PROPOSAL FOR A NEW GUIDELINE

for

OECD GUIDELINES FOR THE TESTING OF CHEMICALS

SEDIMENT-WATER *LUMBRICULUS* TOXICITY TEST USING SPIKED SEDIMENT

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INTRODUCTION

1. Sediment-ingesting endobenthic animals are subject to potentially high exposure to sediment bound substances and should therefore be given preferential attention, e.g. (1) (2) (3). Among these sediment-ingesters, the aquatic oligochaetes play an important role in the sediments of 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 (e.g. *Lumbriculus variegatus*) burrow in the sediment, and ingest sediment particles below the sediment surface. This ensures exposure of the test organisms to the test substance via all possible uptake routes (e.g. contact with, and ingestion of contaminated sediment particles, but also via porewater and overlying water).

2. This Test Guideline is designed to assess the effects of prolonged exposure of the endobenthic oligochaete *Lumbriculus variegatus* (Müller) to sediment-associating chemicals. It is based on existing sediment toxicity and bioaccumulation test protocols, e.g. (3)(4)(5)(6)(7)(8)(9)(10). The method is described for static test conditions. The exposure scenario used in this guideline is spiking of sediment with the test substance. Using spiked sediment is intended to simulate a sediment contaminated with the test compound.

3. Substances that need to be tested towards sediment-dwelling organisms usually persist in this compartment over long time periods. Sediment-dwelling organisms may be exposed via several routes. The relative importance of each exposure route, and the time taken for each to contribute to the overall toxic effects, is dependent on the physical-chemical properties of the chemical concerned. For strongly adsorbing substances (e.g. with log K_{ow} > 5) or for substances covalently binding to sediment, ingestion of contaminated food may be a significant exposure route. In order not to underestimate the toxicity of such substances, the food necessary for reproduction and growth of the test organisms is added to the sediment before application of the test substance (11). 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 conditions in particular laboratories and the varied characteristics of test substances.

4. The test method is aimed to determine effects of a test substance on the reproduction and the biomass of the test organisms. The measured biological parameters are the total number of surviving worms and the biomass (dry weight) at the end of the exposure. These data are analysed either by using a regression model in order to estimate the concentration that would cause an effect of x % (e.g. EC_{50}, EC_{25}, and EC_{10}), or by using statistical hypothesis testing to determine a NOEC/LOEC.

5. The OECD Guideline No. 218: "Sediment-water chironomid toxicity test using spiked sediment" (6) provided many essential and useful details for the performance of the presented sediment toxicity test method. Hence, this document serves as a basis on which modifications necessary for conducting sediment toxicity tests with *Lumbriculus variegatus* were worked out.

Further documents that are referred to are e.g. the ASTM Standard Guide for Determination of the Bioaccumulation of Sediment-Associated Contaminants by Benthic Invertebrates (3), the U.S. EPA Methods for Measuring the Toxicity and Bioaccumulation of Sediment-Associated Contaminants with Freshwater Invertebrates (7), and the ASTM Standard Guide for Collection, Storage, Characterization, and Manipulation of Sediments for Toxicological Testing and for selection of samplers used to collect benthic invertebrates (12). In addition, practical experience obtained during ring-testing the method ((42), ring-test report), and details from literature are major sources of information for drawing up this document.

PREREQUISITE AND GUIDANCE INFORMATION

6. 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 perform the test is provided in (13).

7. Before carrying out a test, 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.

8. The following additional information may be useful before starting the test:

- octanol-water partition coefficient, K_{ow};
- sediment-water partition coefficient, expressed as K_{oc};
- hydrolysis;
- phototransformation in water;
- vapour pressure;
- biodegradability;
- surface tension.

9. Information on certain characteristics of the sediment to be used, e.g. origin of sediment or its constituents, total organic carbon content (TOC), pH, particle size distribution (percent sand, silt, and clay), and - for natural sediments - nitrogen content, ammonia concentration of the pore water after the equilibration period, percent water content, and on micropollutants in the sediment should be acquired before the start of the test (7).

PRINCIPLE OF THE TEST

10. Adult worms of similar physiological (synchronised) state are exposed to a series of toxicant concentrations applied to the sediment phase of a sediment-water system. Artificial sediment and

reconstituted water should be used as media. Test vessels without the addition of the test substance serve as controls. The test substance is spiked into the sediment in bulk for each concentration level in order to minimise variability between replicates of each concentration level, and the test organisms are subsequently introduced into the test vessels in which the sediment and water concentrations have been equilibrated (see paragraph 30). The test animals are exposed to the sediment-water systems for a period of 28 days. In view of the low nutrient content of the artificial sediment, the sediment should be amended with a food source (see Annex 4) to ensure that the worms will grow and reproduce under control conditions. In this way it is ensured that the test animals are exposed through the water and sediment as well as by their food.

11. The preferred endpoint of this type of study is the EC_x (e.g. EC_{50}, EC_{25}, and EC_{10}; effect concentration, affecting x % of the test organisms) for reproduction and biomass, respectively, compared to the control. It should however be noted, that considering the high uncertainty of low EC_x (e.g. EC_{10}, EC_{25}) with extremely high 95%-confidence limits (e.g. (35)) and the statistical power calculated during hypothesis testing, the EC_{50} is regarded the most robust endpoint. In addition, the No Observed Effect Concentration (NOEC), and the Lowest Observed Effect Concentration (LOEC) may be calculated for biomass, and reproduction, if the test design and the data support these calculations.

REFERENCE SUBSTANCES

12. To assess health and sensitivity of the test organisms, reference toxicity tests may be conducted in regular intervals using a reference toxicant. Potassium chloride (KCl) or copper sulphate ($CuSO_4$) have been used as reference toxicants with *L. variegatus* (3)(7). 96 h-reference toxicity tests in water only may satisfactorily demonstrate the condition of the test animals (3)(7). Information on the toxicity of pentachlorophenol (PCP) in complete tests (28 d exposure to spiked sediment) are included in Annex 6, and in the report on the ring test of the method (42).

VALIDITY OF THE TEST

13. For a test to be valid, the following requirements should be fulfilled:

- A ring-test (42) has shown that for *Lumbriculus variegatus*, the average number of living worms per replicate in the controls should have increased by a factor of at least 1.8 at the end of exposure compared to the number of worms per replicate at the start of exposure.

- The pH of the overlying water should be between 6 and 9 at the start and at the end of the test.

- The oxygen concentration in the overlying water should not be below 60% of air saturation value (ASV) at test temperature at the end of the test.

DESCRIPTION OF THE METHOD

Test system

14. Static systems are recommended. Semi-static or flow-through systems with intermittent or continuous renewal of overlying water should only be used in exceptional cases as for instance if water quality specifications become inappropriate for the test organisms (e.g. dissolved oxygen levels fall too low, the concentration of excretory products rises too high or minerals leach from sediment and affect pH and/or water hardness). However, since regular renewal of overlying water is expected to affect chemical equilibrium (e.g. losses of test compound from the test system), other methods for ameliorating the quality of overlying water, such as aeration, will normally suffice and are therefore recommended.

Test vessels

15. The exposure should be conducted in glass beakers of e.g. 250 mL measuring 6 cm in diameter. Other suitable vessels may be used, but they should guarantee a suitable depth of overlying water and sediment. Each vessel should receive a layer of approximately 1.5 – 3 cm of formulated sediment. The ratio of the depth of the sediment layer to the depth of the overlying water should be 1:4. The vessels should be of suitable capacity in compliance with the loading rate, i.e. the number of test worms added per weight unit of sediment, (see also paragraph 40). Test vessels and other apparatus that will come into contact with the test substance should be made entirely of glass or other chemically inert material (e.g. Teflon or stainless steel).

Test species

16. The test species used in this type of study is the freshwater oligochaete *Lumbriculus variegatus* (Müller). This species is tolerant to a wide range of sediment types, and is widely used for sediment toxicity and bioaccumulation testing (e.g. (3), (5), (7), (9), (14), (15), (16), (17), (18), (19), (20), (21), (31), (39), (40), (41)). The origin of the test animals, the confirmation of species identity (e.g. (22)) as well as the culture conditions should be reported. Identification of species is not required prior to every test if the organisms come from an in-house culture.

Culturing of the test organisms

17. In order to have a sufficient number of worms for conducting sediment toxicity tests, it is useful to keep the worms in permanent laboratory culture. Guidance for laboratory culture methods for *Lumbriculus variegatus*, and sources of starter cultures are given in Annex 5. For details on culturing these species see references (3), (7), (19).

18. 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.

Apparatus

19. 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. Teflon®, stainless steel and/or glass should be used for any equipment having contact with the test media. For substances with high adsorption coefficients, silanised glass may be required. In these situations the equipment will have to be discarded after use.

Water

20. Reconstituted water according to OECD Guideline 203 (23) is recommended for use as overlying water in the tests; it can also be used for the laboratory cultures of the worms (see Annex 2 for preparation). If required, natural water may be used alternatively. The chosen water must be of a quality that will allow the growth and reproduction of the test species for the duration of the acclimation and test periods without showing any abnormal appearance or behaviour. *Lumbriculus variegatus* can survive, grow, and reproduce in this water (31), and maximum standardisation of test and culture conditions is provided. If a reconstituted water is used, the recipe should be reported, and the water should be characterised prior to use at least by pH and hardness (expressed as mg $CaCO_3$/L). Analysis of the water for micropollutants prior to use might provide useful information (see, e.g., Annex 3).

21. The pH of the overlying water should be in the range of 6.0 to 9.0 (see paragraph 13). If ammonia development is expected, it may be desirable to keep the pH between 6.0 and 8.0. For testing of e.g. weak organic acids, it may be advisable to adjust the pH by buffering the water to be used in the test, as described e.g. by (43). The total hardness should be between 90 and 400 mg $CaCO_3$ per liter for natural water. Annex 3 summarises additional criteria of an acceptable dilution water according to OECD Guideline No. 210 (26).

Sediment

22. Since uncontaminated natural sediments 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, a formulated sediment (also called reconstituted, artificial or synthetic sediment) should preferably be used. When using a formulated sediment, maximum standardisation as well as intra- and interlaboratory comparability of test conditions are provided.

23. The following formulated sediment is based on the artificial sediment according to (6) and (25). It is recommended for use in this test ((6)(10)(29)(30)(31)):

(a) 4-5 % (dry weight) peat: as close to pH 5.5 to 6.0 as possible; it is important to use peat in powder form, finely ground (particle size ≤ 0.5 mm), and only air-dried.
(b) 20 ± 1 % (dry weight) kaolin clay (kaolinite content preferably above 30 %).
(c) 75-76 % (dry weight) quartz sand (fine sand, grain size: ≤ 2 mm, but > 50 % of the particles should be in the range of 50-200 µm).
(d) Deionised water, 30–50 % of sediment dry weight, in addition to the dry sediment components.
(e) Calcium carbonate of chemically pure quality ($CaCO_3$) is added to adjust the pH of the final mixture of the sediment.
(f) The total organic carbon content (TOC) of the final mixture should be 2 % (± 0.5 %) of sediment dry weight and should be adjusted by the use of appropriate amounts of peat and sand, according to (a) and (c).
(g) Food, e.g. powdered leaves of Stinging Nettle (*Urtica* sp.), or a mixture of powdered leaves of *Urtica* sp. with alpha-cellulose (1 : 1), at 0.4 - 0.5 % of sediment d.w., in addition to the dry sediment components; for details see Annex 4.

24. The source of peat, kaolin clay, food material, and sand should be known. The OECD guideline 218 (6) lists alternative plant materials to be used as a source of nutrition: leaves of mulberry (*Morus alba*), white clover (*Trifolium repens*), spinach (*Spinacia oleracea*) or cereal leaves (e.g. *Cerophyl*®).

25. The chosen food source should be added prior to or during spiking the sediment with the test substance. The chosen food source should allow for at least acceptable reproduction in the controls. Analysis of the artificial sediment or its constituents for micropollutants prior to use might provide useful information. An example for the preparation of the formulated sediment is described in Annex 4. Mixing of dry constituents is also acceptable if it is demonstrated that after addition of overlying water a separation of sediment constituents (e.g. floating of peat particles) does not occur, and that the peat or the sediment is sufficiently conditioned (see also paragraph 26, and Annex 4). The artificial sediment should be characterised at least by origin of the constituents, grain size distribution (percent sand, silt, and clay), total organic carbon content (TOC), water content, and pH. Measurement of redox potential is optional.

26. If required, e.g. for specific testing purposes, natural sediments from unpolluted sites may also serve as test and/or culture sediment (3). However, if natural sediment is used, it should be characterised at least by origin (collection site), pH and ammonia of the pore water, total organic carbon content (TOC) and nitrogen content, particle size distribution (percent sand, silt, and clay), and percent water content (7), and it should be free from any contamination and other organisms that might compete with, or prey on the test organisms. Measurement of redox potential is optional. It is also 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. At the end of this conditioning period, the overlying water should be removed and discarded.

27. The sediment to be used must be of a quality that will allow the survival and reproduction of the control organisms for the duration of the exposure period without showing any abnormal appearance or behaviour. The control worms should burrow in the sediment, and they should ingest the sediment. Reproduction in the controls should at least be according to the validity criterion as described in paragraph 13. The presence or absence of fecal pellets on the sediment surface, which indicate sediment ingestion by the worms, should be recorded and can be helpful for the interpretation of the test results with respect to exposure pathways. Additional information on sediment ingestion can be obtained by using methods described in (16)(17)(32)(33), which specify sediment ingestion or particle selection in the test organisms.

28. Manipulation procedures for natural sediments prior to use in the laboratory are described in (3)(7)(12). The preparation and storage of the artificial sediment recommended to be used in the *Lumbriculus* test is described in Annex 4.

Application of the test substance

29. The test substance should be dissolved in a suitable organic solvent (e.g. acetone, n-hexane, cyclohexane) at a volume of as small as possible in order to prepare the stock solution. The stock solution should be diluted with the same solvent to prepare the test solutions. 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. Each of the test solutions is then mixed with quartz sand (e.g. 10 g of quartz sand per test vessel). In order to soak the quartz sand completely, a volume of 0.24 mL per g of sand has been found sufficient. Thereafter, the solvent must be evaporated completely, and the dry sand is mixed with the suitable amount of formulated sediment of the corresponding concentration level. 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). The major advantage of this procedure is that virtually no solvent is introduced to the sediment (7). Care should be taken to ensure that the test chemical added to sediment is thoroughly and evenly distributed within the sediment. If necessary, subsamples may be analysed to confirm the target concentrations in the sediment, and to determine degree of homogeneity. It may also be useful to analyse subsamples of the test solutions to confirm the target concentrations in the sediment. Since a solvent is used for coating the test substance on the quartz sand, a solvent control should be employed which is prepared with the same amount of the solvent as the test sediments. The method used for spiking, and the reasons for choosing a specific spiking procedure should be reported. Additional guidance on spiking procedures is given in Environment Canada (1995) (34).

30. Once the spiked sediment has been prepared and topped with the test water, it is desirable to allow partitioning of the test substance from the sediment to the aqueous phase (e.g. (3)(7)(9)). This should preferably be done under the conditions of temperature and aeration used in the test. Appropriate equilibration time is sediment and chemicals specific, and can be in the order of hours to days and in rare cases up to several weeks (4-5 weeks) (e.g. (19)(36)). As this would leave time for degradation of many chemicals, 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.

31. At the end of this equilibration period, the concentration of the test substance should be measured at least in the overlying water and the bulk sediment, at least at the highest concentration and a lower one (see paragraph 54 for sampling details). These analytical determinations of the test substance should allow for calculation of mass balance and expression of results based on measured initial concentrations. If intermediate measurements are made (e.g. on day 7 of exposure), and if the analytical method needs large samples which cannot be taken from test vessels without influencing the test system, analytical determinations should be performed on samples from additional test vessels of appropriate dimensions, that are treated in the same way (including the presence of test organisms) but not used for biological observations.

PERFORMANCE OF THE TEST

Preliminary test

32. If no information is available on the toxicity of the test substance towards *Lumbriculus variegatus*, it may be useful to conduct a preliminary experiment in order to determine the range of concentrations to be tested in the definitive test, and to optimise the test conditions of the definitive test. For this purpose a series of widely spaced concentrations of the test substance are used. The worms are exposed to each concentration of the test substance for a period (e.g. 28 d as in the definitive test) which allows estimation of appropriate test concentrations; no replicates are required. The behaviour of the worms, for example sediment avoidance, which may be caused by the test chemical and/or by the sediment, should be observed and recorded during a preliminary test. Concentrations higher than 1000 mg/kg sediment dry weight should not be tested in the preliminary test.

Definitive test

33. In the definitive test, at least five concentrations should be used and selected e.g. based on the result of the preliminary range-finding test (paragraph 32), and as described in paragraphs 36, 37, 38, and 39.

34. A control (for replication see paragraphs 37, 38, and 39) containing all constituents except for the test substance is run in addition to the test series. If any solubilising agent is used for application of the test substance, it should have no significant effect on the test organisms as revealed by an additional solvent-only control.

Test design

35. The test design relates to the selection of the number and spacing of the test concentrations, the number of vessels at each concentration and the number of worms added per vessel. Designs for EC_x estimation, for estimation of NOEC, and for conducting a limit test are described in paragraphs 36, 37, 38, and 39.

36. The effect concentration (e.g. EC_{50} EC_{25}, EC_{10}) and the concentration range, over which the effect of the test substance is of interest, should be bracketed by the concentrations included in the test. Extrapolating much below the lowest concentration affecting the test organisms or above the highest tested concentration should be done only in exceptional cases, and a full explanation should be given in the report.

37. If the EC_x is to be estimated, at least five concentrations and three replicates for each concentration should be tested; six replicates are recommended for the control or - if used - the solvent control in order to improve the estimation of control variability. In any case, it is advisable that sufficient test concentrations are used to allow a good model estimation. The factor between concentrations should not be greater than two (an exception can be made in cases when the concentration response curve has a shallow slope). The number of replicates at each treatment can be reduced if the number of test concentrations with responses in the range of 5 – 95% are increased. Increasing the number of replicates or reducing the size of the test concentration intervals tends to lead to narrower confidence intervals for the test.

38. If the LOEC/NOEC values are to be estimated, five test concentrations with at least four replicates (six replicates are recommended for the control or - if used - the solvent control in order to improve the estimation of control variability) should be used, and the factor between concentrations should not be greater than two. Some information on the statistical power found during hypothesis testing in the ring test of the method is given in Annex 6.

39. A limit test may be performed (using one test concentration and controls) if no effects are expected up to 1000 mg/kg sediment d.w. (e.g. from a preliminary range-finding test). The purpose of the limit test is to perform a test at a concentration sufficiently high to enable decision makers to exclude possible toxic effects of the substance, and the limit is set at a concentration which is not expected to appear in any situation. 1000 mg/kg (dry weight) is recommended. Usually, at least six replicates for both the treatment and controls are necessary. Some information on the statistical power found during hypothesis testing in the ring test of the method is given in Annex 6.

Exposure conditions

Test organisms

40. The test is conducted with at least 10 worms for each replicate used for determination of biological parameters. This number of worms corresponds to approximately 50 - 100 mg of wet

biomass. Assuming a dry content of 17.1% (37), this results in approximately 9 - 17 mg of dry biomass per vessel. U.S. EPA (2000 (7)) recommends to use a loading rate not exceeding 1 : 50 (dry biomass : TOC). For the formulated sediment described in paragraph 22, this corresponds to approximately 43 g sediment (dry weight) per 10 worms at a TOC content of 2.0% of dry sediment. In cases where more than 10 worms are used per vessel, the amount of sediment and overlying water should be adjusted accordingly.

41. In any one test, worms of similar size should be selected. They should all come from the same source, and should be animals of similar physiological state (see Annex 5). It is recommended that a sub-sample of the batch or stock of worms is weighed before the test in order to estimate the mean weight.

42. The worms to be used in a test are removed from the culture (see Annex 5 for details). Adult animals that do not show signs of recent fragmentation are transferred to glass dishes (e.g. petri dishes) containing clean water. They are subsequently synchronised as described in Annex 5. After regenerating for a period of 10 to 14 d, intact complete worms of similar size, which are actively swimming or crawling upon a gentle mechanical stimulus, should be used for the test. If the test conditions differ from the culture conditions (e.g. in temperature, light regime, and overlying water), an acclimation phase of e.g. 24 h at temperature, light regime, and using the same overlying water as in the test should be sufficient to adapt the worms to the test conditions. The adapted oligochaetes should be allocated randomly to the test vessels.

Feeding

43. Since food is added to the sediment prior to (or during) application of the test substance, the worms are not fed additionally during the test.

Light and temperature

44. The photoperiod in the culture and the test is usually 16 hours (3)(7). Light intensity should be kept low (e.g. 100-1000 lx) to imitate natural conditions at the sediment surface, and measured at least once during the exposure period. The temperature should be 20 °C ± 2°C throughout the test. On one given measuring date the difference of temperature between test vessels should not be higher than ±1°C. The test vessels should be placed in the test incubator or the test area in a randomised way, e.g. in order to minimise temperature induced bias of reproduction.

Aeration

45. The water body should be gently aerated (e.g. 2 bubbles per second) via a pasteur pipette positioned approx. 2 cm above the sediment surface. Care should be taken that the dissolved oxygen concentration does not fall below 60% of air saturation value (ASV). Air supply should be controlled and - if necessary - adjusted at least once daily on workdays.

Water quality measurements

46. The following water quality parameters should be measured in the overlying water:

- Temperature: at least in one test vessel of each concentration level and one test vessel of the controls once per week and at the start and the end of the exposure period; if possible, temperature in the surrounding medium (ambient air or water bath) may be recorded additionally e.g. in hourly intervals;
- Dissolved oxygen content: at least in one test vessel of each concentration level and one test vessel of the controls once per week and at the start and the end of the exposure period; expressed as mg/L and % ASV (air saturation value);
- Air supply: should be controlled at least once daily on workdays and - if necessary - adjusted;
- pH: at least in one test vessel of each concentration level and one test vessel of the controls once per week and at the start and the end of the exposure period;
- Total water hardness: at least in one replicates of the controls and one test vessel at the highest concentration at the start and the end of the exposure period; expressed as mg/L $CaCO_3$;
- Total ammonia content: at least in one replicate of the controls and in one test vessel of each concentration level at the start of the exposure period, and subsequently 3 x per week; expressed as mg/L NH_4^+ or NH_3 or total ammonia-N.

Biological parameters at the end of the test

47. The test vessels should be observed in order to assess visually any behavioural differences in the worms (e.g. sediment avoidance, fecal pellets visible on the sediment surface) compared with the controls. Observations should be recorded.

48. Each replicate is examined (additional vessels designated for chemical analyses may be excluded from examination). An appropriate method should be used to recover all worms from the test vessel. Care should be taken that all worms are recovered uninjured. One possible method is sieving the worms from the sediment. A stainless steel mesh of appropriate mesh size can be used. Most of the overlying water is carefully decanted, and the remaining sediment and water is agitated to result in a slurry, which can be passed through the sieve. Using a 500 µm mesh, most of the sediment particles will pass the sieve very quickly; however, sieving should be done quickly as well, in order to prevent the worms from crawling into or through the mesh. Using a 250 µm mesh will prevent the worms

from crawling into or through the mesh; however, care should be taken that as little as possible of the sediment particles is retained on the mesh. The sieved slurry of each replicate vessel may be passed through the sieve a second time in order to ensure that all worms are recovered. An alternative method could be warming of the sediment by placing the test vessels in a water bath at 50 - 60°C; the worms will leave the sediment and can be collected from the sediment surface by use of a fire-polished widemouth pipette. Another alternative method could be to produce a sediment slurry and pour this slurry onto a shallow pan of suitable size. From the shallow layer of slurry the worms can be picked up by a steel needle or watchmakers' tweezers (to be used rather like a fork than forceps to avoid injuring the worms) and transferred to clean water. After separating the worms from the sediment slurry, these are rinsed in test medium and counted.

49. Independently of the method used, laboratories should demonstrate that their personnel are able to recover an average of at least 90% of the organisms from whole sediment. For example, a certain number of test organisms could be added to control sediment or test sediments, and recovery could be determined after 1 h (7).

50. The total number of living and dead individuals per replicate should be recorded and assessed. The following groups of worms are considered to be dead:

a) there is no reaction after a gentle mechanical stimulus
b) there are signs of decomposition (in combination with "a")
c) number of missing worms

Additionally, the living worms can be assigned to one of three groups:

a) large complete worms (adults) without regenerated body regions
b) complete worms with regenerated, lighter-coloured body regions (i.e., with new posterior part, with new anterior part, or with both new posterior and anterior parts)
c) incomplete worms (i.e., recently fragmented worms with non-regenerated body regions)

These observations are not mandatory, but can be used for additional interpretation of the biological results.

51. Immediately after counting/assessment, the worms found in each replicate are transferred to dried, pre-weighed and labelled weigh pans (one per replicate), and killed using a drop of ethanol per weigh pan. The weigh pans are placed in a drying oven at 100 ± 5°C to dry overnight, after which they are weighed after cooling in a desiccator, and worm dry weight is determined (preferably in g, at least 4 post-decimal digits).

52. In addition to the total dry weight, the ash-free dry weight may be determined as described in (38) in order to account for inorganic components originating from ingested sediment present in the alimentary tract of the worms.

53. The biomass is determined as total biomass per replicate including adult and young worms. Dead worms should not be taken into account for the determination of biomass per replicate.

Sampling for chemical analysis

Sampling Schedule

54. Samples for chemical analysis of the test compound should be taken at least of the highest concentration and a lower one, at least at the end of the equilibration phase (before adding the test organisms), and at the end of the test. At least the sediment and the overlying water should be sampled for analysis.

55. The overlying water should be sampled by carefully decanting or siphoning off the overlying water so as to minimise perturbation of the sediment. The volume of the samples should be recorded.

56. After the overlying water has been removed, the sediment should be homogenised and transferred to a suitable container. The weight of the wet sediment sample is recorded.

57. If analysis of the test substance in the pore water is required additionally, the homogenised and weighed sediment samples should be centrifuged to obtain the pore water. For example, approximately 200 mL of wet sediment can be filled into 250 mL centrifugation beakers. Thereafter the samples should be centrifuged, e.g. at 10000 ± 600 x *g* for 30 - 60 min at a temperature not exceeding the temperature used in the test without filtration to isolate the porewater. After centrifugation, the supernatant is decanted, and the volume recorded. The weight of the remaining sediment pellet is recorded. It may facilitate the estimation of the mass balance or recovery of the test substance in the water-sediment system, if the sediment dry weight is determined at each sampling date. In some cases it might not be possible to analyse concentrations in the pore water as the sample size is too small.

58. Failing immediate analysis, all samples should be stored by an appropriate method, e.g. under the conditions recommended for the particular test substance. Obtain information on the proper storage conditions for the particular test substance - for example, duration and temperature of storage, extraction procedures, etc. - before beginning the study.

Analytical method

59. 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 and sediment samples are satisfactory for the particular method. Also, check that the test substance is not detectable in the control chambers in concentrations higher than the limit of quantitation. If necessary, correct the nominal concentrations for the recoveries of controls. 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 substance on the sampling device).

60. The recovery of test substance, the limit of quantitation, and the limit of detection in sediment and water should be recorded and reported.

DATA AND REPORTING

Treatment of results

61. The main parameters to be evaluated statistically are the biomass and the total number of worms per replicate. Additionally, reproduction (as increase of worm numbers) and growth (as increase of dry biomass) should be evaluated statistically. In this case, an estimate of the dry weight of the worms at start of exposure should be obtained e.g. by measurement of the dry weight of a representative subsample of the batch of synchronised worms to be used for the test.

62. Additionally, mortalities should be evaluated as far as possible. In order to estimate mortalities, the number of worms that do not react to a gentle mechanical stimulus or showed signs of decomposition should be considered dead. Mortalities should at least be recorded and considered when interpreting the test results.

EC_x

63. EC_x-values for the parameters described in paragraph 61 are calculated using appropriate statistical methods (e.g. probit analysis, logistic or Weibull function, trimmed Spearman-Karber method, or simple interpolation). An EC_x is obtained by inserting a value corresponding to x% of the control mean into the equation found. To compute the EC_{50} or any other EC_x, the per-treatment means ($\overline{X}$) should be subjected to regression analysis.

NOEC/LOEC

64. If a statistical analysis is intended to determine the NOEC/LOEC, per-vessel statistics (individual vessels are considered replicates) are necessary. Appropriate statistical methods should be used. In general, adverse effects of the test item compared to the control are investigated using one-tailed (smaller) hypothesis testing at $p \leq 0.05$. Examples are given in the following paragraphs.

65. Normal distribution of data can be tested e.g. with the Kolmogorov-Smirnov goodness-of-fit test, the Range-to-standard-deviation ratio test (R/s-test) or the Shapiro-Wilk test, (two-sided, $p \leq 0.05$). Cochran's test, Levene test or Bartlett's test, (two-sided, $p \leq 0.05$) may be used to test variance homogeneity. If the prerequisites of parametric test procedures (normality, variance homogeneity) are fulfilled, One-way Analysis of Variance (ANOVA) and subsequent multi-comparison tests can be performed. Pairwise comparisons (e.g. Dunnett's t-test) or step-down trend tests (e.g. Williams' test) can be used to calculate whether there are significant differences ($p \leq 0.05$) between the controls and the various test item concentrations. Otherwise, non-parametric methods (e.g. Bonferroni-U-test according to Holm or Jonckheere-Terpstra trend test) should be used to determine the NOEC and the LOEC.

Limit test

66. If a limit test (comparison of control and one treatment only) has been performed and the prerequisites of parametric test procedures (normality, homogeneity) are fulfilled, metric responses (total worm number, and biomass as worm dry weight) can be evaluated by the Student test (t-test). The unequal-variance t-test (Welch t-test) or a non parametric test, such as the Mann-Whitney-U-test may be used, if these requirements are not fulfilled. Some information on the statistical power found during hypothesis testing in the ring test of the method is given in Annex 6.

67. To determine significant differences between the controls (control and solvent control), the replicates of each control can be tested as described for the limit test. If these tests do not detect significant differences, all control and solvent control replicates may be pooled. Otherwise all treatments should be compared with the solvent control.

68. Effect concentrations should be expressed in mg/kg sediment dry weight. If the recovery of test compound measured in the sediment, or in sediment and overlying water at start of exposure, is between 80 and 100% of the nominal concentrations, the effect concentrations (EC_x, NOEC, LOEC) may be expressed based on nominal concentrations. If recovery deviates from the nominal concentrations by more than ± 20% of the nominal concentrations, the effect concentrations (EC_x, NOEC, LOEC) should be based on the initially measured concentrations at the beginning of the exposure, e.g. taking into account the mass balance of the test compound in the test system (see paragraph 31). In cases where the measured concentrations deviate from the nominal concentrations by more than ± 20% of the nominal values, additional information can be obtained from analysis of stock and/or application solutions in order to confirm that the test sediments were prepared correctly.

Interpretation of results

69. The results should be interpreted with caution if there were deviations from this guideline, and where measured concentrations of test concentrations occur at levels close to the detection limit of the analytical method used.

Test report

70. The test report should include at least the following information:

Test substance:

- chemical identification data (common name, chemical name, structural formula, CAS number, etc.) including purity and analytical method for quantification of test substance; source of the test substance, identity and concentration of any solvent used.

- any information available on the physical nature and physical-chemical properties as obtained prior to start of the test, (e.g. water solubility, vapour pressure, partition coefficient in soil (or in sediment if available), log K_{ow}, stability in water, etc.);

Test species:

- scientific name, source, any pre-treatment, acclimation, culture conditions, etc..

Test conditions:

- test procedure used (e.g. static, semi-static or flow-through);

- 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 at start of exposure, number of test concentrations, length of conditioning, equilibration and exposure periods, sampling frequency);

- method of test substance pre-treatment and spiking/application;

- the nominal test concentrations, details about the sampling for chemical analysis, and the analytical methods by which concentrations of the test substance were obtained;

- sediment characteristics as described in paragraphs 25 - 26, and any other measurements made; preparation of formulated sediment;

- preparation of the test water (if reconstituted water is used) and characteristics (oxygen concentration, pH, conductivity, hardness, and any other measurements made) at the start of the test;

- detailed information on feeding including type of food, preparation, amount and feeding regimen;

- light intensity and photoperiod(s);

– depth of sediment and overlying water;

– methods used for determination of all biological parameters (e.g. sampling, inspection, weighing of test organisms) and all abiotic parameters (e.g. water and sediment quality parameters);

– volumes and/or weights of all samples for chemical analysis;

– detailed information on the treatment of all samples for chemical analysis, including details of preparation, storage, spiking procedures, extraction, and analytical procedures (and precision) for the test substance, and recoveries of the test substance.

Results:

– water quality within the test vessels (pH, temperature, dissolved oxygen concentration, hardness, ammonia concentrations, and any other measurements made);

– total organic carbon content (TOC), dry weight to wet weight ratio, pH of the sediment, and any other measurements made;

– total number, and number of complete and incomplete worms in each test chamber at the end of the test;

– dry weight of the worms of each test chamber at the end of the test, dry weight of a subsample of the worms at start of the test;

– any observed abnormal behaviour in comparison to the controls (e.g., sediment avoidance, presence or absence of fecal pellets);

– any observed mortalities;

– estimates of toxic endpoints (e.g. EC_x, NOEC and/or LOEC), and the statistical methods used for their determination;

– the nominal test concentrations, the measured test concentrations and the results of all analyses made to determine the concentration of the test substance in the test vessels.

Evaluation of results:

– compliance of the results with the validity criteria as listed in paragraph 13;

– discussion of the results, including any influence on the outcome of the test resulting from deviations from this guideline.

ANNEX 1

DEFINITIONS AND UNITS

For the purpose of this guideline the following definitions are used:

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}; also sometimes expressed as P_{ow}) is the ratio of the solubility of a substance in n-octanol and water at equilibrium and represents the lipophilicity of a substance (OECD Guideline 117). The K_{ow} or its logarithm of K_{ow} (log K_{ow}) is used as an indication of the potential of a substance 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 exposure phase is the time during which the test organisms are exposed to the test substance.

Formulated sediment or reconstituted, artificial 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 placed over sediment in the test vessel.

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

The EC_x is the concentration of the test substance in the sediment that results in a x % (e.g. 50%) effect on a biological parameter within a stated exposure period.

The Lowest Observed Effect Concentration (LOEC) is the lowest tested concentration of a test substance at which the substance is observed to have a significant toxic effect (at $p \leq 0.05$) when compared with the control. However, all test concentrations above the LOEC must have an effect equal to or greater than those observed at the LOEC. If these two conditions cannot be satisfied, a full explanation must be given for how the LOEC (and hence the NOEC) has been selected.

The No Observed Effect Concentration (NOEC) is the test concentration immediately below the LOEC which, when compared with the control, has no statistically significant effect ($p \leq 0.05$), within a given exposure period.

MDD: minimum detectable difference from the control values during hypothesis testing; used as a measure of statistical power.

ANNEX 2

COMPOSITION OF THE RECOMMENDED RECONSTITUTED WATER

(adopted from (23))

(a) Calcium chloride solution
Dissolve 11.76 g $CaCl_2$ x 2 H_2O in deionised water; make up to 1 L with deionised water

(b) Magnesium sulphate solution
Dissolve 4.93 g $MgSO_4$ x 7 H_2O 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 μScm^{-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 3

SOME PHYSICAL-CHEMICAL CHARACTERISTICS OF AN ACCEPTABLE DILUTION WATER

SUBSTANCE	CONCENTRATIONS
Particular matter	< 20 mg/L
Total organic carbon	< 2 µg/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

(adopted from (26))

ANNEX 4

RECOMMENDED ARTIFICIAL SEDIMENT – GUIDANCE ON PREPARATION AND STORAGE

Sediment constituents

Constituent	Characteristics	% of sediment dry weight
Peat	Sphagnum moss peat, air dried, no visible plant remains, finely ground (particle size ≤ 0.5 mm)	5 ± 0.5
Quartz sand	Grain size: ≤ 2 mm, but > 50% of the particles should be in the range of 50-200 µm	75 - 76
Kaolinite clay	Kaolinite content ≥ 30%	20 ± 1
Urtica powder	*Folia urticae*, leaves of *Urtica dioica* (stinging nettle), finely ground (particle size ≤ 0.5 mm); in accordance with pharmacy standards, for human consumption; in addition to dry sediment	0.4 - 0.5%
Organic carbon	Adjusted by addition of peat and sand	2 ± 0.5
Calcium carbonate	$CaCO_3$, pulverised, chemically pure, in addition to dry sediment	0.05 - 1
Deionised Water	Conductivity ≤ 10 µS/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. A suspension of the required amount of peat powder in deionised water is prepared 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. pH is measured again and should be 6.0 ± 0.5. Then the peat suspension is mixed with the other constituents (sand and kaolin clay) and deionised water to obtain an homogeneous sediment with a water content in a range of 30–50 per cent of dry weight of the sediment. The pH

of the final mixture 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 formulated 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. 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. 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 spiked 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.

Storage

The dry constituents of the artificial sediment may be stored in a dry, cool place at room temperature. The prepared sediment spiked with the test substance should be used in the test immediately. Samples of spiked sediment may be stored under the conditions recommended for the particular test substance until analysis.

ANNEX 5

CULTURE METHODS FOR *LUMBRICULUS VARIEGATUS*

Lumbriculus variegatus (MÜLLER), Lumbriculidae, Oligochaeta

Lumbriculus variegatus (Lumbriculidae, Oligochaeta) is an inhabitant of freshwater sediments and is widely used in ecotoxicological testing. It can easily be cultured under laboratory conditions. An outline of culture methods is given in the following.

Culture methods

Culture conditions for *Lumbriculus variegatus* are outlined in detail in Phipps et al. (1993) (5), Brunson et al. (1998) (19), ASTM (2000) (3), U.S. EPA (2000) (7). A short summary of these conditions is given below. A major advantage of *L. variegatus* is its quick reproduction, resulting in rapidly increasing biomass in laboratory cultured populations (e.g. (3)(5)(7)(15)).

The worms can be cultured in large aquaria (57 - 80 L) at 23°C with a 16 L:8 D photoperiod (100 - 1000 lx) 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 approximately two months.

Each worm culture is started with 500 - 1000 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. .

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

Alternatively *Lumbriculus variegatus* can also be cultured in a system consisting of a layer of quartz sand as used for the artificial sediment (1 - 2 cm depth), and reconstituted water. Glass or stainless steel containers with a height of 12 to 20 cm can be used as culture vessels. The water body should be gently aerated (e.g. 2 bubbles per second) via a pasteur pipette positioned approx. 2 cm above the sediment surface. To avoid accumulation e.g. of ammonia, the overlying water should be exchanged using a flow-through system, or, at least once a week, manually. The oligochaetes can be held at room temperature with a photo period of 16 hours light (intensity 100 - 1000 lx) and 8 hours dark. In the semi-static culture (water renewal once per week), the worms are fed with TetraMin twice a week (e.g. 0.6 - 0.8 mg per cm^2 of sediment surface), which can be applied as a suspension of 50 mg TetraMin per mL H_2O demin..

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 (approximately 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 (3) and (7), or in the following.

An issue to be regarded critically when using *L. variegatus* in sediment 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., (16)(17)). This means that in *L. variegatus* exposure via ingestion of contaminated sediment does not take place continuously.

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 (39), (40), (41). 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 ± 2°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 dental picks for handling these worms.

Sources of starter cultures for *Lumbriculus variegatus* (addresses in the U.S. adopted from (7))

Europe

ECT Oekotoxikologie GmbH
Böttgerstr. 2-14
D-65439 Flörsheim/Main
Germany

Bayer Crop Science AG
Development – Ecotoxicology
Alfred-Nobel-Str. 50
D-40789 Monheim
Germany

University of Joensuu
Laboratory of Aquatic Toxicology
Dept. of Biology
Yliopistokatu 7, P.O. Box 111
FIN-80101 Joensuu
Finland

Dresden University of Technology
Institut für Hydrobiologie
Fakultät für Forst-, Geo- und Hydrowissenschaften
Mommsenstr. 13
D-01062 Dresden
Germany

C.N.R.- I.R.S.A.
Italian National Research Council
Water Research Institute
Via Mornera 25
I-20047 Brugherio MI

U.S.A.

U.S. Environmental Protection Agency
Mid-Continent Ecological Division
6201 Congdon Boulevard
Duluth, MN 55804

Michigan State University
Department of Fisheries and Wildlife
No. 13 Natural Resources Building
East Lansing, MI 48824-1222

U.S. Environmental Protection Agency
Environmental Monitoring System Laboratory
26 W. Martin Luther Dr.
Cincinnati, OH 45244

Wright State University
Institute for Environmental Quality
Dayton, OH 45435

Columbia Environmental Research Center
U.S. Geological Survey
4200 New Haven Road
Columbia, MO 65201

Great Lakes Environmental Research
Laboratory, NOAA
2205 Commonwealth Boulevard
Ann Arbor, MI 48105-1593

ANNEX 6
SUMMARY OF THE RING TEST RESULTS
"SEDIMENT TOXICITY TEST WITH *LUMBRICULUS VARIEGATUS*"

Table 1: Results of individual ring test runs: Mean worm numbers in the controls and solvent controls at the end of the test; SD = standard deviation; CV = coefficient of variation.

	mean worm number in the controls	**SD**	**CV (%)**	**n**	**mean worm number in the solvent controls**	**SD**	**CV (%)**	**n**
	32.3	7.37	22.80	3	**39.0**	3.61	9.25	3
	40.8	6.55	16.05	6	**36.0**	5.29	14.70	3
	41.5	3.54	8.52	2	**38.5**	7.05	18.31	4
	16.3	5.99	36.67	6	**30.8**	6.70	21.80	4
	24.3	10.69	43.94	3	**26.3**	3.06	11.60	3
	28.5	8.29	29.08	4	**30.7**	1.15	3.77	3
	28.3	3.72	13.14	6	**28.8**	2.56	8.89	6
	25.3	5.51	21.74	3	**27.7**	1.53	5.52	3
	23.8	2.99	12.57	4	**21.3**	1.71	8.04	4
	36.8	8.80	23.88	6	**35.0**	4.20	11.99	6
	33.0	3.58	10.84	6	**33.5**	1.73	5.17	4
	20.7	2.73	13.22	6	**15.0**	6.68	44.56	4
	42.0	7.07	16.84	6	**43.7**	0.58	1.32	3
	18.2	3.60	19.82	6	**21.7**	4.04	18.65	3
	32.0	3.95	12.34	6	**31.3**	4.79	15.32	4
interlaboratory								
mean	**29.59**		**20.10**		**30.61**		**13.26**	
SD	**8.32**		**10.03**		**7.57**		**10.48**	
n	**15**				**15**			
min	**16.3**				**15.0**			
max	**42.0**				**43.7**			
CV (%)	**28.1**				**24.7**			

Table 2: Results of individual ring test runs: Mean total dry weights of worms per replicate in the controls and solvent controls at the end of the test; SD = standard deviation; CV = coeff. of variation.

	total dry weight of worms per replicate (controls)	**SD**	**CV (%)**	**n**	**total dry weight of worms per replicate (solvent controls)**	**SD**	**CV (%)**	**n**
	24.72	6.31	25.51	3	**27.35**	4.08	14.93	3
	30.17	2.04	6.75	6	**33.83**	10.40	30.73	3
	23.65	3.61	15.25	2	**28.78**	4.68	16.28	4
	12.92	6.83	52.91	6	**24.90**	6.84	27.47	4
	21.31	4.17	19.57	3	**25.87**	5.30	20.49	3
	22.99	4.86	21.16	4	**24.64**	5.09	20.67	3
	18.91	1.91	10.09	6	**19.89**	1.77	8.89	6
	24.13	1.63	6.75	3	**25.83**	2.17	8.41	3
	22.15	3.18	14.34	4	**22.80**	2.60	11.40	4
	35.20	8.12	23.07	6	**31.42**	8.45	26.90	6
	41.28	5.79	14.02	6	**41.42**	4.37	10.55	4
	15.17	5.78	38.09	6	**10.50**	3.42	32.53	4
	35.69	8.55	23.94	6	**38.22**	1.23	3.21	3
	19.57	5.21	26.65	6	**28.58**	6.23	21.81	3
	29.40	2.16	7.34	6	**31.15**	2.70	8.67	4
interlaboratory								
mean	**25.15**		**20.36**		**27.68**		**17.53**	
SD	**7.87**		**12.56**		**7.41**		**9.10**	
n	**15**				**15**			
min	**12.9**				**10.5**			
max	**41.3**				**41.4**			
CV (%)	**31.3**				**26.8**			

Table 3: Toxicity of PCP: Summary of endpoints in the ring test; interlaboratory means for EC_{50}, NOEC and LOEC; SD = standard deviation; CV = coefficient of variation.

biological parameter		interlaboratory mean (mg/kg)	min	max	interlaboratory factor	SD	CV (%)	geometr. mean (mg/kg)
total number of worms	**EC_{50}**	**23.0**	4.0	37.9	9.4	10.7	46.3	19.9
	NOEC	**9.9**	2.1	22.7	10.7	7.2	72.3	7.6
	LOEC	**27.9**	4.7	66.7	14.2	19.4	69.4	20.9
	MDD (%)	**22.5**	7.1	39.1				
total dry weight of worms	**EC_{50}**	**20.4**	7.3	39.9	5.5	9.1	44.5	18.2
	NOEC	**9.3**	2.1	20.0	9.4	6.6	70.4	7.4
	LOEC	**25.7**	2.1	50.0	23.5	16.8	65.5	19.4
	MDD (%)	**24.8**	10.9	44.7				
mortality/survival	**LC_{50}**	**25.3**	6.5	37.2	5.7	9.4	37.4	23.1
	NOEC	**16.5**	2.1	40.0	18.8	10.3	62.4	12.8
	LOEC	**39.1**	4.7	66.7	14.2	18.1	46.2	32.6
reproduction (increase of number of worms per replicate)	**EC_{50}**	**20.0**	6.7	28.9	4.3	7.6	37.9	18.3
	NOEC	**7.9**	2.1	20.0	9.4	5.2	66.0	6.4
	LOEC	**22.5**	2.1	50.0	23.5	15.4	68.6	16.0
	MDD (%)	**29.7**	13.9	47.9				
growth (biomass increase per replicate)	**EC_{50}**	**15.3**	5.7	29.9	5.2	7.1	46.5	13.7
	NOEC	**8.7**	2.1	20.0	9.4	6.0	68.1	6.9
	LOEC	**24.0**	2.1	50.0	23.5	15.7	65.5	17.3
	MDD (%)	**32.2**	13.6	65.2				

MDD: minimum detectable difference from the control values during hypothesis testing; used as a measure of statistical power

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