

**PROPOSAL FOR A NEW GUIDELINE**

for

**OECD GUIDELINES FOR THE TESTING OF CHEMICALS**

**BIOACCUMULATION IN TERRESTRIAL OLIGOCHAETES**

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## INTRODUCTION

1. Within the OECD test guideline programme, adopted standardised bioaccumulation guidelines exist for fish and sediment oligochaetes bioaccumulation tests up to now (OECD 1996; OECD 2007). However, the extrapolation of aquatic bioaccumulation data to terrestrial organisms like earthworms is difficult, if possible at all. Therefore, model calculations based on a compound's lipophilicity (e.g., Connell & Markwell 1990) are currently used for the assessment of bioaccumulation of chemicals in the soil ecosystem, as e.g. in the EU Technical Guidance Document (EC 2003). The need for a compartment-specific test method was already addressed more than a decade ago (e.g., Phillips 1993). Such a method is especially important for the evaluation of secondary poisoning in terrestrial food chains (Beek et al. 2000). Several national guidelines address the issue of bioaccumulation in organisms other than fish (e. g., ASTM 2000, and U.S. EPA 2000). More importantly, a guideline describing the measurement of bioaccumulation from contaminated soils in earthworms (*Eisenia fetida*, Savigny) and potworms has recently been adopted (ASTM 2004). The development, standardisation and implementation of an internationally accepted, standardised method for the experimental determination of bioaccumulation in spiked soil can therefore considerably improve the risk assessment for chemicals in terrestrial ecosystems (e.g., EPPO 2003, Füll et al. 2003).

2. Soil-ingesting invertebrates are subject to high exposure to soil bound substances and should therefore be given preferential attention. Among these animals, terrestrial oligochaetes play an important role for the structure and the function of the soil ecosystem (Didden 1993; Edwards & Bohlen 1996). They live in the soil and, partly, at the soil surface (especially the litter layer) and often represent the most abundant species if considered in terms of biomass (Petersen & Luxton 1982). By bioturbation of the soil and by serving as prey these animals can have a strong influence on the bioavailability of such substances to other organisms like invertebrate (e.g. predatory mites and beetles; e.g. Schlosser & Riepert (1992)) or vertebrate (e.g. foxes and gulls) predators (Romijn et al. 1993; Dietrich et al. 1995). Some species of terrestrial oligochaetes that are 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* (ASTM 2004) provides many essential and useful details for the performance of the presented soil bioaccumulation method. Further documents that are referred to in this draft guideline are the OECD Guideline 305: Bioconcentration: Flow-through Fish Test (OECD 1996) and the OECD Draft Guideline: Bioaccumulation: Sediment test using benthic oligochaetes (OECD 2007). Practical experience with soil bioaccumulation studies and their standardisation, and details from literature (e.g., Belfroid et al. 1994; Füll 1996; Widianarko & Van Straalen 1996; Marinussen et al. 1997; Khalil 1990; Posthuma et al. 1996; Römbke et al. 1998; Amorim 2000; Bruns et al. 2001, Jager et al 2003b, Van Straalen et al. 2005, Vijver et al 2005) are also major sources of information for drawing up this draft.

4. The method described is sufficiently detailed so that the test can be carried out whilst allowing for adaptations in the experimental design depending on the varied characteristics of test items and the conditions in particular laboratories. It is mostly applicable to stable, neutral organic chemicals, which tend to adsorb to soils. The testing of bioaccumulation of soil-associating, stable metallo-organic compounds may also be possible with this method. It is also applicable to metals and other trace elements.

## **PREREQUISITE AND GUIDANCE INFORMATION**

5. Tests for bioaccumulation, i.e. the increase in concentration of a substance in terrestrial oligochaetes relative to the surrounding medium (in this case the soil), have been performed with heavy metals (see e.g. Sample et al. (1999)) and persistent, organic substances having log  $K_{ow}$  values between 3.0 and 6.0 (e.g. Jager et al 2003b). Such tests can also be applied to

- substances that show a log  $K_{ow}$  of more than 6.0 (superlipophilic 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.
- Metals (excluding essential metals, which are biologically regulated in organisms).

6. Information on the test substance such as safety precautions, proper storage conditions and analytical methods should be obtained before beginning the study. Before carrying out a test for bioaccumulation with terrestrial oligochaetes, the following information about the test compound 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;
- (f) known metabolites.

Other information on the test item such as common name, chemical name (preferably IUPAC name), structural formula, CAS registry number, purity should be known before beginning the study.

7. In order to mimic environmentally relevant exposure concentrations, to test at non-toxic concentrations, and to facilitate the analysis of the test item and its major metabolites in soil and worm samples, it is recommended to use radiolabelled organic test items. The method described here may have to be modified, e.g., to provide sufficient biomass, for measuring non-radiolabelled organic test items or metals. If total radioactive residues are measured (e.g. by liquid scintillation counting following extraction, combustion or tissue solubilisation), the bioaccumulation factor (BAF) is based on the parent compound including any retained metabolites. It is also possible to combine a metabolism study with a bioaccumulation study by analysis and quantification of the percentage of parent compound and its metabolites in samples taken at the end of the uptake phase

or at the peak level of bioaccumulation. In any case, the BAF calculation should be based on the concentration of the parent compound in the organisms and not only on total radioactive residues.

8. In addition to the knowledge of the properties of the test item given in paragraphs 5 - 6 other information required is the toxicity to the species to be used in the test, such as an effect concentration ( $EC_x$ ) or lethal concentration ( $LC_x$ ) for the time required for the uptake phase (e.g. EC 2003). This is to ensure that selected exposure concentrations are much lower than toxic levels. If available, preference should be given to toxicity values derived from long-term studies on sublethal endpoints (OECD 2004a & b). If such data are not available, an acute toxicity test under conditions identical with the bioaccumulation test conditions may provide useful information, see e.g. Elmegaard & Jagers op Akkerhuis (2000).

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 must 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 item is used, the specific radioactivity (i.e. e.g.,  $Bq\ mol^{-1}$ ) and the percentage of radioactivity associated with impurities must be known. The specific radioactivity of the test compound should be as high as possible in order to detect test concentrations as low as possible.

10. The test can be performed with artificial soil (see paragraph 21 and Annex 4) or with field soils (see paragraph 21). Information on characteristics of the test soil (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 acquired before the start of the test (OECD 1984; ASTM 2004).

## **PRINCIPLE OF THE TEST**

11. The parameters which characterise the bioaccumulation of a substance include first of all the bioaccumulation factor (BAF), the uptake rate constant ( $k_u$ ) and the elimination rate constant ( $k_e$ ). Detailed definitions of these parameters 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 item (for preparation of a suitable artificial soil see Annex 4; guidance on application of test item is given in paragraph 22). In addition to the animals exposed to the test item, groups of control worms are held under identical conditions without the test item. The absence of any adverse effects of the test item towards the test organisms in the bioaccumulation test can then be confirmed by matching the control group. Furthermore, the dry weight and the lipid content of the test organisms should be 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 item. An elimination phase is necessary to gain information on the rate at which the test substance is excreted by the test organisms (e.g. Franke et al. 1994). An elimination phase is always required unless uptake of the test item during the exposure phase has been insignificant (e.g. there is no statistically significant difference between the concentration of the test item in test and control worms). If a steady-state (see Annex 1 for definition) has not been reached during the uptake phase, the determination of the kinetic results (BAF<sub>k</sub>, uptake and elimination rate constant(s)) should be based on the results of the uptake and elimination phase. The change of the concentration of the test item in/on the worms is monitored throughout both phases of the test. In case a radiolabelled test item is used, results of total radioactive concentration in soil and worm samples can be obtained within less than 2 days after sampling.

13. During the uptake phase, measurements are taken by time-series sampling for 14 days (enchytraeids) or 21 days (earthworms) to reach steady-state as described e.g. by Sousa et al., (2000), Bruns et al. (2001). The steady state occurs when a plot of the concentration in the worms against time becomes parallel to the time axis and three successive analyses of concentrations made on samples taken at intervals of at least two days varying no more than  $\pm 20\%$  of each other based on statistical comparisons (e.g., analysis of variance, regression analysis).

14. The elimination phase is started by transferring exposed oligochaetes to vessels containing the same substrate without the test substance. During the elimination phase, measurements are taken by time-series sampling for 14 days (enchytraeids) or 21 days (earthworms) unless analytical determination at earlier dates showed absence of the test item. The concentration of the test item in the worms at the end of the elimination phase is reported as an additional endpoint. The bioaccumulation factor ( $BAF_{ss}$ ) is calculated preferably both as the ratio of concentration in the 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 are 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 possible models). The goodness of fit of any model can be determined from e.g., the correlation coefficient or the coefficient of determination (coefficients close to 1 indicate a good fit).

16. To reduce variability in test results for those substances with high lipophilicity, bioaccumulation factors can be expressed additionally in relation to lipid content (expressed in  $\text{kg soil OC kg}^{-1}$  worm lipid content). This approach is based on experiences and theoretical correlations, where - for some chemical classes - there is a clear relationship between a substance's potential for bioaccumulation and its lipophilicity, which has been well established for fish as model organisms (e.g., Nendza 1991). There is also a corresponding relationship between the lipid content of the test fish and the observed bioaccumulation of such substances. For benthic organisms, similar correlations have been found (e.g., Gabric et al. 1990, Landrum 1989). Also for terrestrial oligochaetes this correlation has been utilised (e.g., Connell & Markwell 1990, Belfroid et al. 1993, 1994, 1995). If sufficient worm tissue is available, the lipid content of the test animals may be determined on the same biological material as is used to determine the concentration of the test item. Alternatively, control animals may be used to measure the lipid content, which can then be used to normalise BAF values.

## 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 (fw).

## DESCRIPTION OF THE METHOD

### Test species

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

### Apparatus

19. Care should be taken to avoid the use of materials, for all parts of the equipment, that can dissolve, absorb test items or leach other substances 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, (see also paragraph 32). Stainless steel, plastic or glass should 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 (OECD 1996). 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 soil to be used must 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 Guideline 207 (OECD 1984) 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.

However, natural soils from unpolluted sites may serve as test and/or culture soil (e.g. the German standard soil Lufa). 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 (ASTM 2004). Analysis of the soil or its constituents for micropollutants prior to use might provide useful information. If field soil from agricultural land is used, this must not have been treated (e.g. with pesticides and fertilizers) for 5 months prior to use in a test. Manipulation procedures for natural soils prior to use in ecotoxicological tests with oligochaetes in the laboratory are described in ASTM (2004). For natural soils the storage time in the laboratory should be kept as short as possible.

### **Application of the test item**

22. The test item should be incorporated into the soil. The physicochemical properties of the test item should be taken into consideration. Water soluble test items are mixed directly into the soil. The spiking procedure for poorly water soluble test items recommended for the artificial soil involves coating of one or more of the soil constituents with the test item. For example, the quartz sand, or a portion thereof, can be soaked with a solution of the test item in a suitable solvent, which is then slowly evaporated. The coated fraction can then be mixed into the wet soil. The major advantage of this procedure is that no solvent is introduced to the soil. When using natural soil, the test chemical may be added by spiking a dried portion of the soil as described above for the artificial soil, or by stirring the test item into the wet soil, with subsequent evaporating of any solubilising agent used. In general, the contact of wet soil with solvents should be minimised. According to ASTM (2004), the following considerations are necessary. 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, then no

negative control needs to be applied (see paragraph 39). The solvent control must contain the highest concentration of solvent added to the soil and must use solvent from the same batch used to make the stock solution. Toxicity and volatility of the solvent, and the solubility of the test item in the chosen solvent should be the main criteria for the selection of a suitable solubilising agent.

23. For substances that are poorly soluble in water and organic solvents, 2.0 - 2.5 g of finely ground quartz sand per test vessel can be mixed with the quantity of test substance to obtain the desired test concentration. This mixture of quartz sand and test substance is added to the pre-moistened soil and thoroughly mixed after adding an appropriate amount of de-ionised water to obtain the required moisture content. The final mixture is 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.

24. The concentration of the test item in the soil must be determined after spiking. The homogenous distribution of the test item in the soil should be confirmed before introducing the test organisms. The method used for spiking, and the reasons for choosing a specific spiking procedure should be reported (Environment Canada 1995).

25. An equilibrium between the soil and the soil pore water phase should ideally be established within a suitable period of time. For many poorly water soluble organic chemicals the time required to reach true equilibrium between adsorbed and dissolved fractions may range from days to months. Therefore, an arbitrary time period of 4 d at 20°C is recommended for ageing the spiked soil. Depending on the purpose of the study, e.g., when environmental conditions are to be mimicked, the spiked soil may be aged for a longer period (e.g. for metals 3 weeks at 20°C).

### **Culturing of the test organisms**

26. In order to have a sufficient number of worms for conducting bioaccumulation tests the worms have to be kept in permanent laboratory culture. Summaries of laboratory culture methods for *Eisenia fetida* and *Eisenia andrei*, *Enchytraeus albidus* and *Enchytraeus luxuriosus* are outlined in Annex 5. For details of culturing these species see e.g. OECD (1984) or OECD (2004a & b).

27. To ensure that the tests are performed with animals of the same species, the establishment of single species cultures is strongly recommended. Ensure that the cultures and especially the worms used in the tests are free from observable diseases and abnormalities.

## PERFORMANCE OF THE TEST

28. The test organisms are exposed to the test item during the uptake phase. The uptake phase should be run for up to 14 (enchytraeids) or 21 (earthworms) days unless it can be demonstrated earlier that steady state has been reached.

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

### Test organisms

30. The number of worms per sample must provide a mass of worm tissue such that the mass of test item per sample at the beginning of the uptake phase and at the end of the elimination phase, respectively, is significantly higher than the substance's analytical detection limit in biological material. This refers to the experience that in bioaccumulation tests, in the mentioned stages of uptake and elimination phase the concentration in the test animals is usually relatively low. Since the individual weight in many species of terrestrial enchytraeids is very low (e.g. 5 - 10 mg wet weight per individual for *Enchytraeus albidus* or even less for *Enchytraeus crypticus* or *Enchytraeus luxuriosus*), the worms of each single replicate test vessel may be pooled for weighing and chemical analysis. The preferred number of enchytraeid worms per replicate is 20. If the analytical detection limit of the test item is high, a higher number of worms may be necessary. For test species with higher individual weight (*Eisenia fetida* and *Eisenia andrei*), replicates containing one individual may be used.

31. The earthworms to be 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 weight and age of an animal sometimes appears to have a significant effect on BAF-values (e.g. due to varying lipid content and/or presence of eggs), these parameters should be recorded accurately. It is recommended that a sub-sample of the worms to be used is weighed before the test in order to estimate the mean wet and dry weight.

32. Use a high soil-to-worm ratio in order to minimise the decrease of test item concentration in the soil during the uptake phase. For *Eisenia fetida* and *Eisenia andrei* a minimum amount of 50 g DW of soil per worm, and for enchytraeids, a minimum of 10 – 20 g DW of soil per test vessel are recommended. The vessels should contain a soil layer of 2 – 3 cm (enchytraeids) or 4 – 5 cm (earthworms).

33. The worms to be 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. 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 acclimatisation, earthworms are transferred to glass dishes (e.g. petri dishes) containing clean water, and subsequently weighed before adding them 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.

34. Burrowing behaviour can have an influence on the exposure of the test organisms. Therefore, burrowing behaviour of the test organisms should be observed and recorded. In tests with earthworms, the animals (control and treatments) should burrow in the soil within a period of 24 h after addition to the test vessels. If the earthworms fail to burrow in the soil (more than 10%), 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 at improved conditions. For enchytraeids, the

exposure situation is different from the exposure situation for earthworms. Enchytraeids mainly live in the interstitial pores of the soil, and frequently their integument may be only partly in contact with the surrounding substrate. Therefore, exposure of burrowing and non-burrowing enchytraeids is assumed to be virtually equal, and non-burrowing of the enchytraeids does not necessarily require the repetition of the test.

### **Feeding**

35. Feeding should be considered when a soil with low total organic carbon is used. Food should then be added to the soil before the test is started. When artificial soil according to OECD (1984) is used, a weekly rate for earthworms of 10 mg of dried dung and for enchytraeids of 2 - 2.5 mg of ground oat flakes per g soil dry weight is recommended (Bruns et al. 2001a). Preferably the same type of food as in the cultures should be used.

### **Light and temperature**

36. 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) (ASTM 2004). The test temperature should be  $20 \pm 2^\circ\text{C}$  throughout the test.

### **Test concentrations**

37. A single concentration is sufficient for determining the bioaccumulation potential. If required, evaluation of a possible concentration dependency of bioaccumulation will require testing more concentrations. For metals, the concentration should also be above the background levels in tissue and soil. If the effect concentration of the test item is close to the analytical detection limit, the use of radiolabelled test item with high specific radioactivity is recommended (see also paragraphs 8 and 9).

## **Replicates**

38. The minimum number of replicates should be three per sampling point.

39. If any solubilising agent is used for application of the test item, a solvent control containing all constituents except for the test item should be run in addition to the treated replicates (4 replicates should be sampled at start, 4 at the end of the uptake phase, and 4 at the end of the elimination phase. As an option, 4 replicates of a negative control (no solvent) may also be provided for 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. In case no solvent other than water is used, at least 12 replicates of a negative control (4 sampled at start, 4 at end of uptake and 4 at end of elimination) should be provided for biological (dry-to-wet weight ratio, lipid content) and background analysis. It is recommended to establish a sufficient number of reserve control replicates (e.g. 8).

## **Frequency of soil quality measurements**

40. Soil pH, soil moisture content and the temperature (continuously) in the test chamber should be measured at the start and the end of the uptake and the elimination phase. Once per week the soil moisture content should be controlled by reweighing 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**

41. Examples of activity schedules for uptake and elimination phase in earthworm and enchytraeid tests are given in Annex 3.

42. Sample the soil from the test vessels for determination of test item concentration before inserting the worms, and during both uptake and elimination phases. During the test the

concentrations of test item are determined in the worms, and soil in order to monitor the distribution of the test item in the compartments of the test system.

43. Sample the worms and soil on at least six occasions during the uptake as well as the elimination phase. If the stability of a test item can be demonstrated, the number of soil analyses may be reduced. However, it is recommended to analyse at least three replicates at the beginning and the end of the uptake phase in order to show homogeneity of test item distribution. In addition, soil samples from other dates should be kept until the end of the study.

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 the excess water as described in paragraph 33. Transfer the worms carefully to a pre-weighed vessel, weigh them instantly and kill them immediately thereafter, using the most appropriate method (e.g., using liquid nitrogen, or freezing at  $-20 \pm 2^{\circ}\text{C}$ ).

The earthworms should be allowed to purge their gut for 24 h before being killed. The weight of the worms must be determined before and after purging in order to assess a possible decrease in biomass during the test (see validity criterion in paragraph 17). Enchytraeids are not allowed to purge their gut before weighing and tissue analysis.

44. During the elimination phase, the worms purge their gut in clean soil. This means, measurements 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., Mount et al. 1999). Similar findings have been reported for earthworms in studies on the accumulation of radiolabelled cadmium and zinc (Vijver et al. 2005). The concentration in the worms of this first sample of the elimination phase may then 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.

45. Preferably analyse the soil and worm samples immediately (i.e. within 1 - 2 d) after removal in order to prevent degradation or other losses, and to calculate the approximate uptake and elimination rates as the test proceeds. Failing immediate analysis, the samples should be stored by an appropriate method, e.g. by deep-freezing ( $\leq -18$  C). Obtain information on the proper storage conditions for the particular test item - for example, duration and temperature of storage, extraction procedures, etc. - before beginning the study.

46. Since the whole procedure is governed essentially by the accuracy, precision and sensitivity of the analytical method used for the test item, check experimentally that the precision and reproducibility of the chemical analysis, as well as the recovery of the test item from soil and worm samples are satisfactory for the particular method. Also, check that the test item is not detectable in the control vessels in concentrations higher than background. When  $C_a$  is  $> 0$  in the control worms, this has to be included in the calculation of kinetic parameters (see Annex 2). Handle all samples throughout the test in such a manner so as to minimise contamination and loss (e.g. resulting from adsorption of the test item on the sampling device).

47. The overall recovery and the recovery of test item in worms, soil, and, if employed, in traps containing absorbents to retain evaporated test item, should be recorded and reported.

48. Since the use of radiolabelled instead of non-labelled substances is recommended for the presented test method, it is possible to analyse for total radioactivity only (i.e. parent and metabolites). However, if analytically feasible, quantification of parent compound and metabolites at steady state or at the end of the uptake phase can provide important information. The samples should then be cleaned 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.

49. Due to low individual biomass, in contrast to earthworm samples it is not possible to determine the concentration of test item in each individual enchytraeid worm. Therefore, pooling of the individuals sampled from a given test vessel is acceptable. If pooling involves the reduction

of the number of replicates, it does, however, restrict 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 to accommodate the desired pooling, procedure and power, should be included in the test.

50. It is recommended, that the BAF is expressed both as a function of total dry weight, and, when required (i.e. for highly lipophilic substances), as a function of the lipid content (see paragraph 16). Suitable methods should be used for determination of lipid content (e.g., Gardner et al. 1985, Randall et al. 1991). These methods use a chloroform/methanol extraction technique. However, to avoid the use of chlorinated solvents, a modification of the Bligh & Dyer method (Bligh & Dyer 1959) as described in De Boer et al. (1999) 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 that produced for analysis for the test item, since the lipids often have to be removed from the extract before it can be analysed chromatographically (OECD 1996). 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 item.

## DATA AND REPORTING

### Treatment of results

51. The uptake curve of the test item 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, that is, become approximately parallel to the time axis, calculate the steady state  $BAF_{ss}$  from:

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


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

52. When no steady state is reached, the BAF, and the rate constants should be determined as described below.

53. Determine the accumulation factor ( $BAF_K$ ) as the ratio  $k_s/k_e$ . 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 (see Annex 2).

## Test report

54. The test report must include the following information:

### Test item:

- 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, the precise position of the labelled atoms, the specific radioactivity, and the percentage of radioactivity associated with impurities.

### 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<sub>max</sub>, 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 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 occasion; for earthworms, the wet weights at start of the test, and at each sampling occasion before and after gut purging;
- the lipid content of the test organisms (if determined on testing occasion);
- curves, showing the uptake and elimination kinetics of the test item 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  $\text{g kg}^{-1}$  wet and dry weight of whole body,  $C_s$  expressed in  $\text{g kg}^{-1}$  wet and dry weight). If a biota-soil accumulation factor (BSAF; see Annex 1 for definition) 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  $\text{g kg}^{-1}$  lipid content of the organism, and  $C_s$  may be expressed as  $\text{g kg}^{-1}$  organic carbon (OC) of the soil;
- BAF (expressed in  $\text{kg soil kg}^{-1}$  worm), soil uptake rate constant  $k_s$  (expressed in  $\text{g soil kg}^{-1}$  of worm  $\text{d}^{-1}$ ), and elimination rate constant  $k_e$  (expressed in  $\text{d}^{-1}$ ); BSAF (expressed in  $\text{kg soil OC kg}^{-1}$  worm lipid content) may be reported additionally;
- if measured: percentages of parent compound, metabolites, and bound residues (i.e. the percentage of test item that can not be extracted with common extraction methods) detected in soil and test animals;
- methods used for statistical analyses of the 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 item from the test animals.

## ANNEX 1

### DEFINITIONS AND UNITS

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

Bioconcentration is the increase in concentration of the test item in or on an organism, resulting exclusively from uptake via the body surface, relative to the concentration of the test item in the surrounding medium.

Biomagnification is the increase in concentration of the test item 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 item 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 item in/on the test organism ( $C_a$  in  $\text{g kg}^{-1}$  dry weight) divided by the concentration of the substance in the surrounding medium ( $C_s$  as  $\text{g kg}^{-1}$  of dry weight of soil). In order to refer to the units of  $C_a$  and  $C_s$ , the BAF has the units of  $\text{kg soil kg}^{-1}$  worm.

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

The biota-soil accumulation factor (BSAF) is the lipid-normalised concentration of the test item in/on the test organism divided by the organic carbon-normalised concentration of the test item in the soil at steady state.  $C_a$  is then expressed as  $\text{g kg}^{-1}$  lipid content of the organism, and  $C_s$  as  $\text{g kg}^{-1}$  organic content of the soil; the BSAF has the units of  $\text{kg OC kg}^{-1}$  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 items which are taken up slowly, more appropriate intervals would be seven days (OECD 1996).

Bioaccumulation factors calculated directly from the ratio of the soil uptake constant and the elimination rate constant ( $k_s$  and  $k_e$ , respectively - see below) are termed kinetic bioaccumulation factor ( $\text{BAF}_k$ ).

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 item.

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  $\text{g soil kg}^{-1}$  of worm  $\text{d}^{-1}$ .

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 is calculated in the same manner for the end of the uptake phase (day 21). However, it should be noted if the BAF is based on steady state concentrations or not.

The preferred 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 by Spacie & Hamelink (1982)(64). Given a set of sequential time concentration data and the model equations

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

or

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

where  $C_a$  = concentration of substance in worms [g kg<sup>-1</sup> wet or dry weight]

$k_s$  = uptake rate constant in tissue [g soil kg<sup>-1</sup> of worm d<sup>-1</sup>]

$C_s$  = concentration of substance in soil [g kg<sup>-1</sup> of wet or dry weight]

$k_e$  = elimination rate constant [d<sup>-1</sup>]

$t_c$  = 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 ( $C_{a,0}$ ) has to be included in these equations, to make them read:

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

and

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

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. Widianarko & van Straalen 1996, Sousa et al. 2000):

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

where  $C_s$  = concentration of substance in the soil [g kg<sup>-1</sup> wet or dry weight]

$k_0$  = degradation rate constant in soil [d<sup>-1</sup>]

$C_0$  = initial concentration of substance in soil [g kg<sup>-1</sup> of wet or dry weight]

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

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

where  $C_a$  = concentration of substance in worms [g kg<sup>-1</sup> wet or dry weight]

$k_s$  = uptake rate constant in tissue [g soil kg<sup>-1</sup> of worm d<sup>-1</sup>]

$k_0$  = degradation rate constant in soil [d<sup>-1</sup>]

$k_e$  = elimination rate constant [d<sup>-1</sup>]

$t_c$  = time at the end of the uptake phase

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

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

may be reduced to:

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

or

$$C_a/C_s = k_s/k_e = \text{BAF} \quad \text{[equation 8]}$$

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

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.

$$C_a(t) = C_{a,ss} * e^{-k_e t} \quad \text{[equation 9]}$$

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., Spacie & Hamelink 1982 (64), Franke et al. 1994 (26)). 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., Van Gestel & Ma 1990, Jager et al. 2000, Jager et al. 2003, Vijver et al. 2005).

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 together. For a description of a method that may allow for such a combined calculation of uptake and elimination rate constants, references Janssen et al. (1991)(39), Van Brummelen & Van Straalen (1996)(68) and Sterenborg et al. (2003)(66) may be consulted.

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

$m = 1$  for uptake phase and  $2$  for elimination phase

Nevertheless, these model equations should be used with caution, especially when changes in the test chemical's bioavailability, or (bio)degradation occur during the test (see e.g. Widianarko & van Straalen 1996).

## ANNEX 3

**EXAMPLES OF ACTIVITY SCHEDULES FOR  
SOIL BIOACCUMULATION TESTS**

Earthworm test

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

Day	Activities
-6	Conditioning of the prepared soil for 48 h;
-4	Spiking of the soil fraction with the test item 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 item concentration; 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; 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; 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.

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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).

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#### b) Elimination phase

Day	Activities
-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; transfer worms from remaining exposed replicates to vessels containing clean soil; taking soil and worm samples after 4 - 6 h for determination of test item concentration;
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; 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; 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).

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## Enchytraeid test

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

Day	Activities
-6	Conditioning of the prepared soil for 48 h;
-4	Spiking of the soil fraction with the test item 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 item concentration; 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 <u>soil and worm samples</u> 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; 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; 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	Activities
-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; transfer worms from remaining exposed replicates to vessels containing clean soil; taking soil and worm samples after 4 - 6 h for determination of test item concentration;
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; 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; 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).	

## 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 micropollutants can influence the test, an artificial soil 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 (OECD 1984);
Quartz sand:	70%	Industrial quartz sand (air dried); grain size: more than 50% of the particles should be in the range of 50-200 $\mu\text{m}$ , but all particles should be $\leq 2$ mm;
Kaolinite clay:	20%	Kaolinite content $\geq 30$ %;
Calcium carbonate:	$\leq 1\%$	$\text{CaCO}_3$ , 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 (Van Gestel 1992). 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%) (Hund-Rinke et al., 2000 (33), Roembke et al., 2000 (57)), 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 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 ( $6.0 \pm 0.5$ ), a sufficient amount of  $\text{CaCO}_3$  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 (ISO 1998). 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  $\text{WHC}_{\text{max}}$  by using the solution of the test item and/or by adding deionised or reconstituted water (see paragraphs 22 - 25). 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 3 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*; Bouche 1972). According to Jaenike (1982) 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 temperature optimum 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 are widely used in standardised ecotoxicological tests for about 20 years, their culturing is well established (OECD 1984; Venter & Reinecke 1988).

Both species can be bred in a wide range of animal wastes. The breeding medium recommended by ISO (1998) 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 (1984), 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 (ww) 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 *E. fetida*) indicate 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. Bell 1958). *Enchytraeus albidus* is found in marine, limnic and terrestrial habitats, mainly in decaying organic matter (seaweed, compost) and rarely in meadows (Kasprzak 1982). 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 (Römbke & Moser 1999). 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 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 *E. luxuriosus*. This species is a true soil inhabitant, which has been newly described by Schmelz & Collado (1999). If other species of *Enchytraeus* are used, they must 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 like *E. 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 *E. albidus*. When using this species instead of *E. albidus*, the size of the test vessel can but needs not to be smaller. In addition, it has to be considered that this species reproduces very rapidly having a generation time of less than 20 days at  $20 \pm 2^\circ\text{C}$  (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) filled with a mixture of artificial soil and commercially available, uncontaminated garden soil free of additives. Compost material must be avoided since it could contain toxic substances like heavy metals. Fauna should be removed from the breeding soil before use by 3 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  $6.0 \pm 0.5$ . The culture is kept in an incubator at a temperature of  $15 \pm 2^\circ\text{C}$  without light. In any case, a temperature higher than  $23^\circ\text{C}$  must 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 must be avoided (e.g. if a lid is used, the number of lid holes must be high enough to provide sufficient exchange of air). The breeding soil has to 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 (Scheffczyk, 2008, pers. comm.). If food from the last feeding date remains in the container, the amount of food given must 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<sup>®</sup>.

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 they reproduce continuously.

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