

OECD GUIDELINES FOR THE TESTING OF CHEMICALS

PROPOSAL FOR A NEW GUIDELINE

Bioaccumulation in Sediment-dwelling Benthic Oligochaetes

INTRODUCTION

1. Sediment-ingesting endobenthic animals may be exposed to sediment bound substances (1). Among these sediment-ingesters, aquatic oligochaetes play an important role in the bottoms of the aquatic systems. They live in the sediment and often represent the most abundant species especially in habitats with environmental conditions adverse to other animals. By bioturbation of the sediment and by serving as prey these animals can have a strong influence on the bioavailability of such substances to other organisms, e.g. benthivorous fish. In contrast to epibenthic organisms, endobenthic aquatic oligochaetes burrow in the sediment, and ingest sediment particles below the sediment surface. Because of that, these organisms are exposed to chemicals via many uptake routes including contact with, ingestion of contaminated sediment particles, porewater and overlying water. Some species of benthic oligochaetes that are currently used in ecotoxicological testing are described in Annex 6.

2. The parameters which characterise the bioaccumulation of a substance include first of all the bioaccumulation factor (BAF), the sediment uptake rate constant (k_s) and the elimination rate constant (k_e). Detailed definitions of these parameters are provided in Annex 1.

3. To assess the bioaccumulation potential of chemicals in general, and to investigate the bioaccumulation of substances which tend to partition into or onto the sediments, a compartment-specific test method is needed (1) (2) (3) (4).

4. This Test Guideline is designed to assess bioaccumulation of sediment-associated chemicals in endobenthic oligochaete worms. The test substance is spiked into the sediment. Using spiked sediment is intended to simulate a contaminated sediment.

5. This guideline is based on existing sediment toxicity and bioaccumulation test methods (1) (4) (5) (7) (8) (9) (10). Other useful documents are: the discussions and results of an international workshop (12), and the outcome of an international ring test (62).

6. This test applies to stable, neutral organic chemicals, which tend to associate with sediments. Bioaccumulation of sediment-associated, stable metallo-organic compounds can also be measured with this method (13). It is not applicable to metals and other trace elements (12).

PREREQUISITE AND INFORMATION ON TEST SUBSTANCE

7. There are only a few well established Quantitative Structure-Activity Relationships (QSAR) concerning bioaccumulation processes presently available (14). The most widely used relationship is the correlation between the lipophilicity of stable organic substances (expressed as $\log K_{ow}$; see Annex 1 for definition) and their bioaccumulation and bioconcentration, respectively, which has been developed for the description of a substance partitioning between water and fish. Correlations for the sediment compartment have also been established using this relationship (15) (16) (17) (18). The use of $\log K_{ow}$ - \log BCF correlation as a major QSAR is recommended for a preliminary estimation of the bioaccumulation potential of sediment-associated chemicals.

8. This test is applicable to:

- stable, organic substances having log Kow values between 3.0 and 6.0 (5) (19);
- superlipophilic substances that show a log Kow of more than 6.0 (5);
- substances which belong to a class of organic substances known for their bioaccumulation potential in living organisms, e.g. surfactants or highly adsorptive substances

9. Information on the test substance such as safety precautions, proper storage conditions and analytical methods should be obtained before beginning the study. Guidance for testing substances with physical-chemical properties that make them difficult to test is provided in (21). Before carrying out a test for bioaccumulation with aquatic oligochaetes, the following information about the test compound should be known:

- common name, chemical name (preferably IUPAC name), structural formula, CAS registry number, purity;
- solubility in water [Guideline 105; (22)];
- octanol-water partition coefficient, K_{ow} [Guidelines 107, 117; (22)];
- sediment-water partition coefficient, expressed as K_{oc} [Guideline 121 (22)];
- hydrolysis [Guideline 111; (22)];
- phototransformation in water [(23)];
- vapour pressure [Guideline 104; (22)];
- ready biodegradability [Guidelines 301 A to F; (22)];
- surface tension [Guideline 115; (22)];
- critical micelles concentration (24).

10. Radiolabelled test substances can facilitate the analysis of water, sediment and biological samples, and may be used to determine whether metabolites identification and quantification should be made. The method described here was validated in an international ring test (62) for ^{14}C -labelled compounds. If total radioactive residues are measured 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, it is recommended that BAF calculation be based on the concentration of the parent compound in the organisms and not only on total radioactive residues.

11. In addition to the properties of the test substance, other information required is the toxicity to the oligochaetes species to be used in the test, preferably an LC50 for the time necessary for the uptake phase. Preference should be given to toxicity values derived from long-term studies on sublethal endpoints. If such data are not available, an acute toxicity test under conditions identical with the bioaccumulation test conditions may provide useful information.

12. An appropriate analytical method of known accuracy, precision, and sensitivity for the quantification of the substance in the test solutions, in the sediment, 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 substance in water, sediment, 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 must also 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 (12).

13. Information on characteristics of the sediment to be used (e.g. origin of sediment or its constituents, pH and ammonia of the pore water (field sediments), organic carbon content (TOC), particle size distribution (percent sand, silt, and clay), and percent dry weight) should be available (7).

PRINCIPLE OF THE TEST

14. The test consists of two phases; the uptake (exposure) phase and the elimination (post-exposure) phase. During the uptake phase, worms are exposed to sediment spiked with the test substance, topped with reconstituted water and equilibrated as appropriate (12). Groups of control worms are held under identical conditions without the test substance.

15. For the elimination phase the worms are transferred to a sediment-water-system free of test substance. An elimination phase is necessary to gain information on the rate at which the test substance is excreted by the test organisms (19) (20). An elimination phase is always required unless uptake of the test substance during the exposure phase has been insignificant (e.g. there is no statistical difference between the concentration of the test substance in test and control worms). If a steady state has not been reached during the uptake phase. In this case, determination of the kinetic results – BAF_k , uptake and elimination rate constant(s) – may be done using the results of the elimination phase. Change of the body burden (i.e. the concentration of the test substance in/on the worms) is monitored throughout both phases of the test.

16. During the uptake phase, measurements are made until BAF has reached a plateau or steady state. By default, the duration of the uptake phase should be 28 days. Practical experience has shown that a 12 to 14 day uptake phase is sufficient for several stable, neutral organic substances to reach steady-state (7) (9) (10).

17. However, if the steady state is not reached within 28 d, the elimination phase is started by transferring exposed oligochaetes to vessels containing the same medium without the test substance. The elimination phase is terminated when either the 10% level of steady state concentration or of the concentration measured on day 28 in the worms is reached, or after a maximum duration of 10 d. The residue level in the worms at the end of the elimination phase is reported as endpoint, e.g. as Non-eliminated residues (NER). The bioaccumulation factor (BAF_{ss}) is calculated preferably both as the ratio of concentration in worms (C_a) and in the sediment (C_s) at apparent steady state, and as a kinetic bioaccumulation factor, BAF_k as the ratio of the rate constant of uptake from sediment (k_s) and the elimination rate constant (k_e) assuming first-order kinetics. If first-order kinetics are not applicable, more complex models should be employed (Annex 1 and reference (26).

18. If a steady state is not achieved within 28 days, the uptake phase may be extended subjecting groups of exposed worms – if available – to further measurements until steady state is reached; the elimination phase should be started on day 28. If a steady state is not reached within 28 days, calculate BAF_k from the uptake rate and elimination rate constant(s). For calculation see Annex 2.

19. The uptake rate constant, the elimination rate constant (or constants, where more complex 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 models). The goodness of fit of any model is determined from e.g., the correlation coefficient or the coefficient of determination.

20. To reduce variability in test results for organic substances with high lipophilicity, bioaccumulation factors should be expressed additionally in relation to the lipid content of the test organisms (in kg sediment OC kg^{-1} worm lipid content). This approach is based on experiences and theoretical correlations for the aquatic compartment, where – for some chemical classes – there is a clear

relationship between the potential of a substance to bioaccumulate and its lipophilicity, which has been well established for fish as model organisms (14) (26) (27). There is also a relationship between the lipid content of the test fish and the observed bioaccumulation of such substances. For benthic organisms, similar correlations have been found (14) (15) (16) (17)). If sufficient worm tissue is available, the lipid content of the test animals may be determined on the same biological material as the one used to determine the concentration of the test substance. However, it is practical to use acclimatised control animals at least at start or – preferably – at the end of the uptake phase to measure the lipid content, which can then be used to normalise the BAF values.

VALIDITY OF THE TEST

21. For a test to be valid the following conditions apply:

- The cumulative mortality of the worms (controls and treatments) until the end of the test should not exceed 20% of the initial number.
- In addition, it should be demonstrated that the worms burrow in the sediment to allow for maximum exposure.

DESCRIPTION OF THE METHOD

Test species

22. Several species of aquatic oligochaetes can be used for the test. The most commonly used species are listed in Annex 6.

23. Toxicity tests (96 h, in water only) should be conducted at regular intervals (e.g. every month) with a reference toxicant such as potassium chloride (KCl) or copper sulfate (CuSO₄) (1) to demonstrate the health conditions of the test animals (1) (7). If reference toxicity tests are not conducted at regular intervals, the batch of organisms to be used in a sediment bioaccumulation test should be checked using a reference toxicant. Measurement of the lipid content might also provide useful information on the condition of the animals.

Culture of the test organisms

24. In order to have a sufficient number of worms for conducting bioaccumulation tests the worms may have to be kept in permanent single-species laboratory culture. Laboratory culture methods for the selected test species are summarised in Annex 6. For details see references (9) (10) (18) (37) (38) (39) (40) (41).

Apparatus

25. Care should be taken to avoid the use of materials for all parts of the equipment that can dissolve, absorb test substances or leach other substances and have an adverse effect on the test animals. Standard rectangular or cylindrical chambers, made of chemically inert material and of suitable capacity in compliance with the loading rate, i.e. the number of test worms can be used. The use of soft plastic tubing for administering water or air should be avoided. Teflon[®], stainless steel and/or glass should be used for any equipment having contact with the test media. 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 (5). For radiolabelled test substances, and for volatile chemicals, care should be taken to

avoid stripping and escaping of stripped test substance. Traps (e.g. glass gas washing bottles) containing suitable absorbents to retain any residues evaporating from the test chambers should be employed (12).

Water

26. The overlying water must be of a quality that will allow the survival of the test species for the duration of the acclimation and test periods without them showing any abnormal appearance or behaviour. Reconstituted water according to the OECD Test Guideline 203 (25) is recommended for use as overlying water in the tests as well as in the laboratory cultures of the worms. It has been demonstrated that several test species can survive, grow, and reproduce in this water (9), and maximum standardisation of test and culture conditions is provided. The water should be characterised at least by pH, conductivity and hardness. Analysis of the water for micro-pollutants prior to use might provide useful information (Annex 4).

27. The water should be of constant quality during the period of a test. The pH of the overlying water should be between 6 and 9. The total hardness should be between 90 and 400 mg CaCO₃ per litre at the start of the test (8). Ranges for pH and hardness in the mentioned reconstituted water are given in OECD Guideline 203 (25). If there is an interaction suspected between hardness ions and the test substance, lower hardness water should be used. Annex 3 summarises additional criteria of an acceptable dilution water according to OECD Guideline 210 (28).

Sediment

28. The sediment 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 into the sediment. Additional information on sediment ingestion can be obtained by using methods described in (29) (30) (30) (33), which specify sediment ingestion or particle selection in the test organisms. If observable, at least the presence or absence of fecal pellets on the sediment surface, which indicate sediment ingestion by the worms, should be recorded and considered for the interpretation of the test results with respect to exposure pathways. If possible, sediment avoidance should also be recorded.

29. An artificial sediment based on the artificial soil described in the OECD Guideline 207 (34) is recommended for use in both the tests and the laboratory cultures of the worms, since natural sediments of appropriate quality may not be available throughout the year. In addition, indigenous organisms as well as the possible presence of micropollutants might influence the test. Several test species can survive, grow, and reproduce in the artificial sediment (9).

30. The artificial sediment should be characterised at least by origin of the constituents, grain size distribution (percent sand, silt, and clay), organic carbon content (TOC), water content, and pH. Measurement of redox potential is optional. However, natural sediments from unpolluted sites may serve as test and/or culture sediment (1). Natural sediments should be characterised at least by origin (collection site), pH and ammonia of the pore water, organic carbon content (TOC), particle size distribution (percent sand, silt, and clay), and percent water content (7). It is recommended that, before it is spiked with the test substance, the natural sediment be conditioned for seven days under the same conditions which prevail in the subsequent test, if ammonia development is expected. At the end of this conditioning period, the overlying water should be removed and discarded. Analysis of the sediment or its constituents for micro-pollutants prior to use might provide useful information.

Preparation

31. Handling of natural sediments prior to their use in the laboratory is described in (1) (6) (7). The preparation of the artificial sediment is described in Annex 4.

Storage

32. The storage of natural sediments in the laboratory should be as short as possible. U.S. EPA (7) recommends a maximum storage period of 8 weeks at $4 \pm 2^\circ\text{C}$ in the dark. There should be no headspace above the sediment in the storage containers. Recommendations for the storage of artificial sediment are given in Annex 4.

Application of the test substance

33. The sediment is spiked with the test substance. The spiking procedure involves coating of one or more of the sediment constituents with the test substance. For example, the quartz sand, or a portion thereof (e.g. 10 g of quartz sand per test vessel), can be soaked with a solution of the test substance in a suitable solvent, which is then slowly evaporated to dryness. The coated fraction can then be mixed into the wet sediment. The amount of sand provided by the test-substance-and-sand mixture has to be taken into account when preparing the sediment, i.e. the sediment should thus be prepared with less sand (7).

34. With a natural sediment, the test substance may be added by spiking a dried portion of the sediment as described above for the artificial sediment, or by stirring the test substance into the wet sediment, with subsequent evaporating of any solubilising agent used. Suitable solvents for spiking wet sediment are ethanol, methanol, ethylene glycol monomethyl ether, ethylene glycol dimethyl ether, dimethylformamide and triethylene glycol (5) (28). Toxicity and volatility of the solvent, and the solubility of the test substance in the chosen solvent should be the main criteria for the selection of a suitable solubilising agent. Additional guidance on spiking procedures is given in Environment Canada (1995) (35). Care should be taken to ensure that the test chemical added to sediment is thoroughly and evenly distributed within the sediment. Replicated sub-samples of the spiked sediment should be analysed to check the concentrations of the test substance in the sediment, and to determine the degree of homogeneity of test substance distribution.

35. Once the spiked sediment with overlying water has been prepared, it is desirable to allow partitioning of the test substance between the sediment and the aqueous phase. This should preferably be done under the conditions of temperature and aeration used in the test. Appropriate equilibration time is sediment and chemical specific, and can be in the order of hours to days and in rare cases up to several weeks (4-5 weeks) (36) (37). In this test, equilibrium is not awaited but an equilibration period of 48 hours to 7 days is recommended. Depending on the purpose of the study, e.g., when environmental conditions are to be mimicked, the spiked sediment may be equilibrated or aged for a longer period (12).

PERFORMANCE OF THE TEST

Preliminary test

36. It may be useful to conduct a preliminary experiment in order to optimise the test conditions of the definitive test, e.g. selection of test substance concentration(s) and duration of the uptake and elimination phases. The behaviour of worms, for example sediment avoidance, i.e. the worms escape from the sediment which may be caused by the test chemical and/or by the sediment itself, should be observed and recorded during a preliminary test. Sediment avoidance may also be used as a sub-lethal parameter in a preliminary test for estimating the test substance concentration(s) to be used in a bioaccumulation test.

Exposure conditions

Duration of the uptake phase

37. The test organisms are exposed to the test substance during the uptake phase. The uptake phase should be run for up to 28 days (1) (7) (12) unless it can be demonstrated that equilibrium has been reached earlier. The steady state occurs when: (i) a plot of the bioaccumulation factors at each sampling period against time is parallel to the time axis; (ii) three successive analyses of BAF made on samples taken at intervals of at least two days vary no more than $\pm 20\%$ of each other; and (iii) there are no significant differences between the three sampling periods (based on statistical comparisons e.g. analysis of variance and regression analysis). If the steady state has not been reached by 28 days, the uptake phase may be ended by starting the elimination phase, and the BAF_K can be calculated from the uptake and elimination rate constants.

Duration of the elimination phase

38. The first sample should be taken between 4 and 24 h after start of elimination phase, since during the initial period, rapid changes in tissue residue may occur. It is recommended to terminate the elimination phase either when the concentration of test substance is less than 10% of steady-state concentration, or after a maximum duration of 10 days. The residue level in the worms at the end of the elimination phase is reported as a secondary endpoint. The period may, however, be governed by the period over which the concentration of the test substance in the worms remains above the analytical detection limit.

Test organisms

Numbers of test worms

39. The number of worms per sample must provide a mass of worm tissue such that the mass of test substance per sample at the beginning of the uptake phase and at the end of the elimination phase, respectively, is significantly higher than the detection limit for the test substance in biological material. In the mentioned stages of uptake and elimination phases the concentration in the test animals is usually relatively low (7) (9) (18). Since the individual weight in many species of aquatic oligochaetes is very low (5-10 mg wet weight per individual for *Lumbriculus variegatus* and *Tubifex tubifex*), the worms of a given replicate test chamber may be pooled for weighing and test substance analysis. For test species with higher individual weight (e.g. *Branchiura sowerbyi*) replicates containing one individual may be used, but in such cases the number of replicates should be increased to five per sampling point (12). It should however be noted that *B. sowerbyi* was not included in the ring test (62), and is therefore not recommended as a preferable species in the draft guideline.

40. Worms of similar size should be used (for *L. variegatus* see Annex 6). They should come from the same source, and should be adult animals of the same age class (see Annex 6). The weight and age of an animal may have a significant effect on the BAF-values (e.g. due to different lipid content and/or presence of eggs); these parameters should be recorded accurately. To measure the mean wet and dry weight a sub-sample of worms should be weighed before starting the test.

41. With *Tubifex tubifex* and *Lumbriculus variegatus*, reproduction is expected during the test period. A lack of reproduction in a bioaccumulation test should be recorded, and considered when interpreting the test results.

Loading

42. High sediment-to-worm and water-to-worm ratios should be used in order to minimise the reduction of test substance concentration in the sediment during the uptake phase, and to avoid decreases in dissolved oxygen concentration. The chosen loading rate should also correspond to naturally occurring population densities of the chosen species (42). For example, for *Tubifex tubifex*, a loading rate of 1-4 mg of worm tissue (wet weight) per gram of wet sediment is recommended (9) (12). References (1) and (7) recommend a loading rate of ≤ 1 g dry weight of worm tissue per 50 g sediment organic carbon for *L. variegatus*.

43. The worms to be used in a test are removed from the culture by sieving the culture sediment. Adult animals are transferred to glass dishes (e.g. petri dishes) containing clean water. If the test conditions differ from the culture conditions, an acclimation phase of 24 h should be sufficient. Prior to weighing, excess water should be removed from the worms. This can be done by gently placing the worms on a pre-moistened paper tissue. It is not recommended to use absorbing paper to dry the worms as this may cause stress or damage to the worms. Brunson et al. (1998) recommend using non-blotted worms of approximately 1.33 times the target biomass. These additional 33% correspond to the difference between blotted and non-blotted worms (37).

44. At the start of the uptake phase (day 0 of the test), the test organisms are removed from the acclimatisation chamber and distributed randomly to vessels (e.g. petri dishes) containing reconstituted water by adding groups of two worms to each vessel, until each vessel contains ten worms. Each of these groups of worms are then randomly transferred to separate test vessels, e.g. using soft steel forceps. The test vessels are subsequently incubated under test conditions.

Feeding

45. In view of the low nutrient content of the artificial sediment, the sediment should be amended with a food source. In order not to underestimate the exposure of the test organisms, e.g. by selectively feeding uncontaminated food, the food necessary for reproduction and growth of the test organisms should be added to the sediment once before or during application of the test substance (see Annex 5).

Sediment-water ratio

46. The recommended sediment-water ratio is 1:4. This ratio allows to maintain oxygen concentration and avoid the build-up of ammonia in the overlying water (12). The oxygen content in the overlying water should be maintained at $\geq 40\%$ saturation.

Light and temperature

47. The photoperiod in the culture and the test is 16 hours (1) (7). Light intensity should be kept at about 500-1000 lx; these values mimic natural conditions at the sediment surface. Temperature should be $20 \pm 2^\circ\text{C}$ throughout the test.

Test concentrations

48. One test concentration (as low as possible) is used for determination of the uptake kinetics, but a second (higher) concentration may be used. In that case, samples are taken and analysed at steady state or after 28 d to confirm the BAF measured at the lower concentration (12). The higher concentration should be selected so that adverse effects can be excluded (e.g. by choosing approximately 1% of the lowest known chronic effect concentration EC_x as derived from relevant chronic toxicity studies). The lower test concentration should be significantly higher than the detection limit in sediment and biological samples by

the analytical method used. If the effect concentration of the test substance is close to the analytical detection limit, the use of radiolabelled test substance with high specific radioactivity is recommended.

Treated and Control Replicates

49. The minimum number of treated replicates for kinetic measurements should be three per sampling point (12) throughout uptake and elimination phase. Additional replicates should be employed e.g. for optional additional sampling dates. For the elimination phase, a matching number of replicates is prepared with non-spiked sediment and overlying water, so that worms can be transferred at the end of the uptake phase from designated treated vessels to non-treated vessels. The total number of treated replicates should be sufficient for both uptake and elimination phase.

50. Alternatively, the worms designated for sampling during the elimination phase may be exposed in one large container containing spiked sediment of the same batch as used for uptake kinetics. It should be demonstrated that the test conditions (e.g. sediment depth, sediment water ratio, loading, temperature, water quality) are comparable to the replicates designated for the uptake phase. At the end of the uptake phase, water, sediment and worm samples should be taken from this container for analysis, and the worms should be removed carefully and transferred to the replicates prepared for the elimination phase.

51. If no solvent other than water is used, at least 9 replicates of a negative control (at least 3 sampled at start, 3 at end of uptake and 3 at end of elimination) should be provided for biological and background analysis. If any solubilising agent is used for application of the test substance, a solvent control should be run (at least 3 replicates should be sampled at start, 3 at the end of the uptake phase, and 3 at the end of the elimination phase). In this case, at least 4 replicates of a negative control (no solvent) should 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 possible influence of the solvent on the test organisms. Details are given in Annex 3.

Frequency of water quality measurements

52. As a minimum, the following water quality parameters should be measured in the overlying water:

- Temperature in one treated vessel of each treatment level per sampling date, and in one control vessel once per week and at the start and the end of the uptake and elimination period; temperature in the surrounding medium (ambient air or water bath) may also be recorded e.g. in hourly intervals;
- Dissolved oxygen content in one treated vessel of each treatment level per sampling date, and in one control vessel once per week and at the start and the end of the uptake and elimination period; expressed as mg/L and % ASV (air saturation value);
- Air supply controlled at least once per day (workdays) and adjusted if needed;
- pH in one treated vessel of each treatment level per sampling date, and in one control vessel once per week and at the start and the end of the uptake and elimination period;
- Total water hardness at least in one treated vessel and one control test vessel at the start and the end of the uptake and elimination period, expressed as mg/L CaCO₃;
- Total ammonia content at least in one treated vessel and one control test vessel at the start and the end of the uptake and elimination period; expressed as mg/L NH₄⁺ or NH₃ or total ammonia-N.

Sampling and analysis of worms, sediment, and water

Sampling Schedule

53. Examples of sampling schedules for a 28-day uptake phase and a 10-day elimination phase are given in Annex 3.
54. Sample the water and sediment from the test chambers for determination of test substance concentration before adding the worms, and during both uptake and elimination phases. During the test the concentrations of test substance are determined in the worms, sediment, and water in order to monitor the distribution of the test substance in the compartments of the test system.
55. Sample the worms, sediment, and water on at least six occasions during the uptake as well as the elimination phase.
56. Continue sampling until a plateau (steady state) has been established (see Annex 1) or for 28 days. If the plateau has not been reached within 28 days, begin the elimination phase. When beginning the elimination phase, transfer the designated worms to replicate chambers containing untreated sediment and water.

Sampling and sample preparation

57. Obtain water samples by decanting, siphoning or pipetting a volume sufficient for measuring the quantity of the test substance in the sample.
58. The remaining overlying water is carefully decanted or siphoned from the test chamber(s). Sediment samples should be taken carefully, causing minimal disturbance of the worms.
59. Remove the worms from the test replicate at the sampling time by suspending the sediment with overlying water and spreading the contents of each replicate on a shallow tray and picking the worms using soft steel forceps. Rinse them quickly with water in a shallow glass or steel tray. Remove the excess water. Transfer the worms carefully to a pre-weighed vessel and weigh them. Kill the worms by freezing at $-20 \pm 2^\circ\text{C}$. The presence and number of cocoons and/or juveniles should be recorded.
60. In general, the worms should be weighed and killed immediately after sampling without a gut purging phase to obtain a conservative BAF which includes contaminated gut content, and to avoid losses of body residues during any gut-purging period in water only (9). Compounds with $\log K_{ow}$ above 5 are not expected to be eliminated significantly during any gut-purging period in water only, while chemicals with $\log K_{ow}$ lower than 4 may be lost in notable amounts (44).
61. During the elimination phase, the worms purge their gut in clean sediment. This means, measurements immediately before the elimination phase include contaminated gut sediment, while after the initial 4-24 h of the elimination phase, most of the contaminated gut content is assumed to be replaced by clean sediment (12) (44). The concentration in the worms of this sample may then be considered as the tissue concentration after gut purge. To account for dilution of the test substance concentration by uncontaminated sediment 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.
62. If the purpose of a specific study is to measure the bioavailability and true tissue residues in the test organisms, then at least a sub-sample of test animals, preferably sampled during steady state, should be weighed, purged in clean water for a period of 6 hours (44), and weighed again before analysis. Data on worm weight and body concentration of this sub-sample can then be compared to values obtained from un-

purged worms. The worms designated for measurement of elimination should not be purged before the transfer to clean sediment to minimise additional stress for the animals.

63. Preferably analyse the water, sediment, 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. Immediate analysis also avoids delay in determining when a plateau has been reached.

64. Failing immediate analysis, the samples should be stored under appropriate conditions. Obtain information on the proper storage conditions for the particular test substance before beginning the study, (e.g. duration and temperature of storage, extraction procedures, etc.).

Quality of analytical method

65. Since the whole procedure is governed essentially by the accuracy, precision and sensitivity of the analytical method used for the test substance, check experimentally that the precision and reproducibility of the chemical analysis, as well as the recovery of the test substance from water, sediment and worm samples are satisfactory for the particular method. Also, check that the test substance is not detectable in the control chambers in concentrations higher than background. If necessary, correct the values of C_w , C_s and C_a for the recoveries and background values of controls. Handle all samples throughout the test in such a manner so that contamination and loss are minimised (e.g. resulting from adsorption of the test substance on the sampling device).

66. The overall recovery and the recovery of test substance in worms, sediment, water, and, if employed, in traps containing absorbents to retain evaporated test substance, should be recorded and reported.

67. Since the use of radiolabelled substances is recommended, it is possible to analyse for total radioactivity (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.

68. Due to low individual biomass, it is often not possible to determine the concentration of test substance in each individual worm, unless *Branchiura sowerbyi* (40-50 mg wet weight per worm) is used as test species (12). Therefore, pooling of the individuals sampled from a given test vessel is acceptable, but it does restrict the statistical procedures which can be applied to the data. If a specific statistical procedure and power are important considerations, then an adequate number of test animals and/or replicate test chambers to accommodate the desired pooling, procedure and power, should be included in the test.

69. It is recommended that the BAF is expressed both as a function of total wet weight, total dry weight, and, when required (i.e. for highly lipophilic substances) as a function of the lipid content. Suitable methods should be used for determination of lipid content (45) (46). The chloroform/methanol extraction technique may be recommended as standard method (45). However, to avoid the use of chlorinated solvents, a ring-tested modification of the Bligh & Dyer method (47) (48) as described in (48) might be used. Since the various methods do not give identical values (45), it is important to detail the method used. When possible, i.e. if sufficient worm tissue is available, the lipid content is measured in the same sample or extract as that produced for analysis for the test substance, since the lipids often have to be removed from the extract before it is analysed by chromatography (5). However, it is practical to use acclimatised control animals at least at start or - preferably - at the end of the uptake phase to measure the lipid content, e.g. in three samples.

DATA AND REPORTING

Treatment of results

70. The uptake curve of the test substance is obtained by plotting in arithmetic scale the concentration of test substance in/on the worms during the uptake phase against time. If the curve has reached a plateau, calculate the steady state BAF_{ss} :

$$\frac{C_a \text{ at steady state or at day 28 (mean)}}{C_s \text{ at steady state or at day 28 (mean)}}$$

71. Determine the kinetic bioaccumulation factor (BAF_K) as the ratio k_s/k_e . The elimination constant (k_e) is usually determined from the elimination curve (i.e. a plot of the concentration of the test substance in the worms during the elimination phase). The uptake rate constant k_s is then calculated from the uptake curve kinetics. 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 (see Annex 2). If the elimination is obviously not first-order, then more complex models should be employed (26) (27) (49).

72. The Biota-Sediment Accumulation Factor (BSAF) is determined by normalising the BAF_K for the worm lipid content and the sediment total organic carbon content.

Interpretation of results

73. The results should be interpreted with caution where measured concentrations of test concentrations occur at levels close to the detection limit of the analytical method used.

74. Clearly defined uptake and elimination curves are an indication of good quality bioaccumulation data. Generally the confidence limits for the BAF values from well-designed studies should not exceed 25% (5).

Test report

75. The test report must include the following information.

Test substance

- physical nature and, physicochemical properties e.g. $\log K_{ow}$, water solubility;
- substance identification data; source of the test substance, 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 (e.g. static, semi-static or flow-through);
- type and characteristics of illumination used and photoperiod(s);

- test design (e.g. number, material and size of test chambers, water volume, sediment mass and volume, water volume replacement rate (for flow-through or semi-static procedures), any aeration used before and during the test, number of replicates, number of worms per replicate, number of test concentrations, length of uptake and elimination phases, sampling frequency);
- method of test substance preparation and application as well as reasons for choosing a specific method;
- the nominal test concentrations;
- source of the constituents of the artificial water and sediment or - if natural media are used - origin of the water and the sediment, description of any pre-treatment, results of any demonstration of the ability of the test animals to live and/or reproduce in the media used, sediment characteristics (pH and ammonia of the pore water (natural sediments), organic carbon content (TOC), particle size distribution (percent sand, silt, and clay), percent water content, and any other measurements made) and water characteristics (pH, hardness, conductivity, temperature, dissolved oxygen concentration, residual chlorine levels (if measured), and any other measurements made);
- the nominal and measured dry weight in % of wet weight (or dry weight-to-wet weight ratio) of the artificial sediment; the measured dry weight in % of wet weight (or dry weight-to-wet weight ratio) for field sediments;
- water quality within the test chambers as characterised by temperature, pH, ammonium, total hardness, and dissolved oxygen concentration;
- detailed information on the treatment of water, sediment, and worm samples, including details of preparation, storage, spiking procedures, extraction, and analytical procedures (and precision) for the test substance and lipid content, and recoveries of the test substance.

Results

- mortality of the control worms and the worms in each test chamber and any observed abnormal behaviour (e.g., sediment avoidance, presence or absence of fecal pellets, lack of reproduction);
- the measured dry weight in % of wet weight (or dry weight-to-wet weight ratio) of the sediment and the test organisms (useful for normalisation);
- the lipid content of the worms;
- curves showing the uptake and elimination kinetics of the test substance in the worms, and the time to steady state;
- C_a , C_s and C_w (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, and C_w in mg L^{-1}). If a biota-sediment 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 should additionally be expressed as g kg^{-1} lipid content of the organism, and C_s should be expressed as g kg^{-1} organic carbon (OC) of the sediment;
- BAF (expressed in $\text{kg sediment kg}^{-1}$ worm), sediment uptake rate constant k_s (expressed in g sediment g^{-1} of worm d^{-1}), and elimination rate constant k_e (expressed in d^{-1}); BSAF (expressed in $\text{kg sediment OC kg}^{-1}$ worm lipid content) may be reported additionally;
- Non-Eliminated Residues (NER) at end of elimination phase;
- if measured: percentages of parent compound, metabolites, and bound residues (i.e. the percentage of test substance that can not be extracted with common extraction methods) detected in the 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 27;
- unexpected or unusual results, e.g. incomplete elimination of the test substance from the test animals; in such cases results from any preliminary study may provide useful information.

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 exclusively from uptake via the body surface, 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 substance in the food or prey. Biomagnification can lead to a transfer or accumulation of the test substance 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 substance 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 g^{-1} wet or dry weight) divided by the concentration of the substance in the surrounding medium (C_s as g kg^{-1} of wet or dry weight of sediment). In order to refer to the units of C_a and C_s , the BAF has the units of $\text{kg sediment kg}^{-1}$ worm (15).

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 wet or dry weight of sediment) being constant during this period of time.

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

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 the BAF at each sampling period 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 (5).

Bioaccumulation factors calculated directly from the ratio of the sediment uptake rate constant divided by the elimination constant kinetic rate constants (k_s and k_e , respectively - see below) are termed kinetic bioaccumulation factor (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 sediment 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 (OECD Guideline 117), 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 conditioning period is used to stabilise the microbial component of the sediment and to remove e.g. ammonia originating from sediment components; it takes place prior to spiking of the sediment with the test substance. Usually, the overlying water is discarded after conditioning.

The equilibration period is used to allow for distribution of the test substance between the solid phase, the pore water and the overlying water; it takes place after spiking of the sediment with the test substance and prior to addition of the test organisms.

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

The sediment uptake rate constant (k_s) is the numerical value defining the rate of increase in the concentration of the test substance in/on the test organism resulting from uptake from the sediment phase. k_s is expressed in g sediment g⁻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 substance, 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 substance in/on the test organism, following the transfer of the test organisms from a medium containing the test substance to a substance-free medium; k_2 is expressed in d⁻¹.

Artificial sediment, or formulated, reconstituted or synthetic sediment, is a mixture of materials used to mimic the physical components of a natural sediment.

Spiked sediment is sediment to which test substance has been added.

Overlying water is the water lying on top of the sediment in the test vessel.

Pore water or interstitial water is the water occupying space between sediment or soil particles.

ANNEX 2

Calculation of uptake and elimination parameters

Main endpoint of a bioaccumulation test is the bioaccumulation factor, BAF. The BAF can be calculated by dividing the concentration in the test organism, C_a , by the concentration in the sediment, 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 day 28. However, it should be noted whether the BAF is based on steady state concentrations or not.

The preferred means for obtaining the bioaccumulation factor (BAF_K), the sediment uptake rate constant (k_s) and the elimination rate constant (k_e) is to use non-linear parameter estimation methods on a computer. Given the time series of average accumulation factors (C_a , mean values of each sampling date/ C_s , mean values of each sampling date = AF) of the uptake phase based on worm and sediment wet weight, and the model equation

$$AF(t) = BAF_K \times (1 - e^{-k_e t}) \quad [\text{equation 1}]$$

where $AF(t)$ is the ratio of concentration in worms and the concentration in sediment in the sediment at any given time point (t) of the uptake phase, these computer programs calculate values for BAF_K , k_s and k_e .

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

$$BAF_K = \frac{k_s}{k_e} \quad [\text{equation 2}]$$

where k_s = uptake rate constant in tissue [g sediment g^{-1} of worm d^{-1}]
 k_e = elimination rate constant [d^{-1}]

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

The Biota-Sediment Accumulation Factor (BSAF) should be calculated as follows:

$$BSAF = BAF_K \times \frac{f_{oc}}{f_{lip}}$$

where f_{oc} is the fraction of sediment organic carbon based on dry weight, and f_{lip} is the fraction of worm lipid, both based either on dry weight, or on wet weight.

Given a time series of concentration values, the elimination kinetics can be modelled using the following model equations and a computer calculation based non-linear parameter estimation method.

The mean measured body residue at the end of the uptake phase is recommended as the default starting point. The use of the value modelled/estimated from the uptake phase should only be used, e.g. if the measured value deviates significantly from the modelled body residue. See also paragraph 0 for alternative pre-exposure of worms designated for elimination; with this approach, samples of these pre-exposed worms on day 0 of the elimination phase are thought to provide a realistic body residue to start the elimination kinetics with.

If the data points plotted against time indicate a constant exponential decline of the test substance concentration in the animals, a one-compartment model (equation 4) can be used to describe the time course of elimination.

$$C_a(t) = C_{a,ss} \times e^{-k_e t} \quad \text{[equation 3]}$$

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 substances in the later phases of the elimination (9) (19) (26). The two phases can be interpreted by the assumption, that there are two different compartments in the organism, from which the test substance is lost with different velocity. In these cases specific literature should be studied (15) (16) (17) (26).

A two-compartment elimination is described e.g. by the following equation (26):

$$C_a = A \times e^{-k_a \times t} + B \times e^{-k_b \times t} \quad \text{[equation 4]}$$

A and B represent the size of the compartments (in percent of overall tissue residue), where A is the compartment with rapid loss of substance, and B the compartment with slow loss of test substance. The sum of A and B equals 100% of the whole animal compartment volume at steady state. k_a and k_b represent the corresponding elimination constants [d^{-1}]. If the two compartment model is fitted to the depuration data, the uptake rate constant k_s may be determined as follows (63) (64):

$$k_s = \frac{(A \times k_a + B \times k_b) \times BAF}{A + B} \quad \text{[equation 5]}$$

Nevertheless, these model equations should be used with caution, especially when changes in the test chemical's bioavailability occur during the test (36).

The Non-Eliminated Residues (NER) should be calculated as a secondary endpoint by multiplying the ratio of the average concentration in the worms (C_a) on day 10 of the elimination phase and the average concentration in the worms (C_a) at steady state (day 28 of uptake phase) by 100:

$$NER_{10d} [\%] = \frac{C_a \text{ at end of elimination (average)} \times 100}{C_a \text{ at steady state (average)}}$$

ANNEX 3

Example of a Sampling Schedule for a 28-day Bioaccumulation Test**a) Uptake phase (including a 4 d- equilibration phase)**

| Day | Activities |
|---|--|
| -6 | Preparation of peat suspension for sediment; conditioning of the suspension for 48 h; |
| -4 | Spiking of the sediment or sediment fraction; mixing of all sediment constituents; removing sediment samples of treated and solvent control sediment for determination of test item concentration; addition of overlying water; incubation at test conditions (equilibration phase); |
| -3/-2 | Separation of the test organisms from the culture for acclimatisation; |
| 0 | Measurement of temperature, pH, oxygen content in the overlying water of the test chambers; removing replicates for taking samples of water and sediment for determination of test substance concentration; randomised distribution of the worms to the test chambers; retaining of sufficient sub-samples of worms for determination of analytical background values; controlling air supply, if closed test system is used; |
| 1 | Remove replicates for sampling; controlling air supply, worm behaviour, pH, temperature and oxygen content in sampled test chambers; taking water, sediment and worm samples for determination of test substance concentration; |
| 2 | Controlling air supply, worm behaviour and temperature; |
| 3 | Same as day 1; |
| 4 - 6 | Same as day 2; |
| 7 | Same as day 1; compensate evaporated water if necessary; |
| 8 - 13 | Same as day 2; |
| 14 | Same as day 1; compensate evaporated water if necessary; |
| 15 - 20 | Same as day 2; |
| 21 | Same as day 1; compensate evaporated water if necessary; |
| 22 - 27 | Same as day 2; |
| 28 | Same as day 1; measurement of temperature, pH, oxygen content in the overlying water of the test chambers; end of uptake phase; retaining of sufficient subsamples of worms for determination of analytical background values, wet and dry weight, and lipid content; transfer worms from remaining exposed replicates to vessels containing clean sediment for elimination phase (no gut-purging); sampling of water, sediment and worms from solvent controls; sampling of trapping solutions, if installed. |
| Pre-exposure activities (equilibration phase) should be scheduled taking into account the properties of the test substance. If required, conditioning of the prepared sediment under overlying water at $20 \pm 2^\circ\text{C}$ for 7 days; in this case, earlier preparation of the sediment! | |
| Activities described for day 2 should be performed daily (at least on workdays). | |

b) Elimination phase

| Day | Activities |
|---|---|
| -6 | Preparation of peat suspension for sediment; conditioning of the suspension for 48 h; |
| -4 | Mixing of all sediment constituents; removing sediment samples of treated and solvent control sediment for determination of test item concentration; addition of overlying water; incubation at test conditions; |
| 0 (day 28 of uptake phase) | Measurement of temperature, pH, oxygen content in the overlying water of the test chambers; transfer worms from remaining exposed replicates to vessels containing clean sediment; after 4 - 6 h removing replicates for taking samples of water, sediment and worms for determination of test substance concentration; randomised distribution of the worms to the test chambers; |
| 1 | Remove replicates for sampling; controlling air supply, worm behaviour, pH, temperature and oxygen content in sampled test chambers; taking water, sediment and worm samples for determination of test substance concentration; |
| 2 | Controlling air supply, worm behaviour and temperature; |
| 3 | Same as day 1; |
| 4 | Same as day 2; |
| 5 | Same as day 1; |
| 6 | Same as day 2; |
| 7 | Same as day 1; compensate evaporated water if necessary; |
| 8 - 9 | Same as day 2; |
| 10 | Same as day 1; end of elimination phase; measurement of temperature, pH, oxygen content in the overlying water of the test chambers; sampling of water, sediment and worms from solvent controls; sampling of trapping solutions, if installed. |
| Preparation of the sediment prior to start of elimination phase should be done in the same manner as before the uptake phase. | |
| Activities described for day 2 should be performed daily (at least on workdays). | |

ANNEX 4

Some Physical-Chemical Characteristics of an Acceptable Dilution Water

| SUBSTANCE | CONCENTRATIONS |
|--|----------------|
| Particular matter | < 20 mg/L |
| Total organic carbon | < 2µg/L |
| Unionised ammonia | < 1 µg/L |
| Residual chlorine | < 10 µg/L |
| Total organophosphorous pesticides | < 50 ng/L |
| Total organochlorine pesticides plus polychlorinated biphenyls | <50 ng/L |
| Total organic chlorine | < 25 ng/L |

COMPOSITION OF THE RECOMMENDED RECONSTITUTED WATER (25)

- (a) Calcium chloride solution
Dissolve 11.76 g $\text{CaCl}_2 \times 2 \text{H}_2\text{O}$ in deionised water; make up to 1 L with deionised water
- (b) Magnesium sulphate solution
Dissolve 4.93 g $\text{MgSO}_4 \times 7 \text{H}_2\text{O}$ in deionised water; make up to 1 L with deionised water
- (c) Sodium bicarbonate solution
Dissolve 2.59 g NaHCO_3 in deionised water; make up to 1 L with deionised water
- (d) Potassium chloride solution
Dissolve 0.23 g KCl in deionised water; make up to 1 L with deionised water

All chemicals must be of analytical grade.

The conductivity of the distilled or deionised water should not exceed $10 \mu\text{Scm}^{-1}$.

25 ml each of solutions (a) to (d) are mixed and the total volume made up to 1 L with deionised water. The sum of the calcium and magnesium ions in this solutions is 2.5 mmol/L.

The proportion Ca:Mg ions is 4:1 and Na:K ions 10:1. The acid capacity $K_{s4.3}$ of this solution is 0.8 mmol/L.

Aerate the dilution water until oxygen saturation is achieved, then store it for approximately two days without further aeration before use.

ANNEX 5

ARTIFICIAL SEDIMENT - PREPARATION AND STORAGE RECOMMENDATIONS

In contrast to the requirements in the OECD Guideline 207 (34) the peat content of the artificial sediment is recommended to be 2% instead of 10% of dry weight, in order to correspond to a low to moderate organic content of natural sediments (50).

Percentage of dry constituents of the artificial sediment:

| Constituent | Characteristics | % of dry sediment |
|-------------------|--|-------------------|
| Peat | Sphagnum moss peat, degree of decomposition: "medium", air dried, no visible plant remains, finely ground (particle size ≤ 0.5 mm) | 2 ± 0.5 |
| Quartz sand | Grain size: ≤ 2 mm, but $> 50\%$ of the particles should be in the range of 50-200 μm | 76 |
| Kaolinite clay | Kaolinite content $\geq 30\%$ | 22 ± 1 |
| Urtica powder | <i>Folia urticae</i> , powdered leaves of <i>Urtica sp.</i> (stinging nettle), finely ground (particle size ≤ 0.5 mm), or a mixture of powdered leaves of <i>Urtica sp.</i> with alpha-cellulose (1 : 1); in accordance with pharmacy standards, for human consumption; in addition to dry sediment | 0.4 - 0.5% |
| Calcium carbonate | CaCO_3 , pulverised, chemically pure, in addition to dry sediment | 0.05 - 1 |
| Deionised Water | Conductivity $\leq 10 \mu\text{S/cm}$, in addition to dry sediment | 30 - 50 |

If elevated ammonia concentrations are expected, e.g. if the test substance is known to inhibit the nitrification, it may be useful to replace 50% of the nitrogen-rich urtica powder by cellulose (e.g., α -Cellulose powder, chemically pure, particle size ≤ 0.5 mm).

Preparation

The peat is air-dried and ground to a fine powder (grain size ≤ 0.5 mm, no visible plant remains). A suspension of the required amount of peat powder is prepared using a portion of the deionised water to be added to the dry sediment (a water volume of 11.5 x dry weight of peat has been found useful to produce a stirrable peat slurry (9)) using a high-performance homogenising device.

The pH of this suspension is adjusted to 5.5 ± 0.5 with CaCO_3 . The suspension is conditioned for at least two days with gentle stirring at 20 ± 2 °C, to stabilise pH and establish a stable microbial component. The pH is measured again and is adjusted to 6.0 ± 0.5 with CaCO_3 if necessary. Then all of the suspension is mixed with the other dry constituents, taking into account any portion used for spiking. The remaining deionised water is added to obtain a homogeneous sediment. The pH is measured again and is adjusted to 6.5 to 7.5 with CaCO_3 if necessary. However, if ammonia development is expected, it may be useful to keep the pH of the sediment below 7.0 (e.g. between 6.0 and 6.5). Samples of the sediment are taken to determine the dry weight and the organic carbon content. If ammonia development is expected, the artificial sediment may be conditioned for seven days under the same conditions which prevail in the subsequent test (e.g. sediment-water ratio 1 : 4, height of sediment layer as in test vessels) before it is spiked with the test substance, i.e. it should be topped with water, which should be aerated. At the end of the conditioning period, the overlying water should be removed and discarded. Samples of the sediment are taken to determine dry weight and total organic carbon (e.g. 3 samples).

Thereafter, the spiked quartz sand is mixed with the sediment for each treatment level, the sediment is distributed to the replicate test vessels, and topped with the test water (e.g. sediment-water ratio 1 : 4, height of sediment layer as in test vessels). The vessels are then incubated at the same conditions which prevail in the subsequent test. This is where the equilibration period starts. The overlying water should be aerated.

The chosen food source should be added prior to or during spiking the sediment with the test substance. It can be mixed initially with the peat suspension (see above). However, excessive degradation of the food source prior to addition of the test organisms - e.g. in case of long equilibration period - can be avoided by keeping the time period between food addition and start of exposure as short as possible. In order to ensure that the food is in sufficient contact with the test compound, the food source should be mixed with the sediment not later than on the day the test substance is spiked to the sediment. Exceptions may be made where the length of the equilibration period leads to excessive microbial degradation of the food before the test organisms are added. Samples of the sediment are taken to determine dry weight and total organic carbon (e.g. 3 samples of spiked or control sediment).

The dry weight of the components (peat, sand, kaolin) should be reported in g and in in per cent of total dry weight.

The volume of water to be added to the dry components during preparation of the sediment should also be reported in per cent of total dry weight (e.g. 100% dry weight + 46% water means 1000 g d.w. receive a total of 460 mL water, which results in 1460 g wet sediment).

Storage

The dry constituents of the artificial sediment may be stored in a dry, cool place at room temperature. The prepared, wet sediment may be stored (for further use in the culture only) at 4 ± 2 °C in the dark for a period of 2 to 4 weeks from the day of preparation (9).

Sediment spiked with the test substance should be used immediately unless there is information indicating that the particular sediment can be stored without affecting the toxicity and bioavailability of the test substance. Samples of spiked sediment may be stored under the conditions recommended for the particular test substance until analysis.

ANNEX 6

OLIGOCHAETES SPECIES RECOMMENDED FOR BIOACCUMULATION TESTING

Tubifex tubifex (MÜLLER), Tubificidae, Oligochaeta

The tubificid oligochaete (Tubificidae, Oligochaeta) *Tubifex tubifex* (Müller) lives in freshwater sediments in tubes which are lined with mucus. In these tubes the worms dwell head down, ingesting sediment particles utilising the associated microorganisms and organic debris. The posterior portion usually undulates in the overlying water for respiration purposes. Although this species inhabits a wide range of sediment types, *Tubifex tubifex* prefers relatively fine grain sizes (52). The suitability of this species for ecotoxicological testing is described for example in (9)(33)(38)(40)(51)(53)(54)(55).

Culture methods

In order to have a sufficient number of *Tubifex tubifex* for conducting bioaccumulation tests the worms have to be kept in permanent laboratory culture. A system consisting of artificial sediment based on the artificial soil according to Guideline 207 (34) and reconstituted water according to OECD Guideline 203 (25) is recommended for *T. tubifex* culture (9).

Glass or stainless steel containers with a height of 12 to 20 cm can be used as culture vessels. Each culture container is loaded with a layer of wet artificial sediment prepared as described in Annex 4. The depth of the sediment layer should allow for natural burrowing behaviour of the worms (2 cm minimum depth for *T. tubifex*). Reconstituted water is added to the system. Care should be taken to minimise disturbing the sediment. The water body is gently aerated (e.g. 2 bubbles per second) with 0.45 µm-filtered air via a pasteur pipette positioned 2 cm above the sediment surface. The recommended culture temperature is $20 \pm 2^\circ\text{C}$.

The worms are added to the culture system with a maximum loading of 20,000 individuals/m² sediment surface. A higher loading may cause a reduction in growth and reproduction rates (42).

In artificial sediment cultures, the worms have to be fed. A diet consisting of finely ground fish food, e.g. TetraMin[®] can serve as additional nutrition (9); Klerks 1994, personal communication. The feeding rates should allow for sufficient growth and reproduction and should keep build-up of ammonia and fungal growth in the culture at a minimum. Food may be administered twice a week. Practical experience has shown that application of food suspended in deionised water may facilitate homogeneous food distribution on the sediment surface in the culture containers.

To avoid accumulation of ammonia, the overlying water should be exchanged using a flow-through system, or, at least once a week, manually. Sediment should be changed every three months in the stock cultures.

Sampling of worms from the culture can be done by sieving the culture sediment through a 1 mm sieve if only adults are required. For retaining cocoons a 0.5 mm mesh, and for juvenile worms a 0.25 mm sieve is

suitable. The sieves can be placed into reconstituted water after the sediment has passed through. The worms leave the mesh and can then be picked from the water using a soft steel forceps or a pipette with fire-polished edges.

Only intact and clearly identified specimens of *Tubifex tubifex* (e.g. (56)) are used to start a test or new cultures. Diseased or injured worms as well as cocoons infested with fungal hyphae have to be discarded.

A synchronised culture can provide worms of a specified age in suitable intervals when desired. New culture vessels are set up in the chosen intervals (e.g. every two weeks), starting with animals of a certain age (e.g. cocoons). At the culture conditions described here the worms are adult after 8 - 10 weeks. The cultures can be harvested, when the worms have laid new cocoons, e.g. after ten weeks. The sampled adults can be used for tests, and new cultures can be started with the cocoons.

***Lumbriculus variegatus* (MÜLLER), Lumbriculidae, Oligochaeta**

Lumbriculus variegatus (Lumbriculidae, Oligochaeta) is also an inhabitant of freshwater sediments and is widely used in ecotoxicological testing. Information on the biology, culture conditions, and sensitivity of the species can be obtained from (1)(7)(10)(30). *Lumbriculus variegatus* can also be cultured in the artificial sediment recommended for *T. tubifex* according to (9) within certain limitations. Since, in nature *L. variegatus* prefers more coarse sediments than *T. tubifex* (52), laboratory cultures with the artificial sediment used for *T. tubifex* may cease after 4 to 6 months. Practical experience has shown that *L. variegatus* can be held in a sandy substratum (e.g. quartz sand, fine gravel) in a flow-through system using fish food as nutritional source over several years without renewing the substratum. A major advantage of *L. variegatus* over other aquatic oligochaete species is its quick reproduction, resulting in rapidly increasing biomass in laboratory cultured populations (1)(7)(10)(11).

Culture methods

Culture conditions for *Lumbriculus variegatus* are outlined in detail in Phipps et al. (1993) (11), Brunson et al. (1998) (37), ASTM (2000) (1), U.S. EPA (2000) (7). A short summary of these conditions is given below.

The worms can be cultured in large aquaria (57 - 80 L) at 23°C with a 16L:8D photoperiod (100 - 1000 lux) using daily renewed natural water (45 - 50 L per aquarium). The substrate is prepared by cutting unbleached brown paper towels into strips, which may then be blended with culture water for a few seconds to result in small pieces of paper substrate. This substrate can then directly be used in the *Lumbriculus* culture aquaria by covering the bottom area of the tank, or be stored frozen in deionised water for later use. New substrate in the tank will generally last for about two months.

Each worm culture is started with 500 - 1,000 worms, and fed a 10 mL suspension containing 6 g of trout starter food 3 times per week under renewal or flow-through conditions. Static or semi-static cultures should receive lower feeding rates to prevent bacterial and fungal growth. Food and paper substrate should be analysed for the chemicals to be used in bioaccumulation tests.

Under these conditions the number of individuals in the culture generally doubles in about 10 to 14 d.

Lumbriculus variegatus can be removed from the cultures e.g. by transferring substrate with a fine mesh net, or organisms using a fire polished wide mouth (about 5 mm diameter) glass pipette, to a separate beaker. If substrate is co-transferred to this beaker, the beaker containing worms and substrate is left overnight under flow-through conditions, which will remove the substrate from the beaker, while the worms remain at the bottom of the vessel. They can then be introduced to newly prepared culture tanks, or

processed further for the test as outlined in (1) and (7). Injuries or autotomy in the worms should be prevented, e.g. by using pipettes with fire polished edges, or stainless steel picks for handling these worms.

An issue to be regarded critically when using *L. variegatus* in sediment bioaccumulation tests is its reproduction mode (architomy or morphallaxis). This asexual reproduction results in two fragments, which do not feed for a certain period until the head or tail part is regenerated (e.g., (30)(30)). This means that in *L. variegatus* sediment and contaminant uptake via ingestion may not take place continuously as in tubificids, which do not reproduce by fragmentation.

Therefore, a synchronisation should be performed to minimise uncontrolled reproduction and regeneration, and subsequent high variation in test results. Such variation can occur, when some individuals, which have fragmented and therefore do not feed for a certain time period, are less exposed to the test substance than other individuals, which do not fragment during the test, e.g. (32). 10 to 14 days before the start of exposure, the worms should be artificially fragmented (synchronisation). Adult worms should be used, which preferably do not show signs of recent morphallaxis. These worms can be placed onto a glass slide in a drop of culture water, and dissected in the median body region with a scalpel. Care should be taken that the posterior ends are of similar size. The posterior ends should then be left to regenerate new heads in a culture vessel containing the same substrate as used in the culture and reconstituted water until the start of exposure. Regeneration of new heads is indicated when the synchronised worms are burrowing in the substrate (presence of regenerated heads may be confirmed by inspecting a representative subsample under a binocular microscope). The test organisms are thereafter expected to be in a similar physiological state. This means, that when reproduction by morphallaxis occurs in synchronised worms during the test, virtually all animals are expected to be equally exposed to the spiked sediment. Feeding of the synchronised worms should be done once as soon as the worms are starting to burrow in the substrate, or 7 d after dissection. The feeding regimen should be comparable to the regular cultures, but it may be advisable to feed the synchronised worms with the same food source as is to be used in the test. The worms should be held at test temperature, at $20 \pm 2^\circ\text{C}$. After regenerating, intact complete worms of similar size, which are actively swimming or crawling upon a gentle mechanical stimulus, should be used for the test. Injuries or autotomy in the worms should be prevented, e.g. by using pipettes with fire polished edges, or stainless steel picks for handling these worms.

When using *Lumbriculus variegatus* in the test, due to the specific reproduction mode of this species, an increase of the number of worms should occur during the test, if conditions are appropriate (7). A lack of reproduction in a bioaccumulation test with *L. variegatus* should be recorded, and considered when interpreting the test results.

***Branchiura sowerbyi* (BEDDARD), Tubificidae, Oligochaeta (not validated in ring test)**

Branchiura sowerbyi inhabits a variety of sediment types of reservoirs, lakes, ponds and rivers. However, they are more abundant in mud-clay sediments with high organic matter content. Furthermore, the worms are living in the sediment layer. Even the posterior end of the worms is usually burrowed. This species is easily identified from the gill filaments on their posterior part. The adults can reach a length of 9 - 11 cm and a wet weight of 40-50 mg. The worms have a high rate of reproduction, show population doubling times of less than 2 weeks and under the conditions of temperature and feeding described below (Aston et al., 1982, (57)). *B. sowerbyi* has been used both in toxicity and bioaccumulation studies (Marchese & Brinkhurst 1996, (40), Roghair et al. 1996, (60) respectively).

Culture methods

A summary of culture conditions for *Branchiura sowerbyi* is given below (provided by Mercedes R. Marchese, INALI, Argentina, and Carla J. Roghair, RIVM, The Netherlands).

No single technique for culturing the test organisms is required. The organisms can be cultured using uncontaminated, natural sediment (40). Practical experience showed that a medium consisting of natural sediment and sand improves the condition of the worms compared to pure natural sediment (41)(60). 3 L-beakers containing 1,500 mL sediment/water medium, consisting of 375 mL of natural uncontaminated sediment (about 10 % Total Organic Carbon; about 17% of the particles $\leq 63 \mu\text{m}$), 375 mL of clean sand (M32), and 750 mL of reconstituted or dechlorinated tap water can be used for the culture (40)(41)(60). Paper towels also can be used as a substrate for culturing, but population growth is lower than in natural sediment. In semi-static systems the water layer in the beaker is slowly aerated, and the overlying water should be renewed weekly.

Each beaker contains 25 young worms to start with. After two months the adult worms are picked out of the sediment with a pair of tweezers and are put in a new beaker with freshly made sediment/water medium. The old beaker also contains cocoons and young worms. Up to 400 young worms per beaker can be harvested in this way. Adults worms can be used for reproduction for at least one year.

The cultures should be maintained at a temperature of 21 to 25°C. Variation of temperature should be kept below $\pm 2^\circ\text{C}$. The time required for embryonic development from an egg being laid until the young leaves the cocoon is approximately three weeks at 25°C. The egg production obtained per surviving worm in *B. sowerbyi* was found to range from 6.36 (40) to 11.2 (39) in mud at 25°C. The number of eggs per cocoon ranges from 1.8 to 2.8 (57)(59) or up to 8 (58).

Dissolved oxygen, water hardness, temperature, and pH should be measured weekly. Fish food (e.g. TetraMin[®]) can be added as suspension two or three times per week ad libitum. The worms can also be fed with thawed lettuce.

A major advantage of this species is the high individual biomass (up to 40 - 50 mg wet weight per individual). Therefore this species may be used for testing bioaccumulation of non-radiolabelled test compounds. It can be exposed in the systems used for *T. tubifex* or *L. variegatus* with a single individual per replicate (12). Replication, however, should then be increased, unless larger test chambers are used (12).

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Attachment 2: Reference List of Milestones

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