10]}$$

Elimination processes sometimes appear to be biphasic, showing a rapid decline of C_a during the early phases, that changes to a slower loss of test items in the later phases of the elimination, e.g. (27) (68). The two phases can be interpreted by the assumption, that there are two different compartments in the organism, from which the test item is lost with different velocities. In these cases, specific literature should be studied e.g. (38) (39) (40) (78).

Using the model equations above, the kinetic parameters (k_s and k_e) may also be calculated in one run by applying the first order kinetics model to all data from both the uptake and elimination phase simultaneously. For a description of a method that may allow for such a combined calculation of uptake and elimination rate constants, references (41), (73) and (70) may be consulted.

$$C_a = \left[\frac{k_s}{k_e} * C_s(1-e^{-k_e t}) * (m=1)\right] + \left[\frac{k_s}{k_e} * C_s(e^{-k_e(t-t_c)} - e^{-k_e t}) * (m=2)\right] \qquad \text{[equation 11]}$$

Note: When uptake and elimination parameters are estimated simultaneously from the combined uptake and the elimination data, "m" as shown in equation 11 is a descriptor that allows the computer program to assign the equation's sub-terms to the data sets of the respective phase and to perform the evaluation correctly (m = 1 for uptake phase; m = 2 for elimination phase).

Nevertheless, these model equations should be used with caution, especially when changes in the test substance´s bioavailability, or (bio)degradation occur during the test (see e.g. (79)).

ANNEX 3

EXAMPLES OF SCHEDULES FOR SOIL BIOACCUMULATION TESTS

Earthworm test

a) Uptake phase with 8 sampling dates used for calculation of kinetics

Day	Activity
-6	Conditioning of the prepared soil for 48 h;
-4	Spiking of the soil fraction with the test substance solution; evaporating of any solvent; mixing of the soil constituents; distributing the soil to the test vessels; equilibration at test conditions for 4 days (3 weeks for metal-spiked soil);
-3 to -1	Separation of the test organisms from the culture for acclimation; preparation and moisturising of the soil constituents;
0	Measuring temperature, and soil pH; removing soil samples from treated vessels and solvent controls for determination of test substance concentration; addition of food ration; weighing and randomised distribution of the worms to the test vessels; retaining of sufficient subsamples of worms for determination of analytical background values, wet and dry weight, and lipid content; weighing of all test vessels to control soil moisture; controlling air supply, if closed test system is used;
1	Controlling air supply, recording worm behaviour and temperature; taking soil and worm samples for determination of test item concentration;
2	Same as day 1;
3	Controlling air supply, worm behaviour and temperature;
4	Same as day 1;
5 - 6	Same as day 3;
7	Same as day 1; addition of food ration; control soil moisture by re-weighing the test vessels and compensate evaporated water;
8 - 9	Same as day 3;
10	Same as day 1;
11 - 13	Same as day 3;
14	Same as day 1; addition of food ration; control soil moisture by re-weighing the test vessels and compensate evaporated water;
15 - 16	Same as day 3;
17	Same as day 1;
18 - 20	Same as day 3;
21	Same as day 1; measuring temperature and soil pH; control soil moisture by re-weighing the test vessels; end of uptake phase; transfer worms from remaining exposed replicates to vessels containing clean soil for elimination phase (no gut-purging); sampling of soil and worms from solvent controls.
	Pre-exposure activities (equilibration phase) should be scheduled taking into account the properties of the test substance. Activities described for day 3 should be performed daily (at least on workdays).

b) Elimination phase

Day	Activity
-6	Preparation and moisturising of the soil constituents; conditioning of the prepared soil for 48 h;
-4	Mixing of the soil constituents; distributing the soil to the test vessels; incubation at test conditions for 4 days;
0 (end of uptake phase)	Measuring temperature and soil pH; weighing and randomised distribution of the worms to the test vessels; addition of food ration; transfer worms from remaining exposed replicates to vessels containing clean soil; taking soil and worm samples after 4 – 6 h for determination of test substance concentration;
1	Controlling air supply, recording worm behaviour and temperature; taking soil and worm samples for determination of test substance concentration;
2	Same as day 1;
3	Controlling air supply, worm behaviour and temperature;
4	Same as day 1;
5 - 6	Same as day 3;
7	Same as day 1; addition of food ration; control soil moisture by re-weighing the test vessels and compensate evaporated water;
8 - 9	Same as day 3;
10	Same as day 1;
11 - 13	Same as day 3;
14	Same as day 1; addition of food ration; control soil moisture by re-weighing the test vessels and compensate evaporated water;
15 - 16	Same as day 3;
17	Same as day 1;
18 - 20	Same as day 3;
21	Same as day 1; measuring temperature and soil pH; control soil moisture by re-weighing the test vessels; sampling of soil and worms from solvent controls.
	Preparation of the soil prior to start of elimination phase should be done in the same manner as before the uptake phase. Activities described for day 3 should be performed daily (at least on workdays).

Enchytraeid test

a) Uptake phase with 8 sampling dates used for calculation of kinetics

Day	Activity
-6	Conditioning of the prepared soil for 48 h;
-4	Spiking of the soil fraction with the test substance solution; evaporating of any solvent; mixing of the soil constituents; distributing the soil to the test vessels; equilibration at test conditions for 4 days (3 weeks for metal-spiked soil);
-3 to -1	Separation of the test organisms from the culture for acclimation; preparation and moisturising of the soil constituents;
0	Measuring temperature, and soil pH; removing soil samples from treated vessels and solvent controls for determination of test substance concentration; addition of food ration to soil; weighing and randomised distribution of the worms to the test vessels; retaining of sufficient subsamples of worms for determination of analytical background values, wet and dry weight, and lipid content; weighing of all test vessels to control soil moisture; controlling air supply, if closed test system is used;
1	Controlling air supply, recording worm behaviour and temperature; taking soil and worm samples for determination of test item concentration;
2	Same as day 1;
3	Controlling air supply, worm behaviour and temperature;
4	Same as day 1;
5 - 6	Same as day 3;
7	Same as day 1; addition of food ration to soil; control soil moisture by re-weighing the test vessels and compensate evaporated water;
9	Same as day 1;
10	Same as day 3;
11	Same as day 1;
12 - 13	Same as day 3;
14	Same as day 1; addition of food ration to soil; measuring temperature and soil pH; control soil moisture by re-weighing the test vessels; end of uptake phase; transfer worms from remaining exposed replicates to vessels containing clean soil for elimination phase (no gut-purging); sampling of soil and worms from solvent controls.
	Pre-exposure activities (equilibration phase) should be scheduled taking into account the properties of the test substance. Activities described for day 3 should be performed daily (at least on workdays).

ANNEX 4

ARTIFICIAL SOIL – PREPARATION AND STORAGE RECOMMENDATIONS

Since natural soils from a particular source may not be available throughout the year, and indigenous organisms as well as the presence of micro-pollutants can influence the test, an artificial substrate, the artificial soil according to OECD Test Guideline 207 (48), is recommended for use in this test. Several test species can survive, grow, and reproduce in this soil, and maximum standardisation as well as intra- and interlaboratory comparability of test and culture conditions are provided.

Soil constituents

Peat:	10%	Sphagnum-peat, in accordance with the OECD Guideline 207 (48);
Quartz sand:	70%	Industrial quartz sand (air dried); grain size: more than 50% of the particles should be in the range of 50-200 µm, but all particles should be ≤ 2 mm;
Kaolinite clay:	20%	Kaolinite content ≥ 30 %;
Calcium carbonate:	≤ 1%	$CaCO_3$, pulverised, chemically pure.

As an option, the organic carbon content of the artificial soil may be reduced, e.g. by lowering the peat content to 4-5% of dry soil 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 (74). It has been demonstrated that *Enchytraeus albidus* and *Eisenia fetida* can comply with the validity criteria on reproduction when tested in field soils with lower organic carbon content, e.g. 2.7% (33), (61), and there is experience that this can also be achieved in artificial soil with 5% peat.

Preparation

The dry constituents of the soil are mixed thoroughly (e.g. in a large-scale laboratory mixer). This should be done about one week before starting the test. The mixed dry soil constituents should be moistened with deionised water at least 48 h before application of the test item in order to equilibrate/stabilise the acidity. For the determination of pH a mixture of soil and 1 M KCl solution in a 1:5 ratio is used. If the pH value is not within the required range (6.0 ± 0.5), a sufficient amount of $CaCO_3$ is added to the soil, or a new batch of soil is prepared.

The maximum water holding capacity (WHC) of the artificial soil is determined according to ISO 11268-2 (35). At least two days before starting the test, the dry artificial soil is moistened by adding enough deionised or reconstituted water to obtain approximately half of the final water content. The final water content should be 40% to 60% of the maximum WHC. At the start of the test, the pre-moistened soil is divided into as many batches as the number of test concentrations and controls used for the test, and the moisture content is adjusted to 40 – 60% of WHC_{max} by using the solution of the test item and/or by adding deionised or reconstituted water. The moisture content is determined at the beginning and at the end of the test (at 105 °C). It should be optimal for the species' requirements (the moisture content can also be checked as follows: when the soil is gently squeezed in the hand, small drops of water should appear between the fingers).

Storage

The dry constituents of the artificial soil may be stored at room temperature until use. The prepared, pre-moistened soil may be stored in a cool place for up to three days prior to spiking; care should be taken to minimise evaporation of water. Soil spiked with the test item should be used immediately unless there is information indicating that the particular soil can be stored without affecting the toxicity and bioavailability of the test item. Samples of spiked soil may then be stored under the conditions recommended for the particular test item until analysis.

ANNEX 5

SPECIES OF TERRESTRIAL OLIGOCHAETES RECOMMENDED FOR TESTING BIOACCUMULATION FROM SOIL

Earthworms

The recommended test species is *Eisenia fetida* (Savigny 1826), belonging to the family Lumbricidae. Since 1972 it is divided into two subspecies (*Eisenia fetida* and *Eisenia andrei* (10)). According to Jaenike (36), they are true, separate species. *Eisenia fetida* is easily recognised by its bright intersegmental yellow stripes whereas *Eisenia andrei* has a uniform, dark red colour. Originating probably from the region of the Black Sea, they are distributed world-wide today, especially in anthropogenically modified habitats like compost heaps. Both can be used for ecotoxicological as well as bioaccumulation tests.

Eisenia fetida and *Eisenia andrei* are commercially available, e.g. as fish bait. In comparison to other lumbricid earthworms, they have a short life-cycle, reaching maturity within ca. 2 – 3 months (at room temperature). Their optimum temperature is approximately at 20 – 24°C. They prefer relatively moist substrates with a nearly neutral pH and a high content of organic material. Since these species have been widely used in standardised ecotoxicological tests for about 25 years, their culturing is well established (48) (77).

Both species can be bred in a wide range of animal wastes. The breeding medium recommended by ISO (35) is a 50:50 mixture of horse or cattle manure and peat. The medium should have a pH value of about 6 to 7 (regulated with calcium carbonate), a low ionic conductivity (less than 6 mS/cm or less than 0.5 % salt concentration) and should not be contaminated excessively with ammonia or animal urine. Also, a commercial gardening soil free of additives, or artificial soil according to OECD (48), or a 50:50 mixture of both can be used. The substrate should be moist but not too wet. Breeding boxes of 10 litre to 50 litre volume are suitable.

To obtain worms of standard age and mass, it is best to start the culture with cocoons. Therefore, adult worms are added to a breeding box containing fresh substrate to produce cocoons. Practical experience has shown that a population density of approximately 100 adult worms per kg substrate (wet weight) leads to good reproduction rates. After 28 days, the adult worms are removed. The earthworms hatched from the cocoons are used for testing when mature after at least 2 months but less than 12 months.

Worms of the species described above can be considered healthy if they move through the substrate, do not try to leave the substrate, and reproduce continuously. Very slow motioning or a yellow posterior end (in the case of *Eisenia fetida*) indicates substrate exhaustion. In this case, fresh substrate and/or a lower number of animals per box is recommended.

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Enchytraeids

The recommended test species is *Enchytraeus albidus* Henle 1837 (white potworm). *Enchytraeus albidus* is one of the biggest (up to 15 mm) species of the annelid oligochaete family Enchytraeidae and it is world-wide distributed e.g. (8). *Enchytraeus albidus* is found in marine, limnic and terrestrial habitats, mainly in decaying organic matter (seaweed, compost) and rarely in meadows (42). This broad ecological tolerance and some morphological variations indicate that there might be different races for this species.

Enchytraeus albidus is commercially available, sold as food for fish. It should be checked whether the culture is contaminated by other, usually smaller species (60). If contamination occurs, all worms should be washed with water in a Petri dish. Large adult specimens of *Enchytraeus albidus* are then selected (by using a stereomicroscope) to start a new culture. All other worms are discarded. Its life cycle is short as maturity is reached between 33 days (at 18 °C) and 74 days (at 12 °C). Only cultures which have been kept in the laboratory for at least 5 weeks (one generation) without problems should be used for a test.

Other species of the *Enchytraeus* genus are also suitable, especially *Enchytraeus luxuriosus.* This species is a true soil inhabitant, which has been newly described in (65). If other species of *Enchytraeus* are used, they should be clearly identified and the rationale for the selection of the species should be reported.

Enchytraeus crypticus (Westheide & Graefe 1992) is a species belonging to the same group as *Enchytraeus luxuriosus*. It has not been found to exist with certainty in the field, having only been described from earthworm cultures and compost heaps (Römbke 2003). Its original ecological requirements are therefore not known. However, recent laboratory studies in various field soils have confirmed that this species has a broad tolerance towards soil properties like pH and texture (Jänsch et al. 2005). In recent years, this species has often been used in ecotoxicological studies because of the simplicity of its breeding and testing, e.g. Kuperman et al. 2003). However, it is small (3 – 12 mm; 7 mm on average (Westheide & Müller 1996), and this makes handling more difficult compared with *Enchytraeus albidus*. When using this species instead of *Enchytraeus albidus*, the size of the test vessel can but needs not to be smaller. In addition, it should be considered that this species reproduces very rapidly having a generation time of less than 20 days at 20 ± 2°C (Achazi et al. 1999) and even quicker at higher temperatures.

Enchytraeids of the species *Enchytraeus albidus* (as well as other *Enchytraeus* species) can be bred in large plastic boxes (e.g. 30 x 60 x 10 cm or 20 x 12 x 8 cm which is suitable for culture of worms of small size) filled with a mixture of artificial soil and commercially available, uncontaminated garden soil free of additives. Compost material should be avoided since it could contain toxic substances like heavy metals.

Fauna should be removed from the breeding soil before use by three times deep-freezing. Pure artificial soil can also be used but the reproduction rate could be slower compared to that obtained with mixed substrates. The substrate should have a pH of 6.0 ± 0.5. The culture is kept in an incubator at a temperature of 15 ± 2°C without light. In any case, a temperature higher than 23°C should be avoided. The artificial/natural soil moisture should be moist but not wet. When the soil is gently pressed by hand, only small drops of water should appear. In any case, anoxic conditions should be avoided (e.g. if a lid is used, the number of lid holes should be high enough to provide sufficient exchange of air). The breeding soil should be aerated by carefully mixing it once per week.

The worms should be fed at least once per week ad libitum with rolled oats which are placed into a cavity on the soil surface and covered with soil. If food from the last feeding date remains in the container, the amount of food given should be adjusted accordingly. If fungi grow on the remaining food, it should be replaced by a new quantity of rolled oats. In order to stimulate reproduction, the rolled oats may be supplemented with commercially available, vitamin amended protein powder every two weeks. After three months, the animals are transferred to a freshly prepared culture or breeding substrate. The rolled oats, which have to be stored in sealed vessels, should be autoclaved or heated before use in order to avoid infections by flour mites (e.g. *Glyzyphagus sp.*, Astigmata, Acarina) or predacious mites (e.g. *Hypoaspis (Cosmolaelaps) miles*, Gamasida, Acarina). After disinfecting, the food is ground up so that it can easily be strewn on the soil surface. Another possible food source is baker's yeast or the fish food TetraMin®.

In general, the culturing conditions are sufficient if worms do not try to leave the substrate, move quickly through the soil, exhibit a shiny outer surface without soil particles clinging to it, are more or less whitish coloured, and if worms of different ages are visible. Actually, worms can be considered healthy if the they reproduce continuously.

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