Council of the European Union

Brussels, 22 July 2015
(OR. en)

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COVER NOTE

From:	European Commission
date of receipt:	10 July 2015
To:	General Secretariat of the Council
Subject:	COMMISSION REGULATION (EU) No …/.. of XXX amending, for the purpose of its adaptation to technical progress, Regulation (EC) No 440/2008 laying down test methods pursuant to Regulation (EC) No 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) (Text with EEA relevance)

Delegations will find attached document D39048/03.

Encl.: D39048/03

EN

ANNEX

The Annex to Regulation (EC) No 440/2008 is amended as follows:

(1)

A note is inserted at the beginning of the Annex, before Part A:

"Note:

Before using any of the following test methods to test a multi-constituent substance (MCS), a substance of unknown or variable composition, complex reaction product or biological material (UVCB), or a mixture and where its applicability for the testing of MCS, UVCB, or mixtures is not indicated in the respective test method, it should be considered whether the method is adequate for the intended regulatory purpose.

If the test method is used for the testing of a MCS, UVCB or mixture, sufficient information on its composition should be made available, as far as possible, e.g. by the chemical identity of its constituents, their quantitative occurrence, and relevant properties of the constituents."

(2) Chapter A.24 is added:

"A.24. Partition Coefficient (n-octanol/water), High PerformanceLiquid Chromatography (HPLC) Method

INTRODUCTION

This test method is equivalent to OECD test guideline (TG) 117 (2004)

1. The partition coefficient (P) is defined as the ratio of the equilibrium concentrations of a dissolved substance in a two-phase system consisting of two largely immiscible solvents. In the case of n-octanol and water,

$$P_{ow} = \frac{C_{n-octanol}}{C_{water}}$$

The partition coefficient being the quotient of two concentrations, is dimensionless and is

usually given in the form of its logarithm to base ten.

2. P_{ow} is a key parameter in studies of the environmental fate of chemical substances. A highly-significant relationship between the P_{ow} of non-ionised form of substances and their bioaccumulation in fish has been shown. It has also been shown that P_{ow} is a useful parameter in the prediction of adsorption on soil and sediments and for establishing quantitative structure-activity relationships for a wide range of biological effects.

3. The original proposal for this test method was based on an article by C.V. Eadsforth and P. Moser (1). The development of the test method and an OECD inter-laboratory comparison test were coordinated by the Umweltbundesamt of the Federal Republic of Germany during 1986 (2).

INITIAL CONSIDERATIONS

4. log P_{ow} values in the range -2 to 4 (occasionally up to 5 and more)[1] can be experimentally determined by the Shake-Flask method (Chapter A.8 of this Annex, OECD Test Guideline 107). The HPLC method covers log P_{ow} in the range of 0 to 6 (1)(2)(3)(4)(5). This method may require an estimation of P_{ow} to assign suitable reference substances and support any conclusions drawn from the data generated by the test. Calculation methods are briefly discussed in the Appendix to this test method. The HPLC operation mode is isocratic.

5. The P_{ow} values depend on the environmental conditions such as temperature, pH, ionic strength etc, and these should be defined in the experiment for the correct interpretation of P_{ow} data. For ionisable substances, another method (e.g. draft OECD guideline on pH metric method for ionised substances (6)) may become available and could be used as an alternative method. Although this draft OECD guideline may appropriate be suitable to determine P_{ow} for those ionisable substances, in some cases it is more appropriate to use the HPLC method at an environmentally relevant pH (see paragraph 9).

PRINCIPLE OF THE METHOD

6. Reverse phase HPLC is performed on analytical columns packed with a commercially available solid phase containing long hydrocarbon chains (e.g. C8, C18) chemically bound onto silica.

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[1] An upper limit is given by the necessity to achieve a complete separation phase after adjustments of the partitition equilibrium and before samples are taken out for analytical determinations. If proper care is taken, the upper limit can be extended to higher values of P_{ow}

7. A chemical injected on such a column partitions between the mobile solvent phase and the hydrocarbon stationary phase as it is transported along the column by the mobile phase. The substances are retained in proportion to their hydrocarbon-water partition coefficient, with hydrophilic substances eluted first and lipophilic substances last. The retention time is described by the capacity factor k given by the expression:

$$k = \frac{t_R - t_0}{t_0}$$

where t_R is the retention time of the test substance, and t_0 is the dead-time, i.e. the average time a solvent molecule needs to pass the column. Quantitative analytical methods are not required and only the determination of retention times is necessary.

8. The octanol/water partition coefficient of a test substance can be computed by experimentally determining its capacity factor k and then inputting k into the following equation:

$$\log P_{ow} = a + b \times \log k$$

where

a, b = linear regression coefficients.

The equation above can be obtained by linearly regressing the log of octanol/water partition coefficients of reference substances against the log of capacity factors of the reference substances.

9. Reverse phase HPLC method enables partition coefficients to be estimated in the log P_{ow} range between 0 and 6, but can be expanded to cover the log P_{ow} range between 6 and 10 in exceptional cases. This may require that the mobile phase is modified (3). The method is not applicable to strong acids and bases, metal complexes, substances which react with the eluent, or surface-active agents. Measurements can be performed on ionisable substances in their non-ionised form (free acid or free base) only by using an appropriate buffer with a pH below the pK_a for a free acid or above the pK_a for a free base. Alternatively, the pH-metric method for the testing of ionisable substances (6) may become available and could be used as an alternative method (6). If the log P_{ow} value is determined for the use in environmental hazard classification or in environmental risk assessment, the test should be performed in the pH range relevant for the natural environment, i.e. in the pH range of 5.0 - 9.

10. In some cases impurities can make the interpretation of the results difficult due to uncertainty in peak assignments. For mixtures which result in an unresolved band, upper and lower limits of log P_{ow}, and the area % of each log P_{ow} peak should be reported. For

mixtures which are a group of homologues, the weighted average log P_{ow} should also be stated (7), calculated based on the single P_{ow} values and the corresponding area % values (8). All peaks that contribute an area of 5% or more to the total peak area should be taken into consideration in the calculation (9):

$$weighted\ average \log P_{ow} = \frac{\sum_i (\log P_{owi})(area\ \%)}{total\ peak\ area\ \%} = \frac{\sum (logP_{owi})(area\ \%_i)}{\sum_i area\ \%}$$

The weighed average log P_{ow} is valid only for substances or mixtures (e.g. tall oils) consisting of homologues (e.g. series of alkanes). Mixtures can be measured with meaningful results, provided that the analytical detector used has the same sensitivity towards all the substances in the mixture and that they can be adequately resolved.

INFORMATION ON THE TEST SUBSTANCE

11. The dissociation constant, structural formula, and solubility in the mobile phase should be known before the method is used. In addition, information on hydrolysis would be helpful.

QUALITY CRITERIA

12. In order to increase the confidence in the measurement, duplicate determinations must be made.

    - Repeatability: The value of log P_{ow} derived from repeated measurements made under identical conditions and using the same set of reference substances should fall within a range of $\pm$ 0.1 log units.

    - Reproducibility: If the measurements are repeated with a different set of reference substances, results may differ. Typically, the correlation coefficient R for the relationship between log k and log P_{ow} for a set of test substances is around 0.9, corresponding to an octanol/water partition coefficient of log P_{ow} $\pm$ 0.5 log units.

13. The inter-laboratory comparison test has shown that with the HPLC method log P_{ow} values can be obtained to within $\pm$ 0.5 units of the Shake-Flask values (2). Other comparisons can be found in the literature (4)(5)(10)(11)(12). Correlation graphs based on structurally related reference substances give the most accurate results (13).

REFERENCE SUBSTANCES

14. In order to correlate the measured capacity factor k of a substance with its P_{ow}, a calibration graph using at least 6 points has to be established (see paragraph 24). It is up to the user to select the appropriate reference substances. The reference substances should normally have log P_{ow} values which encompass the log P_{ow} of the test substance, i.e. at least one reference substance should have a P_{ow} above that of the test substance, and another a P_{ow} below that of the test substance. Extrapolation should only be used in exceptional cases. It is preferable that these reference substances should be structurally related to the test substance. log P_{ow} values of the reference substances used for the calibration should be based on reliable experimental data. However, for substances with high log P_{ow} (normally more than 4), calculated values may be used unless reliable experimental data are available. If extrapolated values are used a limit value should be quoted.

15. Extensive lists of log P_{ow} values for many groups of chemicals are available (14)(15). If data on the partition coefficients of structurally related substances are not available, a more general calibration, established with other reference substances, may be used. Recommended reference substances and their P_{ow} values are listed in Table 1. For ionisable substances the values given apply to the non-ionised form. The values were checked for plausibility and quality during the inter-laboratory comparison test.

Table 1: Recommended reference substances

	CAS Number	Reference substance	log P_{ow}	pKa
1	78-93-3	2-Butanone (Methylethylketone)	0.3	
2	1122-54-9	4-Acetylpyridine	0.5	
3	62-53-3	Aniline	0.9	
4	103-84-4	Acetanilide	1.0	
5	100-51-6	Benzyl alcohol	1.1	
6	150-76-5	4-Methoxyphenol	1.3	pKa = 10.26
7	122-59-8	Phenoxyacetic acid	1.4	pKa = 3.12
8	108-95-2	Phenol	1.5	pKa = 9.92
9	51-28-5	2,4-Dinitrophenol	1.5	pKa = 3.96
10	100-47-0	Benzonitrile	1.6	
11	140-29-4	Phenylacetonitrile	1.6	
12	589-18-4	4-Methylbenzyl alcohol	1.6	
13	98-86-2	Acetophenone	1.7	
14	88-75-5	2-Nitrophenol	1.8	pKa = 7.17
15	121-92-6	3-Nitrobenzoic acid	1.8	pKa = 3.47
16	106-47-8	4-Chloroaniline	1.8	pKa = 4.15
17	98-95-3	Nitrobenzene	1.9	
18	104-54-1	Cinnamyl alcohol (Cinnamic alcohol)	1.9	
19	65-85-0	Benzoic acid	1.9	pKa = 4.19
20	106-44-5	p-Cresol	1.9	pKa = 10.17
21	140-10-3 (trans)	Cinnamic acid	2.1	pKa = 3.89 (cis) *4*.44 (trans)
22	100-66-3	Anisole	2.1	
23	93-58-3	Methyl benzoate	2.1	
24	71-43-2	Benzene	2.1	
25	99-04-7	3-Methylbenzoic acid	2.4	pKa = 4.27
26	106-48-9	4-Chlorophenol	2.4	pKa = 9.1
27	79-01-6	Trichloroethylene	2.4	
28	1912-24-9	Atrazine	2.6	
29	93-89-0	Ethyl benzoate	2.6	
30	1194-65-6	2,6-Dichlorobenzonitrile	2.6	
31	535-80-8	3-Chlorobenzoic acid	2.7	pKa = 3.82
32	108-88-3	Toluene	2.7	
33	90-15-3	1-Naphthol	2.7	pKa = 9.34
34	608-27-5	2,3-Dichloroaniline	2.8	
35	108-90-7	Chlorobenzene	2.8	
36	1746-13-0	Allyl phenyl ether	2.9	
37	108-86-1	Bromobenzene	3.0	
38	100-41-4	Ethylbenzene	3.2	

DESCRIPTION OF THE METHOD

Preliminary estimate of the partition coefficient

16. If it is necessary, the partition coefficient of the test substance may be estimated preferably by using a calculation method (see Appendix, or where appropriate, by using the ratio of the solubility of the test substance in the pure solvents.

Apparatus

17. A liquid-phase chromatograph fitted with a low-pulse pump and a suitable detection system is required. A UV detector, using a wavelength of 210 nm, or an RI detector is applicable to the wide variety of chemical groups. The presence of polar groups in the stationary phase may seriously impair the performance of the HPLC column. Therefore, stationary phases should have a minimal percentage of polar groups (16). Commercial microparticulate reverse-phase packing or ready-packed columns can be used. A guard column may be positioned between the injection system and the analytical column.

Mobile phase

18. HPLC-grade methanol and distilled or de-ionised water are used to prepare the eluting solvent, which is degassed before use. Isocratic elution should be employed. Methanol/water ratios with minimum water content of 25% should be used. Typically a 3:1 (v/v) methanol-water mixture is satisfactory for eluting substances with a log P of 6 within an hour, at a flow rate of 1 ml/min. For substances with a log P above 6 it may be necessary to shorten the elution time (and those of the reference substances) by decreasing the polarity of the mobile phase or the column length.

19. The test substance and the reference substances must be soluble in the mobile phase in sufficient concentration to allow their detection. Additives may be used with the methanol-water mixture in exceptional cases only, since they will change the properties of the column. In these cases it must be confirmed that the retention time of the test and reference substances are not influenced. If methanol-water is not appropriate, other organic solvent-water mixtures can be used, e.g. ethanol-water, acetonitrile-water or isopropyl alcohol (2-propanol)-water.

20. The pH of the eluent is critical for ionisable substances. It should be within the operating pH range of the column, usually between 2 and 8. Buffering is recommended. Care must be taken to avoid salt precipitation and column deterioration which occur with some organic phase/buffer mixtures. HPLC measurements with silica-based stationary phases above pH 8 are not normally advisable since the use of an alkaline mobile phase may cause rapid deterioration in the performance of the column.

Solutes

21. The test and reference substances must be sufficiently pure in order to assign the peaks in the chromatograms to the respective substances. Substances to be used for test or calibration purposes are dissolved in the mobile phase if possible. If a solvent other than the mobile phase is used to dissolve the test and reference substances, the mobile phase should be used for the final dilution prior to injection.

Test conditions

22. The temperature during the measurement should not vary by more than $\pm$ 1 °C.

Determination of dead time t_0

23. The dead time t_0 can be measured by using unretained organic substances (e.g. thiourea or formamide). A more precise dead time can be derived from the retention times measured or a set of approximately seven members of a homologous series (e.g. n-alkyl methyl ketones) (17). The retention times t_R (n_C+1) are plotted against t_R (n_C), where n_C is the number of carbon atoms. A straight line, t_R (n_C +1) = A t_R (n_C) + (1-A)t_0, is obtained, where A, representing k(n_C +1)/k(n_C), is constant. The dead time t_0 is obtained from the intercept (1-A)t_0 and the slope A.

Regression Equation

24. The next step is to plot a correlation log k versus log P for appropriate reference substances with log P values near the value expected for the test substance. In practice, from 6 to 10 reference substances are injected simultaneously. The retention times are determined, preferably on a recording integrator linked to the detection system. The corresponding logarithms of the capacity factors, log k, are plotted as a function of log P. The regression equation is performed at regular intervals, at least once daily, so that account can be taken of possible changes in column performance.

DETERMINATION OF THE P_{OW} OF THE TEST SUBSTANCE

25. The test substance is injected in the smallest detectable quantities. The retention time is determined in duplicate. The partition coefficient of the test substance is obtained by interpolation of the calculated capacity factor on the calibration graph. For very low and very high partition coefficients extrapolation is necessary. Especially in these cases attention must be given to the confidence limits of the regression line. If the retention time of sample is outside the range of retention times obtained for the standards, a limit value should be quoted.

DATA AND REPORTING

Test report

26. The following must be included in the report:

- if determined the preliminary estimate of the partition coefficient, the estimated values and the method used; and if a calculation method was used, its full description including identification of the data base and detailed information on the choice of fragments;
- test and reference substances: purity, structural formula and CAS number,
- description of equipment and operating conditions: analytical column, guard column,
- mobile phase, means of detection, temperature range, pH;
- elution profiles (chromatograms);
- deadtime and how it was measured;
- retention data and literature log P_{ow} values for reference substances used in calibration;
- details on fitted regression line (log k versus log P_{ow}) and the correlation coefficient of the line including confidence intervals;
- average retention data and interpolated log P_{ow} value for the test substance;
- in case of a mixture: elution profile chromatogram with indicated cut-offs;
- log P_{ow} values relative to area % of the log P_{ow} peak;
- calculation using a regression line;
- calculated weighted average log P_{ow} values, when appropriate.

LITERATURE

(1) C.V. Eadsforth and P. Moser. (1983). Assessment of Reverse Phase Chromatographic Methods for Determining Partition Coefficients. Chemosphere. 12, 1459.

(2) W. Klein, W. Kördel, M. Weiss and H.J. Poremski. (1988). Updating of the OECD Test Guideline 107 Partition Coefficient n-Octanol-Water, OECD Laboratory Intercomparison Test on the HPLC Method. Chemosphere. 17, 361.

(3) C.V. Eadsforth. (1986). Application of Reverse H.P.L.C. for the Determination of Partition Coefficient. Pesticide Science. 17, 311.

(4) H. Ellgehausen, C. D'Hondt and R. Fuerer (1981). Reversed-phase chromatography as a general method for determining octan-1-ol/water partition coefficients. Pesticide. Science. 12, 219.

(5) B. McDuffie (1981). Estimation of Octanol Water Partition Coefficients for Organic Pollutants Using Reverse Phase High Pressure Liquid Chromatography. Chemosphere. 10, 73.

(6) OECD (2000). Guideline for Testing of Chemicals – Partition Coefficient (n-octanol/water): pH-metric Method for Ionisable Substances. Draft Guideline, November 2000.

(7) OSPAR (1995). ”Harmonised Offshore Chemicals Notification Format (HOCFN) 1995”, Oslo and Paris Conventions for the Prevention of Marine Pollution Programmes and Measures Committee (PRAM), Annex 10, Oviedo, 20–24 February 1995.

(8) M. Thatcher, M. Robinson, L. R. Henriquez and C. C. Karman. (1999). An User Guide for the Evaluation of Chemicals Used and Discharged Offshore, A CIN Revised CHARM III Report 1999. Version 1.0, 3. August.

(9) E. A. Vik, S. Bakke and K. Bansal. (1998). Partitioning of Chemicals. Important Factors in Exposure Assessment of Offshore Discharges. Environmental Modelling & Software Vol. 13, pp. 529-537.

(10) L.O. Renberg, S.G. Sundstroem and K. Sundh-Nygård. (1980). Partition coefficients of organic chemicals derived from reversed-phase thin-layer chromatography. Evaluation of methods and application on phosphate esters, polychlorinated paraffins and some PCB-substitutes. Chemosphere. 9, 683.

(11) W.E. Hammers, G.J.Meurs and C.L. De-Ligny. (1982). Correlations between liquid chromatographic capacity ratio data on Lichrosorb RP-18 and partition coefficients in the octanol-water system. J. Chromatography 247, 1.

(12) J.E. Haky and A.M. Young. (1984). Evaluation of a simple HPLC correlation method for the estimation of the octanol-water partition coefficients of organic compounds. J. Liq. Chromatography. 7, 675.

(13) S. Fujisawa and E. Masuhara. (1981). Determination of Partition Coefficients of Acrylates Methacrylates and Vinyl Monomers Using High Performance Liquid Chromatography. Journal of Biomedical Materials Research. 15, 787.

(14) C. Hansch and A. J. Leo. (1979). Substituent Constants for Correlation Analysis in Chemistry and Biology. John Willey, New York.

(15) C. Hansch, chairman; A.J. Leo, dir. (1982). Log P and Parameter Database: A tool for the quantitative prediction of bioactivity - Available from Pomona College Medical Chemistry Project, Pomona College, Claremont, California 91711.

(16) R. F. Rekker, H. M. de Kort. (1979). The hydrophobic fragmental constant: An extension to a 1000 data point set. Eur. J. Med. Chem. – Chim. Ther. 14, 479.

(17) G.E. Berendsen, P.J. Schoenmakers, L. de Galan, G. Vigh, Z. Varga-Puchony, and J. Inczédy. (1980). On determination of hold-up time in reversed-phase liquid chromatography. J. Liq. Chromato. 3, 1669.

Appendix

P_{OW} CALCULATION METHODS

INTRODUCTION

1. This appendix provides a short introduction to the calculation of P_{ow}. For further information the reader is referred to textbooks (1)(2).

2. Calculated values of P_{ow} are used for:

- deciding which experimental method to use: Shake Flask method for log P_{ow} between -2 and 4 and HPLC method for log P_{ow} between 0 and 6;
- selecting conditions to be used in HPLC (reference substances, methanol/water ratio);
- checking the plausibility of values obtained through experimental methods;
- providing an estimate when experimental methods cannot be applied.

Principle of calculation methods

3. The calculation methods suggested here are based on the theoretical fragmentation of the molecule into suitable substructures for which reliable log P_{ow} increments are known. The log P_{ow} is obtained by summing the fragment values and the correction terms for intramolecular interactions. Lists of fragment constants and correction terms are available (1)(2)(3)(4)(5)(6). Some are regularly updated (3).

Reliability of calculated values

4. In general, the reliability of calculation methods decreases as the complexity of the substance under study increases. In the case of simple molecules of low molecular weight and with one or two functional groups, a deviation of 0.1 to 0.3 log P_{ow} units between the results of the different fragmentation methods and the measured values can be expected. The margin of error will depend on the reliability of the fragment constants used, the ability to recognise intramolecular interactions (e.g. hydrogen bonds) and the correct use of correction terms. In the case of ionising substances the charge and degree of ionisation must be taken into consideration (10).

Fujita-Hansch π-method

5. The hydrophobic substituent constant, π, originally introduced by Fujita et al. (7) is defined as:

$$^{\pi}X = \log P_{ow}(PhX) - \log P_{ow}(PhH)$$

where PhX is an aromatic derivative and PhH the parent substance.

e.g. $^{\pi}Cl$ = log Pow (C6H5Cl) - log P_{ow} (C6H6)
= 2.84 - 2.13
= 0.71

The π-method is primarily of interest for aromatic substances. π-values for a large number of substituents are available (4)(5).

Rekker method

6. Using the Rekker method (8) the log P_{ow} value is calculated as:

$$Log\ P_{ow} = \sum_i a_i f_i + \sum_j (\text{interaction terms})$$

where a_i is the number of times a given fragment occurs in the molecule and f_i is the log P_{ow} increment of the fragment. The interaction terms can be expressed as an integral multiple of one single constant C_m (so-called "magic constant"). The fragment constants f_i and C_m have been determined from a list of 1054 experimental P_{ow} values of 825 substances using multiple regression analysis (6)(8). The determination of the interaction terms is carried out according to set rules (6)(8)(9).

Hansch-Leo method

7. Using the Hansch and Leo method (4), the log P_{ow} value is calculated as:

$$\text{Log } P_{ow} = \sum_i a_i f_i + \sum_j b_j F_j$$

where f_i is a fragment constant, F_j a correction term (factor), a_i and b_j the corresponding frequency of occurence. Lists of atomic and group fragmental values and of correction terms F_j were derived by trial and error from experimental P_{ow} values. The correction terms have been divided into several different classes (1)(4). Sofware packages have been developed to take into account all the rules and correction terms (3).

COMBINED METHOD

8. The calculation of log P_{ow} of complex molecules can be considerably improved, if the molecule is dissected into larger substructures for which reliable log P_{ow} values are available, either from tables (3)(4) or by existing measurements. Such fragments (e.g. heterocycles, anthraquinone, azobenzene) can then be combined with the Hansch- π values or with Rekker or Leo fragment constants.

Remarks

i) The calculation methods are only applicable to partly or fully ionised substances when the necessary correction factors are taken into account.

ii)If the existence of intramolecular hydrogen bonds can be assumed, the corresponding correction terms (approx. +0.6 to +1.0 log P_{ow} units) must be added (1). Indications on the presence of such bonds can be obtained from stereo models or spectroscopic data.

iii) If several tautomeric forms are possible, the most likely form should be used as the basis of the calculation.

iv) The revisions of lists of fragment constants should be followed carefully.

LITERATURE ON CALCULATION METHODS

(1) W.J. Lyman, W.F. Reehl and D.H. Rosenblatt (ed.). Handbook of Chemical Property Estimation Methods, McGraw-Hill, New York (1982).

(2) W.J. Dunn, J.H. Block and R.S. Pearlman (ed.). Partition Coefficient, Determination and Estimation, Pergamon Press, Elmsford (New York) and Oxford (1986).

(3) Pomona College, Medicinal Chemistry Project, Claremont, California 91711, USA, Log P Database and Med. Chem. Software (Program CLOGP-3).

(4) C. Hansch and A.J. Leo. Substituent Constants for Correlation Analysis in Chemistry and Biology, John Wiley, New York (1979).

(5) Leo, C. Hansch and D. Elkins. (1971) Partition coefficients and their uses. *Chemical . Reviews*. 71, 525.

(6) R. F. Rekker, H. M. de Kort. (1979). The hydrophobic fragmental constant: An extension to a 1000 data point set. *Eur. J. Med. Chem*. – *Chim. Ther.* 14, 479.

(7) Toshio Fujita, Junkichi Iwasa & Corwin Hansch (1964). A New Substituent Constant, π, Derived from Partition Coefficients. *J. Amer. Chem. Soc*. 86, 5175.

(8) R.F. Rekker. The Hydrophobic Fragmental Constant, Pharmacochemistry Library, Vol. 1, Elsevier, New York (1977).

(9) C.V. Eadsforth and P. Moser. (1983). Assessment of Reverse Phase Chromatographic Methods for Determining Partition Coefficients. *Chemosphere.* 12, 1459.

(10) R.A. Scherrer. ACS - Symposium Series 255, p. 225, American Chemical Society, Washington, D.C. (1984)."

(3) Chapter C.3 is replaced by the following:

"C.3. Freshwater Alga and Cyanobacteria, Growth Inhibition Test

INTRODUCTION

1. This test method is equivalent to OECD test guideline (TG) 201 (2006, annex corrected in 2011). The need to extend the test method to include additional species and update it to meet the requirements for hazard assessment and classification of chemicals has been identified. This revision has been completed on the basis of extensive practical experience, scientific progress in the field of algal toxicity studies, and extensive regulatory use, which has occurred since the original adoption.

2. Definitions used are given in Appendix 1.

PRINCIPLE OF THE TEST

3. The purpose of this test is to determine the effects of a chemical on the growth of freshwater microalgae and/or cyanobacteria. Exponentially growing test organisms are exposed to the test chemical in batch cultures over a period of normally 72 hours. In spite of the relatively brief test duration, effects over several generations can be assessed.

4. The system response is the reduction of growth in a series of algal cultures (test units) exposed to various concentrations of a test chemical. The response is evaluated as a function of the exposure concentration in comparison with the average growth of replicate, unexposed control cultures. For full expression of the system response to toxic effects (optimal sensitivity), the cultures are allowed unrestricted exponential growth under nutrient sufficient conditions and continuous light for a sufficient period of time to measure reduction of the specific growth rate.

5. Growth and growth inhibition are quantified from measurements of the algal biomass as a function of time. Algal biomass is defined as the dry weight per volume, e.g. mg algae/litre test solution. However, dry weight is difficult to measure and therefore surrogate parameters are used. Of these surrogates, cell counts are most often used. Other surrogate parameters include cell volume, fluorescence, optical density, etc. A conversion factor between the measured surrogate parameter and biomass should be known.

6. The test endpoint is inhibition of growth, expressed as the logarithmic increase in biomass (average specific growth rate) during the exposure period. From the average specific growth rates recorded in a series of test solutions, the concentration bringing

about a specified x % inhibition of growth rate (e.g. 50%) is determined and expressed as the E_rC_x (e.g. E_rC_{50}).

7. An additional response variable used in this test method is yield, which may be needed to fulfil specific regulatory requirements in some countries. It is defined as the biomass at the end of the exposure period minus the biomass at the start of the exposure period. From the yield recorded in a series of test solutions, the concentration bringing about a specified x % inhibition of yield (e.g., 50 %) is calculated and expressed as the E_yC_x (e.g. E_yC_{50}).

8. In addition, the lowest observed effect concentration (LOEC) and the no observed effect concentration (NOEC) may be statistically determined.

INFORMATION ON THE TEST CHEMICAL

9. Information on the test chemical which may be useful in establishing the test conditions includes structural formula, purity, stability in light, stability under the conditions of the test, light absorption properties, pKa, and results of studies of transformation including biodegradability in water.

10. The water solubility, octanol water partition coefficient (P_{ow}) and vapour pressure of the test chemical should be known and a validated method for the quantification of the chemical in the test solutions with reported recovery efficiency and limit of detection should be available.

VALIDITY OF THE TEST

11. For the test to be valid, the following performance criteria should be met:

- The biomass in the control cultures should have increased exponentially by a factor of at least 16 within the 72-hour test period. This corresponds to a specific growth rate of 0.92 day^{-1}. For the most frequently used species the growth rate is usually substantially higher (see Appendix 2). This criterion may not be met when species that grow slower than those listed in Appendix 2 are used. In this case, the test period should be extended to obtain at least a 16-fold growth in control cultures, while the growth has to be exponential throughout the test period. The test period may be shortened to at least 48 hours to maintain unlimited, exponential growth during the test as long as the minimum multiplication factor of 16 is reached.
- The mean coefficient of variation for section-by-section specific growth rates (days 0-1, 1-2 and 2-3, for 72-hour tests) in the control cultures (See Appendix 1 under "coefficient of variation") must not exceed 35%. See paragraph 49 for the calculation of section-by-section specific growth rate. This criterion applies to the mean value of coefficients of variation calculated for replicate control cultures.
- The coefficient of variation of average specific growth rates during the whole test period in replicate control cultures must not exceed 7% in tests with

Pseudokirchneriella subcapitata and Desmodesmus subspicatus. For other less frequently tested species, the value should not exceed 10%.

REFERENCE CHEMICAL

12. Reference chemical(s), such as 3,5-dichlorophenol used in the international ring test (1), may be tested as a means of checking the test procedure. Potassium dichromate can also be used as a reference chemical for green algae. It is desirable to test a reference chemical at least twice a year.

APPLICABILITY OF THE TEST

13. This test method is most easily applied to water-soluble chemicals which, under the conditions of the test, are likely to remain in the water. For testing of chemicals that are volatile, strongly adsorbing, coloured, having a low solubility in water or chemicals that may affect the availability of nutrients or minerals in the test medium, certain modifications of the described procedure may be required (e.g., closed system, conditioning of the test vessels). Guidance on some appropriate modifications is given in (2) (3) and (4).

DESCRIPTION OF THE TEST METHOD

Apparatus

14. Test vessels and other apparatus which will come into contact with the test solutions should be made entirely of glass or other chemically inert material. The items should be thoroughly washed to ensure that no organic or inorganic contaminants may interfere with the algal growth or composition of the test solutions.

15. The test vessels will normally be glass flasks of dimensions that allow a sufficient volume of culture for measurements during the test and a sufficient mass transfer of CO_2 from the atmosphere (see paragraph 30). Note that the liquid volume must be sufficient for analytical determinations (see paragraph 37).

16. In addition some or all of the following equipment may be required:

- Culturing apparatus: a cabinet or chamber is recommended, in which the chosen incubation temperature can be maintained at ± 2°C.
- Light measurement instruments: it is important to note that the method of measurement of light intensity, and in particular the type of receptor (collector), may affect the measured value. Measurements should preferably be made using a spherical (4 π) receptor (which responds to direct and reflected light from all angles above and below the plane of measurement), or a 2 π receptor (which responds to light from all angles above the measurement plane).
- Apparatus to determine algal biomass. Cell count, which is the most frequently used

surrogate parameter for algal biomass, may be made using an electronic particle counter, a microscope with counting chamber, or a flow cytometer. Other biomass surrogates can be measured using a flow cytometer, fluorimeter, spectrophotometer or colorimeter. A conversion factor relating cell count to dry weight is useful to calculate. In order to provide useful measurements at low biomass concentrations when using a spectrophotometer, it may be necessary to use cuvettes with a light path of at least 4 cm.

Test organisms

17. Several species of non-attached microalgae and cyanobacteria may be used. The strains listed in Appendix 2 have been shown to be suitable using the test procedure specified in this test method.

18. If other species are used, the strain and/or origin should be reported. Confirm that exponential growth of the selected test alga can be maintained throughout the test period under the prevailing conditions.

Growth medium

19. Two alternative growth media, the OECD and the AAP medium, are recommended. The compositions of these media are shown in Appendix 3. Note that the initial pH value and the buffering capacity (regulating pH increase) of the two media are different. Therefore the results of the tests may be different depending on the medium used, particularly when testing ionising chemicals.

20. Modification of the growth media may be necessary for certain purposes, e.g. when testing metals and chelating agents or testing at different pH values. Use of a modified medium should be described in detail and justified (3) (4).

Initial biomass concentration

21. The initial biomass in the test cultures must be the same in all test cultures and sufficiently low to allow exponential growth throughout the incubation period without risk of nutrient depletion. The initial biomass should not exceed 0.5 mg/l as dry weight. The following initial cell concentrations are recommended:

Pseudokirchneriella subcapitata:	5×10^3 - 10^4 cells/ml
Desmodesmus subspicatus	2-5 x 10^3 cells/ml
Navicula pelliculosa	10^4 cells/ml
Anabaena flos-aquae	10^4 cells/ml
Synechococcus leopoliensis	5×10^4 - 10^5 cells/ml

Concentrations of test chemical

22. The concentration range in which effects are likely to occur may be determined on the basis of results from range-finding tests. For the final definitive test at least five

concentrations, arranged in a geometric series with a factor not exceeding 3.2, should be selected. For test chemicals showing a flat concentration response curve a higher factor may be justified. The concentration series should preferably cover the range causing 5-75 % inhibition of algal growth rate.

Replicates and controls

23. The test design should include three replicates at each test concentration. If determination of the NOEC is not required, the test design may be altered to increase the number of concentrations and reduce the number of replicates per concentration. The number of control replicates must be at least three, and ideally should be twice the number of replicates used for each test concentration.

24. A separate set of test solutions may be prepared for analytical determinations of test chemical concentrations (see paragraphs 36 and 38).

25. When a solvent is used to solubilise the test chemical, additional controls containing the solvent at the same concentration as used in the test cultures must be included in the test design**.**

Preparation of inoculum culture

26. In order to adapt the test alga to the test conditions and ensure that the algae are in the exponential growth phase when used to inoculate the test solutions, an inoculum culture in the test medium is prepared 2-4 days before start of the test. The algal biomass should be adjusted in order to allow exponential growth to prevail in the inoculum culture until the test starts. Incubate the inoculum culture under the same conditions as the test cultures. Measure the increase in biomass in the inoculum culture to ensure that growth is within the normal range for the test strain under the culturing conditions. An example of the procedure for algal culturing is described in Appendix 4. To avoid synchronous cell divisions during the test a second propagation step of the inoculum culture may be required.

Preparation of test solutions

27. All test solutions must contain the same concentrations of growth medium and initial biomass of test alga. Test solutions of the chosen concentrations are usually prepared by mixing a stock solution of the test chemical with growth medium and inoculum culture. Stock solutions are normally prepared by dissolving the chemical in test medium.

28. Solvents, e.g. acetone, t-butyl alcohol and dimethyl formamide, may be used as carriers to add chemicals of low water solubility to the test medium (2)(3). The concentration of solvent should not exceed 100 µl/l, and the same concentration of solvent should be added to all cultures (including controls) in the test series.

Incubation

29. Cap the test vessels with air-permeable stoppers. The vessels are shaken and placed in the culturing apparatus. During the test it is necessary to keep the algae in suspension and to facilitate transfer of CO_2. To this end constant shaking or stirring should be used. The cultures should be maintained at a temperature in the range of 21 to 24°C, controlled at ± 2°C. For species other than those listed in Appendix 2, e.g. tropical species, higher temperatures may be appropriate, providing that the validity criteria can be fulfilled. It is recommended to place the flasks randomly and to reposition them daily in the incubator.

30. The pH of the control medium should not increase by more than 1.5 units during the test. For metals and chemicals that partly ionise at a pH around the test pH, it may be necessary to limit the pH drift to obtain reproducible and well defined results. A drift of < 0.5 pH units is technically feasible and can be achieved by ensuring an adequate CO_2 mass transfer rate from the surrounding air to the test solution, e.g. by increasing the shaking rate. Another possibility is to reduce the demand for CO_2 by reducing the initial biomass or the test duration.

31. The surface where the cultures are incubated should receive continuous, uniform fluorescent illumination e.g. of «cool-white» or «daylight» type. Strains of algae and cyanobacteria vary in their light requirements. The light intensity should be selected to suit the test organism used. For the recommended species of green algae, select the light intensity at the level of the test solutions from the range of 60-120 $\mu E \cdot m^{-2}\ s^{-1}$ when measured in the photosynthetically effective wavelength range of 400-700 nm using an appropriate receptor. Some species, in particular *Anabaena flos-aquae*, grow well at lower light intensities and may be damaged at high intensities. For such species an average light intensity in the range 40-60 $\mu E \cdot m^{-2} \cdot s^{-1}$ should be selected. (For light-measuring instruments calibrated in lux, an equivalent range of 4440 – 8880 lux for cool white light corresponds approximately to the recommended light intensity 60-120 $\mu E \cdot m^{-2} \cdot s^{-1}$). Maintain the light intensity within ±15% from the average light intensity over the incubation area.

Test duration

32. Test duration is normally 72 hours. However, shorter or longer test durations may be used provided that all validity criteria in paragraph 11 can be met.

Measurements and analytical determinations

33. The algal biomass in each flask is determined at least daily during the test period. If measurements are made on small volumes removed from the test solution by pipette, these should not be replaced.

34. Measurement of biomass is done by manual cell counting by microscope or an electronic particle counter (by cell counts and/or biovolume). Alternative techniques, e.g. flow cytometry, *in vitro* or *in vivo* chlorophyll fluorescence (5) (6), or optical

density can be used if a satisfactory correlation with biomass can be demonstrated over the range of biomass occurring in the test.

35. Measure the pH of the solutions at the beginning and at the end of the test.

36. Provided an analytical procedure for determination of the test chemical in the concentration range used is available, the test solutions should be analysed to verify the initial concentrations and maintenance of the exposure concentrations during the test.

37. Analysis of the concentration of the test chemical at the start and end of the test of a low and high test concentration and a concentration around the expected EC_{50} may be sufficient where it is likely that exposure concentrations will vary less than 20% from nominal values during the test. Analysis of all test concentrations at the beginning and at the end of the test is recommended where concentrations are unlikely to remain within 80-120 % of the nominal concentration. For volatile, unstable or strongly adsorbing test chemicals, additional samplings for analysis at 24 hour intervals during the exposure period are recommended in order to better define loss of the test chemical. For these chemicals, extra replicates may be needed. In all cases, determination of test chemical concentrations need only be performed on one replicate vessel at each test concentration (or the contents of the vessels pooled by replicate).

38. The test media prepared specifically for analysis of exposure concentrations during the test should be treated identically to those used for testing, i.e. they should be inoculated with algae and incubated under identical conditions. If analysis of the dissolved test chemical concentration is required, it may be necessary to separate algae from the medium. Separation should preferably be made by centrifugation at a low g-force, sufficient to settle the algae.

39. If there is evidence that the concentration of the chemical being tested has been satisfactorily maintained within ± 20 % of the nominal or measured initial concentration throughout the test, analysis of the results can be based on nominal or measured initial values. If the deviation from the nominal or measured initial concentration is not within the range of ± 20 %, analysis of the results should be based on geometric mean concentration during exposure or on models describing the decline of the concentration of the test chemical (3) (7).

40. The alga growth inhibition test is a more dynamic test system than most other short-term aquatic toxicity tests. As a consequence, the actual exposure concentrations may be difficult to define, especially for adsorbing chemicals tested at low concentrations. In such cases, disappearance of the test chemical from solution by adsorption to the increasing algal biomass does not mean that it is lost from the test system. When the result of the test is analysed, it should be checked whether a decrease in concentration of the test chemical in the course of the test is accompanied by a decrease in growth inhibition. If this is the case, application of a suitable model describing the decline of the concentration of the test chemical (7) may be considered. If not, it may be

appropriate to base the analysis of the results on the initial (nominal or measured) concentrations.

Other observations

41. Microscopic observation should be performed to verify a normal and healthy appearance of the inoculum culture and to observe any abnormal appearance of the algae (as may be caused by the exposure to the test chemical) at the end of the test.

Limit test

42. Under some circumstances, e.g. when a preliminary test indicates that the test chemical has no toxic effects at concentrations up to 100 mg/l or up to its limit of solubility in the test medium (whichever is the lower), a limit test involving a comparison of responses in a control group and one treatment group (100 mg/l or a concentration equal to the limit of solubility), may be undertaken. It is strongly recommended that this be supported by analysis of the exposure concentration. All previously described test conditions and validity criteria apply to a limit test, with the exception that the number of treatment replicates should be at least six. The response variables in the control and treatment group may be analysed using a statistical test to compare means, e.g. a Student's t-test. If variances of the two groups are unequal, a t-test adjusted for unequal variances should be performed

DATA AND REPORTING

Plotting growth curves

43. The biomass in the test vessels may be expressed in units of the surrogate parameter used for measurement (e.g. cell number, fluorescence).

44. Tabulate the estimated biomass concentration in test cultures and controls together with the concentrations of test material and the times of measurement, recorded with a resolution of at least whole hours, to produce plots of growth curves. Both logarithmic scales and linear scales can be useful at this first stage, but logarithmic scales are mandatory and generally give a better presentation of variations in growth pattern during the test period. Note that exponential growth produces a straight line when plotted on a logarithmic scale, and inclination of the line (slope) indicates the specific growth rate.

45. Using the plots, examine whether control cultures grow exponentially at the expected rate throughout the test. Examine all data points and the appearance of the graphs critically and check raw data and procedures for possible errors. Check in particular any data point that seems to deviate by a systematic error. If it is obvious that procedural mistakes can be identified and/or considered highly likely, the specific data point is marked as an outlier and not included in subsequent statistical analysis. (A zero algal concentration in one out of two or three replicate vessels may indicate the

vessel was not inoculated correctly, or was improperly cleaned). State reasons for rejection of a data point as an outlier clearly in the test report. Accepted reasons are only (rare) procedural mistakes and not just bad precision. Statistical procedures for outlier identification are of limited use for this type of problem and cannot replace expert judgement. Outliers (marked as such) should preferably be retained among the data points shown in any subsequent graphical or tabular data presentation.

Response variables

46. The purpose of the test is to determine the effects of the test chemical on the growth of algae. This test method describes two response variables, as different jurisdictions have different preferences and regulatory needs. In order for the test results to be acceptable in all jurisdictions, the effects should be evaluated using both response variables (a) and (b) described below.

    (a) Average specific growth rate: this response variable is calculated on the basis of the logarithmic increase of biomass during the test period, expressed per day

    (b) Yield: this response variable is the biomass at the end of the test minus the starting biomass.

47. It should be noted that toxicity values calculated by using these two response variables are not comparable and this difference must be recognised when using the results of the test. EC_x values based upon average specific growth rate (E_rC_x) will generally be higher than results based upon yield (E_yC_x) if the test conditions of this test method are adhered to, due to the mathematical basis of the respective approaches. This should not be interpreted as a difference in sensitivity between the two response variables, simply that the values are different mathematically. The concept of average specific growth rate is based on the general exponential growth pattern of algae in non-limited cultures, where toxicity is estimated on the basis of the effects on the growth rate, without being dependent on the absolute level of the specific growth rate of the control, slope of the concentration-response curve or on test duration. In contrast, results based upon the yield response variable are dependent upon all these other variables. E_yC_x is dependent on the specific growth rate of the algal species used in each test and on the maximum specific growth rate that can vary between species and even different algal strains. This response variable should not be used for comparing the sensitivity to toxicants among algal species or even different strains. While the use of average specific growth rate for estimating toxicity is scientifically preferred, toxicity estimates based on yield are also included in this test method to satisfy current regulatory requirements in some countries.

Average growth rate

48. The average specific growth rate for a specific period is calculated as the logarithmic increase in the biomass from the equation for each single vessel of controls and treatments [1]:

$$\mu_{i-j} = \frac{\ln X_j - \ln X_i}{t_j - t_i} \text{ (day}^{-1}\text{)} \qquad [1],$$

where:

μ_{i-j} is the average specific growth rate from time *i* to j;
X_i is the biomass at time *i*;
X_j is the biomass at time *j*

For each treatment group and control group, calculate a mean value for growth rate along with variance estimates.

49. Calculate the average specific growth rate over the entire test duration (normally days 0-3), using the nominally inoculated biomass as the starting value rather than a measured starting value, because in this way greater precision is normally obtained. If the equipment used for biomass measurement allows sufficiently precise determination of the low inoculum biomass (e.g. flow cytometer) then the measured initial biomass concentration can be used. Assess also the section-by-section growth rate, calculated as the specific growth rates for each day during the course of the test (days 0-1, 1-2 and 2-3) and examine whether the control growth rate remains constant (see validity criteria, paragraph 11). A significantly lower specific growth rate on day one than the total average specific growth rate may indicate a lag phase. While a lag phase can be minimised and practically eliminated in control cultures by proper propagation of the pre-culture, a lag phase in exposed cultures may indicate recovery after initial toxic stress or reduced exposure due to loss of test chemical (including sorption onto the algal biomass) after initial exposure. Hence the section-by-section growth rate may be assessed in order to evaluate effects of the test chemical occurring during the exposure period. Substantial differences between the section-by-section growth rate and the average growth rate indicate deviation from constant exponential growth and that close examination of the growth curves is warranted.

50. Calculate the percent inhibition of growth rate for each treatment replicate from equation [2]:

$$\%I_r = \frac{\mu_C - \mu_T}{\mu_C} \times 100 \qquad [2],$$

where:

$\%I_r$ = percent inhibition in average specific growth rate;
μ_C = mean value for average specific growth rate (μ) in the control group;
μ_T = average specific growth rate for the treatment replicate.

51. When solvents are used to prepare the test solutions, the solvent controls rather than the controls without solvents should be used in calculation of percent inhibition.

Yield

52. Yield is calculated as the biomass at the end of the test minus the starting biomass for each single vessel of controls and treatments. For each test concentration and control,

calculate a mean value for yield along with variance estimates. The percent inhibition in yield (%I_y) may be calculated for each treatment replicate as follows:

$$\% \, I_y = \frac{(Y_c - Y_T)}{Y_c} \times 100 \qquad [3]$$

where:

$\% \, I_y$ = percent inhibition of yield;
Y_C = mean value for yield in the control group;
Y_T = value for yield for the treatment replicate.

Plotting concentration response curve

53. Plot the percentage of inhibition against the logarithm of the test chemical concentration and examine the plot closely, disregarding any such data point that was singled out as an outlier in the first phase. Fit a smooth line through the data points by eye or by computerised interpolation to get a first impression of the concentration-response relationship, and then proceed with a more detailed method, preferably a computerised statistical method. Depending on the intended usage of data; the quality (precision) and amount of data as well as the availability of data analysis tools, it may be decided (and sometimes well justified) to stop the data analysis at this stage and simply read the key figures EC_{50} and EC_{10} (and/or EC_{20}) from the eye fitted curve (see also section below on stimulatory effects). Valid reasons for not using a statistical method may include:

- Data are not appropriate for computerised methods to produce any more reliable results than can be obtained by expert judgement - in such situations some computer programs may even fail to produce a reliable solution (iterations may not converge etc.)
- Stimulatory growth responses cannot be handled adequately using available computer programs (see below).

Statistical procedures

54. The aim is to obtain a quantitative concentration-response relationship by regression analysis. It is possible to use a weighted linear regression after having performed a linearising transformation of the response data - for instance into probit or logit or Weibull units (8), but non-linear regression procedures are preferred techniques that better handle unavoidable data irregularities and deviations from smooth distributions. Approaching either zero or total inhibition, such irregularities may be magnified by the transformation, interfering with the analysis (8). It should be noted that standard methods of analysis using probit, logit, or Weibull transforms are intended for use on quantal (e.g. mortality or survival) data, and must be modified to accommodate growth or biomass data. Specific procedures for determination of EC_x values from continuous data can be found in (9) (10) and (11). The use of non-linear regression analysis is further detailed in Appendix 5.

55. For each response variable to be analysed, use the concentration-response relationship to calculate point estimates of EC_x values. When possible, the 95% confidence limits

for each estimate should be determined. Goodness of fit of the response data to the regression model should be assessed either graphically or statistically. Regression analysis should be performed using individual replicate responses, not treatment group means. If, however nonlinear curve fitting is difficult or fails because of too great scatter in the data, the problem may be circumvented by performing the regression on group means as a practical way of reducing the influence of suspected outliers. Use of this option should be identified in the test report as a deviation from normal procedure because curve fits with individual replicates did not produce a good result.

56. EC_{50} estimates and confidence limits may also be obtained using linear interpolation with bootstrapping (13), if available regression models/methods are unsuitable for the data.

57. For estimation of the LOEC and hence the NOEC, for effects of the test chemical on growth rate, it is necessary to compare treatment means using analysis of variance (ANOVA) techniques. The mean for each concentration must then be compared with the control mean using an appropriate multiple comparison or trend test method. Dunnett's or Williams' test may be useful (12)(14)(15)(16)(17). It is necessary to assess whether the ANOVA assumption of homogeneity of variance holds. This assessment may be performed graphically or by a formal test (17). Suitable tests are Levene's or Bartlett's. Failure to meet the assumption of homogeneity of variances can sometimes be corrected by logarithmic transformation of the data. If heterogeneity of variance is extreme and cannot be corrected by transformation, analysis by methods such as step-down Jonkheere trend tests should be considered. Additional guidance on determining the NOEC can be found in (11).

58. Recent scientific developments have led to a recommendation of abandoning the concept of NOEC and replacing it with regression based point estimates EC_x. An appropriate value for x has not been established for this algal test. A range of 10 to 20 % appears to be appropriate (depending on the response variable chosen), and preferably both the EC_{10} and EC_{20} should be reported.

Growth stimulation

59. Growth stimulation (negative inhibition) at low concentrations is sometimes observed. This can result from either hormesis ("toxic stimulation") or from addition of stimulating growth factors with the test material to the minimal medium used. Note that the addition of inorganic nutrients should not have any direct effect because the test medium should maintain a surplus of nutrients throughout the test. Low dose stimulation can usually be ignored in EC_{50} calculations unless it is extreme. However, if it is extreme, or an EC_x value for low x is to be calculated, special procedures may be needed. Deletion of stimulatory responses from the data analysis should be avoided if possible, and if available curve fitting software cannot accept minor stimulation, linear interpolation with bootstrapping can be used. If stimulation is extreme, use of a hormesis model may be considered (18).

Non toxic growth inhibition

60. Light absorbing test materials may give rise to a growth rate reduction because shading reduces the amount of available light. Such physical types of effects should be separated from toxic effects by modifying the test conditions and the former should be reported separately. Guidance may be found in (2) and (3).

TEST REPORT

61. The test report must include the following:

Test chemical:

- physical nature and relevant physical-chemical properties, including water solubility limit;
- chemical identification data (e.g., CAS Number), including purity (impurities).

Test species:

- the strain, supplier or source and the culture conditions used.

Test conditions:

- date of start of the test and its duration;
- description of test design: test vessels, culture volumes, biomass density at the beginning of the test;
- composition of the medium;
- test concentrations and replicates (e.g., number of replicates, number of test concentrations and geometric progression used);
- description of the preparation of test solutions, including use of solvents etc.
- culturing apparatus;
- light intensity and quality (source, homogeneity);
- temperature;
- concentrations tested: the nominal test concentrations and any results of analyses to determine the concentration of the test chemical in the test vessels. The recovery efficiency of the method and the limit of quantification in the test matrix should be reported;
- all deviations from this test method;
- method for determination of biomass and evidence of correlation between the measured parameter and dry weight;

Results:

- pH values at the beginning and at the end of the test at all treatments;
- biomass for each flask at each measuring point and method for measuring biomass;
- growth curves (plot of biomass versus time);
- calculated response variables for each treatment replicate, with mean values and coefficient of variation for replicates;

- graphical presentation of the concentration/effect relationship;
- estimates of toxicity for response variables e.g., EC_{50}, EC_{10}, EC_{20} and associated confidence intervals. If calculated, LOEC and NOEC and the statistical methods used for their determination;
- if ANOVA has been used, the size of the effect which can be detected (e.g. the least significant difference);
- any stimulation of growth found in any treatment;
- any other observed effects, e.g. morphological changes of the algae;
- discussion of the results, including any influence on the outcome of the test resulting from deviations from this test method.

LITERATURE

(1) International Organisation for Standardisation (1993). ISO 8692 Water quality – Algal growth inhibition test.

(2) International Organisation for Standardisation (1998). ISO/DIS 14442. Water quality – Guidelines for algal growth inhibition tests with poorly soluble materials, volatile compounds, metals and waster water.

(3) OECD (2000). Guidance Document on Aquatic Toxicity Testing of Difficult Substances and mixtures. Environmental Health and Safety Publications. Series on Testing and Assessment, no. 23. Organisation for Economic Co-operation and Development, Paris.

(4) International Organisation for Standardisation (1998). ISO 5667-16 Water quality – Sampling – Part 16: Guidance on Biotesting of Samples.

(5) Mayer, P., Cuhel, R. and Nyholm, N. (1997). A simple in vitro fluorescence method for biomass measurements in algal growth inhibition tests. *Water Research* 31: 2525-2531.

(6) Slovacey, R.E. and Hanna, P.J. (1997). In vivo fluorescence determinations of phytoplancton chlorophyll, Limnology & Oceanography 22: 919-925

(7) Simpson, S.L., Roland, M.G.E., Stauber, J.L. and Batley, G.E. (2003). Effect of declining toxicant concentrations on algal bioassay endpoints. *Environ. Toxicol. Chem.* 22: 2073-2079.

(8) Christensen, E.R., Nyholm, N. (1984). Ecotoxicological Assays with Algae: Weibull Dose-Response Curves. *Env. Sci. Technol*. 19: 713-718.

(9) Nyholm, N. Sørensen, P.S., Kusk, K.O. and Christensen, E.R. (1992). Statistical treatment of data from microbial toxicity tests. *Environ. Toxicol. Chem*. 11: 157-167.

(10) Bruce, R.D.,and Versteeg, D.J. (1992). A statistical procedure for modelling continuous toxicity data. *Environ. Toxicol. Chem*. 11: 1485-1494.

(11) OECD (2006). Current Approaches in the Statistical Analysis of Ecotoxicity Data: A Guidance to Application. Organisation for Economic Co-operation and Development, Paris.

(12) Dunnett, C.W. (1955). A multiple comparisons procedure for comparing several treatments with a control. *J. Amer. Statist. Assoc*. 50: 1096-1121

(13) Norberg-King T.J. (1988). An interpolation estimate for chronic toxicity: The ICp approach. National Effluent Toxicity Assessment Center Technical Report 05-88. US EPA, Duluth, MN.

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

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

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

(17) Draper, N.R. and Smith, H. (1981). Applied Regression Analysis, second edition. Wiley, New York.

(18) Brain, P. and Cousens, R. (1989). An equation to describe dose-responses where there is stimulation of growth at low doses. *Weed Research*, 29, 93-96.

Appendix 1

DEFINITIONS

The following definitions and abbreviations are used for the purposes of this test method:

Biomass is the dry weight of living matter present in a population expressed in terms of a given volume; e.g., mg algae/litre test solution. Usually "biomass" is defined as a mass, but in this test this word is used to refer to mass per volume. Also in this test, surrogates for biomass, such as cell counts, fluorescence, etc. are typically measured and the use of the term "biomass" thus refers to these surrogate measures as well.

Chemical means a substance or mixture

Coefficient of variation is a dimensionless measure of the variability of a parameter, defined as the ratio of the standard deviation to the mean. This can also be expressed as a percent value. Mean coefficient of variation of average specific growth rate in replicate control cultures should be calculated as follows:

1. Calculate % CV of average specific growth rate out of the daily/section by section growth rates for the respective replicate;
2. Calculate the mean value out of all values calculated under point 1 to get the mean coefficient of variation of the daily/section by section specific growth rate in replicate control cultures.

EC_x is the concentration of the test chemical dissolved in test medium that results in an x % (e.g. 50%) reduction in growth of the test organism within a stated exposure period (to be mentioned explicitly if deviating from full or normal test duration). To unambiguously denote an EC value deriving from growth rate or yield the symbol "E_rC" is used for growth rate and "E_yC" is used for yield.

Growth medium is the complete synthetic culture medium in which test algae grow when exposed to the test chemical. The test chemical will normally be dissolved in the test medium.

Growth rate (average specific growth rate) is the logarithmic increase in biomass during the exposure period.

Lowest Observed Effect Concentration (LOEC) is the lowest tested concentration at which the chemical is observed to have a statistically significant reducing effect on growth (at $p < 0.05$) when compared with the control, within a given exposure time. However, all test concentrations above the LOEC must have a harmful effect equal to or greater than those observed at the LOEC. When these two conditions cannot be satisfied, a full explanation must be given for how the LOEC (and hence the NOEC) has been selected.

No Observed Effect Concentration (NOEC) is the test concentration immediately

below the LOEC.

Response variable is a variable for the estimation of toxicity derived from any measured parameters describing biomass by different methods of calculation. For this test method growth rates and yield are response variables derived from measuring biomass directly or any of the surrogates mentioned.

Specific growth rate is a response variable defined as quotient of the difference of the natural logarithms of a parameter of observation (in this test method, biomass) and the respective time period

Test chemical means any substance or mixture tested using this test method.

Yield is the value of a measurement variable at the end of the exposure period minus the measurement variable's value at the start of the exposure period to express biomass increase during the test.

Appendix 2

STRAINS SHOWN TO BE SUITABLE FOR THE TEST

Green algae

Pseudokirchneriella subcapitata (formerly known as *Selenastrum capricornutum*), ATCC 22662, CCAP 278/4, 61.81 SAG

Desmodesmus subspicatus (formerly known as *Scenedesmus subspicatus*), 86.81 SAG

Diatoms

Navicula pelliculosa, UTEX 664

Cyanobacteria

Anabaena flos-aquae, UTEX 1444, ATCC 29413, CCAP 1403/13A

Synechococcus leopoliensis, UTEX 625, CCAP 1405/1

Sources of Strains

The strains recommended are available in unialgal cultures from the following collections (in alphabetical order):

ATCC: American Type Culture Collection
10801 University Boulevard
Manassas, Virginia 20110-2209
USA

CCAP, Culture Collection of Algae and Protozoa
Institute of Freshwater Ecology,
Windermere Laboratory
Far Sawrey, Amblerside
Cumbria LA22 0LP
UK

SAG: Collection of Algal Cultures
Inst. Plant Physiology
University of Göttingen
Nikolausberger Weg 18
37073 Göttingen
GERMANY

UTEX Culture Collection of Algae
Section of Molecular, Cellular and Developmental Biology
School of Biological Sciences
the University of Texas at Austin
Austin, Texas 78712
USA.

Appearance and characteristics of recommended species

	P. subcapitata	***D. subspicatus***	***N. pelliculosa***	***A. flos-aquae***	***S. leopoliensis***
Appearance	Curved, twisted single cells	Oval, mostly single cells	Rods	Chains of oval cells	Rods
Size (L x W) µm	8-14 x 2-3	7-15 x 3-12	7.1 x 3.7	4.5 x 3	6 x 1
Cell volume (µm^3/cell)	40-60[1]	60-80[1]	40-50[1]	30-40[1]	2.5[2]
Cell dry weight (mg/cell)	2-3 x 10^{-8}	3-4 x 10^{-8}	3-4 x 10^{-8}	1-2 x 10^{-8}	2-3 x 10^{-9}
Growth rate[3] (day^{-1})	1.5 -1.7	1.2-1.5	1.4	1.1-1.4	2.0 - 2.4

[1] Measured with electronic particle counter
[2] Calculated from size
[3] Most frequently observed growth rate in OECD medium at light intensity approx. 70 µE m^{-2} s^{-1} and 21 °C

Specific Recommendations on Culturing and Handling of Recommended Test Species

Pseudokirchneriella subcapitata and Desmodesmus subspicatus

These green algae are generally easy to maintain in various culture media. Information on suitable media is available from the culture collections. The cells are normally solitary, and cell density measurements can easily be performed using an electronic particle counter or microscope.

Anabaena flos-aquae

Various growth media may be used for keeping a stock culture. It is particularly important to avoid allowing the batch culture to go past log phase growth when renewing, recovery is difficult at this point.

Anabaena flos-aquae develops aggregates of nested chains of cells. The size of these aggregates may vary with culturing conditions. It may be necessary to break up these

aggregates when microscope counting or an electronic particle counter is used for determination of biomass.

Sonication of sub-samples may be used to break up chains to reduce count variability. Longer sonication than required for breaking up chains into shorter lengths may destroy the cells. Sonication intensity and duration must be identical for each treatment.

Count enough fields on the hemocytometer (at least 400 cells) to help compensate for variability. This will improve reliability of microscopic density determinations.

An electronic particle counter can be used for determination of total cell volume of Anabaena after breaking up the cell chains by careful sonification. The sonification energy has to be adjusted to avoid disruption of the cells.

Use a vortex mixer or similar appropriate method to make sure the algae suspension used to inoculate test vessels is well mixed and homogeneous.

Test vessels should be placed on an orbital or reciprocate shaker table at about 150 revolutions per minute. Alternatively, intermittent agitation may be used to reduce the tendency of Anabaena to form clumps. If clumping occurs, care must be taken to achieve representative samples for biomass measurements. Vigorous agitation before sampling may be necessary to disintegrate algal clumps.

Synechococcus leopoliensis

Various growth media may be used for keeping a stock culture. Information on suitable media is available from the culture collections.

Synechococcus leopoliensis grows as solitary rod-shaped cells. The cells are very small, which complicates the use of microscope counting for biomass measurements. Electronic particle counters equipped for counting particles down to a size of approximately 1 μm are useful. In vitro fluorometric measurements are also applicable.

Navicula pelliculosa

Various growth media may be used for keeping a stock culture. Information on suitable media is available from the culture collections. Note that silicate is required in the medium.

Navicula pelliculosa may form aggregates under certain growth conditions. Due to production of lipids the algal cells sometimes tend to accumulate in the surface film. Under those circumstances special measures have to be taken when sub-samples are taken for biomass determination in order to obtain representative samples. Vigorous shaking, e.g. using a vortex mixer may be required.

Appendix 3

GROWTH MEDIA

One of the following two growth media may be used:

- OECD medium: Original medium of OECD TG 201, also according to ISO 8692
- US. EPA medium AAP also according to ASTM.

When preparing these media, reagent or analytical-grade chemicals should be used and deionised water.

Composition of the AAP-medium (US. EPA) and the OECD TG 201 medium.

Component	AAP		OECD	
	mg/l	mM	mg/l	mM
$NaHCO_3$	15.0	0.179	50.0	0.595
$NaNO_3$	25.5	0.300		
NH_4Cl			15.0	0.280
$MgCl_2 \cdot 6(H_2O)$	12.16	0.0598	12.0	0.0590
$CaCl_2 \cdot 2(H_2O)$	4.41	0.0300	18.0	0.122
$MgSO_4 \cdot 7(H_2O)$	14.6	0.0592	15.0	0.0609
K_2HPO_4	1.044	0.00599		
KH_2PO_4			1.60	0.00919
$FeCl_3 \cdot 6(H_2O)$	0.160	0.000591	0.0640	0.000237
$Na_2EDTA \cdot 2(H_2O)$	0.300	0.000806	0.100	0.000269*
H_3BO_3	0.186	0.00300	0.185	0.00299
$MnCl_2 \cdot 4(H_2O)$	0.415	0.00201	0.415	0.00210
$ZnCl_2$	0.00327	0.000024	0.00300	0.0000220
$CoCl_2 \cdot 6(H_2O)$	0.00143	0.000006	0.00150	0.00000630
$Na_2MoO_4 \cdot 2(H_2O)$	0.00726	0.000030	0.00700	0.0000289
$CuCl_2 \cdot 2(H_2O)$	0.000012	0.00000007	0.00001	0.00000006
pH	7.5		8.1	

The molar ratio of EDTA to iron slightly exceeds unity. This prevents iron precipitation and at the same time, chelation of heavy metal ions is minimised.

In test with the diatom *Navicula pelliculosa* both media must be supplemented with $Na_2SiO_3 \cdot 9H_20$ to obtain a concentration of 1.4 mg Si/l.

The pH of the medium is obtained at equilibrium between the carbonate system of the medium and the partial pressure of CO_2 in atmospheric air. An approximate relationship between pH at 25 °C and the molar bicarbonate concentration is:

$pH_{eq} = 11.30 + \log[HCO_3]$

With 15 mg $NaHCO_3$/l, pH_{eq} = 7.5 (U.S. EPA medium) and with 50 mg $NaHCO_3$/l, pH_{eq} = 8.1 (OECD medium).

Element composition of test media

Element	AAP	OECD
	mg/l	mg/l
C	2.144	7.148
N	4.202	3.927
P	0.186	0.285
K	0.469	0.459
Na	11.044	13.704
Ca	1.202	4.905
Mg	2.909	2.913
Fe	0.033	0.017
Mn	0.115	0.115

Preparation of OECD medium

Nutrient	Concentration in stock solution
Stock solution 1: macro nutrients	
NH_4Cl	1.5 g/l
$MgCl_2 \cdot 6H_2O$	1.2 g/l
$CaCl_2 \cdot 2H_2O$	1.8 g/l
$MgSO_4 \cdot 7H_2O$	1.5 g/l
KH_2PO_4	0.16 g/l
Stock solution 2: iron	
$FeCl_3 \cdot 6H_2O$	64 mg/l
$Na_2EDTA \cdot 2H_2O$	100 mg/l
Stock solution 3: trace elements	
H_3BO_3	185 mg/l
$MnCl_2 \cdot 4H_2O$	415 mg/l
$ZnCl_2$	3 mg/l
$CoCl_2 \cdot 6H_2O$	1.5 mg/l
$CuCl_2 \cdot 2H_2O$	0.01 mg/l
$Na_2MoO_4 \cdot 2H_2O$	7 mg/l
Stock solution 4: bicarbonate	
$NaHCO_3$	50 g/l
$Na_2SiO_3 \cdot 9H_20$	

Sterilise the stock solutions by membrane filtration (mean pore diameter 0.2 μm) or by autoclaving (120 °C, 15 min). Store the solutions in the dark at 4 °C.

Do not autoclave stock solutions 2 and 4, but sterilise them by membrane filtration.

Prepare a growth medium by adding an appropriate volume of the stock solutions 1-4 to water:

Add to 500 ml of sterilised water:

10 ml of stock solution 1

1 ml of stock solution 2

1 ml of stock solution 3

1 ml of stock solution 4

Make up to 1 000 ml with sterilised water.

Allow sufficient time for equilibrating the medium with the atmospheric CO2, if

necessary by bubbling with sterile, filtered air for some hours.

Preparation of U.S. EPA medium

1. Add 1 ml of each stock solution in *2.1–2.7* to approximately 900 ml of deionised or distilled water and then dilute to 1 litre.

2. Macronutrient stock solutions are made by dissolving the following into 500 ml of deionised or distilled water. Reagents *2.1, 2.2, 2.3,* and *2.4* can be combined into one stock solution.

2.1	$NaNO_3$	12.750 g.
2.2	$MgCl_2 \cdot 6H_2O$	6.082 g.
2.3	$CaCl_2 \cdot 2H_2O$	2.205 g.
2.4	Micronutrient Stock Solution(see 3).	
2.5	$MgSO_4 \cdot 7H_2O$	7.350 g.
2.6	K_2HPO_4	0.522 g.
2.7	$NaHCO_3$	7.500 g.
2.8	$Na_2SiO_3 \cdot 9H_2O$	See Note 1.

NOTE 1: Use for diatom test species only. May be added directly (202.4 mg) or by way of stock solution to give 20 mg/l Si final concentration in medium.

3. The micronutrient stock solution is made by dissolving the following into 500 ml of deionised or distilled water:

3.1	H_3BO_3	92.760 mg.
3.2	$MnCl_2 \cdot 4H_2O$	207.690 mg.
3.3	$ZnCl_2$	1.635 mg.
3.4	$FeCl_3 \cdot 6H_2O$	79.880 mg.
3.5	CoCl2·6H2O	0.714 mg.
3.6	Na2$MoO_4 \cdot 2H_2O$	3.630 mg.
3.7	$CuCl_2 \cdot 2H_2O$	0.006 mg.
3.8	$Na_2EDTA \cdot 2H_2O$	150.000 mg. [Disodium (Ethylenedinitrilo) tetraacetate].
3.9	$Na_2SeO_4 \cdot 5H_2O$	0.005 mg See Note 2.

NOTE 2: Use only in medium for stock cultures of diatom species.

4. Adjust pH to 7.5± 0.1 with 0.1 *N* or 1.0 *N* NaOH or HCl.

5. Filter the media into a sterile container through either a 0.22 μm membrane filter if a particle counter is to be used or a 0.45 μm filter if a particle counter is not to be used.

6. Store medium in the dark at approximately 4°C until use.

Appendix 4

EXAMPLE OF A PROCEDURE FOR THE CULTURING OF ALGAE

General observations

The purpose of culturing on the basis of the following procedure is to obtain algal cultures for toxicity tests.

Use suitable methods to ensure that the algal cultures are not infected with bacteria. Axenic cultures may be desirable but unialgal cultures must be established and used.

All operations must be carried out under sterile conditions in order to avoid contamination with bacteria and other algae.

Equipment and materials

See under test method: Apparatus.

Procedures for obtaining algal cultures

Preparation of nutrient solutions (media):

All nutrient salts of the medium are prepared as concentrated stock solutions and stored dark and cold. These solutions are sterilised by filtration or by autoclaving.

The medium is prepared by adding the correct amount of stock solution to sterile distilled water, taking care that no infection occurs. For solid medium 0.8 per cent of agar is added.

Stock culture:

The stock cultures are small algal cultures that are regularly transferred to fresh medium to act as initial test material. If the cultures are not used regularly they are streaked out on sloped agar tubes. These are transferred to fresh medium at least once every two months.

The stock cultures are grown in conical flasks containing the appropriate medium (volume about 100 ml). When the algae are incubated at 20°C with continuous illumination, a weekly transfer is required.

During transfer an amount of "old" culture is transferred with sterile pipettes into a flask of fresh medium, so that with the fast-growing species the initial concentration is about 100 times smaller than in the old culture.

The growth rate of a species can be determined from the growth curve. If this is known, it is possible to estimate the density at which the culture should be transferred to new medium. This must be done before the culture reaches the death phase.

Pre-culture:

The pre-culture is intended to give an amount of algae suitable for the inoculation of test cultures. The pre-culture is incubated under the conditions of the test and used when still exponentially growing, normally after an incubation period of 2 to 4 days. When the algal cultures contain deformed or abnormal cells, they must be discarded.

Appendix 5

DATA ANALYSIS BY NONLINEAR REGRESSION

General considerations

The response in algal tests and other microbial growth tests - growth of biomass - is by nature a continuous or metric variable – a process rate if growth rate is used and its integral over time if biomass is selected. Both are referenced to the corresponding mean response of replicate non-exposed controls showing maximum response for the conditions imposed - with light and temperature as primary determining factors in the algal test. The system is distributed or homogenous and the biomass can be viewed as a continuum without consideration of individual cells. The variance distribution of the type of response for a such system relate solely to experimental factors (described typically by the log-normal or normal distributions of error). This is by contrast to typical bioassay responses with quantal data for which the tolerance (typically binomially distributed) of individual organisms are often assumed to be the dominant variance component. Control responses are here zero or background level.

In the uncomplicated situation, the normalised or relative response, r, decreases monotonically from 1 (zero inhibition) to 0 (100 per cent inhibition). Note, that all responses have an error associated and that apparent negative inhibitions can be calculated as a result of random error only.

Regression analysis

Models

A regression analysis aims at quantitatively describing the concentration response curve in the form of a mathematical regression function Y = f (C) or more frequently F(Z) where Z = log C. Used inversely $C = f^{-1}(Y)$ allows the calculation of, EC_x figures, including the EC_{50}, EC_{10} and EC_{20}, and their 95% confidence limits. Several simple mathematical functional forms have proved to successfully describe concentration - response relationships obtained in algal growth inhibition tests. Functions include for instance the logistic equation, the nonsymmetrical Weibul equation and the log normal distribution function, which are all sigmoid curves asymptotically approaching zero for $C \rightarrow 0$ and one for $C \rightarrow$ infinity.

The use of continuous threshold function models (e.g. the Kooijman model "for inhibition of population growth" Kooijman et al. 1996) is a recently proposed or alternative to asymptotic models. This model assumes no effects at concentrations below a certain threshold EC_0+ that is estimated by extrapolation of the response concentration relationship to intercept the concentration axis using a simple continuous function that is not differentiable in the starting point.

Note that the analysis can be a simple minimisation of sums of residual squares (assuming constant variance) or weighted squares if variance heterogeneity is

compensated.

Procedure

The procedure can be outlined as follows: Select an appropriate functional equation, Y = f(C), and fit it to the data by non-linear regression. Use preferably the measurements from each individual flask rather than means of replicates, in order to extract as much information from the data as possible. If the variance is high, on the other hand, practical experience suggests that means of replicates may provide a more robust mathematical estimation less influenced by systematic errors in the data, than with each individual data point retained.

Plot the fitted curve and the measured data and examine whether the curve fit is appropriate. Analysis of residuals may be a particular helpful tool for this purpose. If the chosen functional relationship to fit the concentration response does not describe well the whole curve or some essential part of it, such as the response at low concentrations, choose another curve fit option - e.g., a non-symmetrical curve like the Weibul function instead of a symmetrical one. Negative inhibitions may be a problem with for instance the log - normal distribution function likewise demanding an alternative regression function. It is not recommended to assign a zero or small positive value to such negative values because this distorts the error distribution. It may be appropriate to make separate curve fits on parts of the curve such as the low inhibition part to estimate EC_{lowx} figures. Calculate from the fitted equation (by "inverse estimation", $C = f^{-1}(Y)$), characteristic point estimates EC_x´s, and report as a minimum the EC_{50} and one or two $EC_{low\ x}$ estimates. Experience from practical testing has shown that the precision of the algal test normally allows a reasonably accurate estimation at the 10 % inhibition level if data points are sufficient - unless stimulation occurs at low concentrations as a confounding factor. The precision of an EC_{20} estimate is often considerably better than that of an EC_{10}, because the EC_{20} is usually positioned on the approximately linear part of the central concentration response curve. Sometimes EC_{10} can be difficult to interpret because of growth stimulation. So while the EC_{10} is normally obtainable with a sufficient accuracy it is recommended to report always also the EC_{20}.

Weighting factors

The experimental variance generally is not constant and typically includes a proportional component, and a weighted regression is therefore advantageously carried out routinely. Weighting factors for a such analysis are normally assumed inversely proportional to the variance:

$$W_i = 1/Var(r_i)$$

Many regression programs allow the option of weighted regression analysis with weighting factors listed in a table. Conveniently weighting factors should be normalised by multiplying them by $n/\Sigma\ w_i$ (n is the number of datapoints) so their sum be one.

Normalising responses

Normalising by the mean control response gives some principle problems and gives rise to a rather complicated variance structure. Dividing the responses by the mean control response for obtaining the percentage of inhibition, one introduces an additional error caused by the error on the control mean. Unless this error is negligibly small, weighting factors in the regression and confidence limits must be corrected for the covariance with the control (Draper and Smith, 1981). Note that high precision on the estimated mean control response is important in order to minimise the overall variance for the relative response. This variance is as follows:

(Subscript i refers to concentration level i and subscript 0 to the controls)

Yi = Relative response = $r_i/r_0 = 1 - I = f(C_i)$

with a variance $Var(Y_i) = Var(r_i/r_0) \cong (\partial Yi / \partial r_i)^2 \cdot Var(r_i) + ((\partial Y_i/\partial r_0)^2 \cdot Var(r_0)$

and since $(\partial Y_i/\partial r_i) = 1/r_0$ and $(\partial Y_i/\partial r_0) = r_i/r_0^2$

with normally distributed data and m_i and m_0 replicates: $Var(r_i) = \sigma^2/m_i$

the total variance of the relative responseY_i thus becomes

$Var(Y_i) = \sigma^2/(r_0^2 \cdot m_i) + r_i^2 \cdot \sigma^2/r_0^4 \cdot m_0$

The error on the control mean is inversely proportional to the square root of the number of control replicates averaged, and sometimes it can be justified to include historic data and in this way greatly reduce the error. An alternative procedure is not to normalise the data and fit the absolute responses including the control response data but introducing the control response value as an additional parameter to be fitted by non linear regression. With a usual 2 parameter regression equation, this method necessitates the fitting of 3 parameters, and therefore demands more data points than non-linear regression on data that are normalised using a pre-set control response .

Inverse confidence intervals

The calculation of non-linear regression confidence intervals by inverse estimation is rather complex and not an available standard option in ordinary statistical computer program packages. Approximate confidence limits may be obtained with standard non-linear regression programs with re-parameterisation (Bruce and Versteeg, 1992)**,** which involves rewriting the mathematical equation with the desired point estimates, e.g. the EC_{10} and the EC_{50} as the parameters to be estimated. (Let the function be I = f (α, β, Concentration) and utilise the definition relationships f (α, β, EC_{10}) = 0.1 and f (α, β, EC_{50}) = 0.5 to substitute f (α, β, concentration) with an equivalent function g(EC_{10}, EC_{50}, concentration).

A more direct calculation (Andersen et al, 1998) is performed by retaining the original equation and using a Taylor expansion around the means of r_i and r_0.

Recently "boot strap methods" have become popular. Such methods use the measured

data and a random number generator directed frequent re-sampling to estimate an empirical variance distribution.

REFERENCES

Kooijman, S.A.L.M.; Hanstveit, A.O.; Nyholm, N. (1996): No-effect concentrations in algal growth inhibition tests. *Water Research*, 30, 1625-1632.

Draper, N.R. and Smith, H. (1981). Applied Regression Analysis, second edition. Wiley, New York.

Bruce, R..D. and Versteeg,, D.J. (1992). A Statistical Procedure for Modelling Continuous Ecotoxicity Data. *Environ. Toxicol. Chem.*11, 1485-1494.

Andersen, J.S., Holst, H., Spliid, H., Andersen, H., Baun, A. & Nyholm, N. (1998). Continuous ecotoxicological data evaluated relative to a control response. *Journal of Agricultural, Biological and Environmental Statistics*, 3, 405-420."

(4) Chapter C.11 is replaced by the following:

"C.11. Activated Sludge, Respiration Inhibition Test (Carbon and Ammonium Oxidation)

INTRODUCTION

1. This test method is equivalent to OECD test guideline (TG) 209 (2010). This test method describes a method to determine the effects of a chemical on micro-organisms from activated sludge (largely bacteria) by measuring their respiration rate (carbon and/or ammonium oxidation) under defined conditions in the presence of different concentrations of the test chemical. The test method is based on the ETAD (Ecological and Toxicological Association of the Dyestuffs Manufacturing industry) test (1) (2), on the previous OECD TG 209 (3) and on the revised ISO Standard 8192 (4). The purpose of the test is to provide a rapid screening method to assess the effects of chemicals on the microorganisms of the activated sludge of the biological (aerobic) stage of waste-water treatment plants. The results of the test may also serve as an indicator of suitable non-inhibitory concentrations of test chemicals to be used in biodegradability tests (for example Chapters C.4 A-F, C.9, C.10, C12 and C.29 of this Annex, OECD TG302C). In this case, the test can be performed as a screening test, similar to a range-finding or limit test (see paragraph 39), considering the overall respiration only. However, this information should be taken with care for ready biodegradability tests (Chapter C.4 A-F and C.29 of this Annex) for which the inoculum concentration is significantly lower than the one used in this test method. Indeed, an absence of inhibition in this respiration test does not automatically result in non-inhibitory conditions in the ready biodegradability test of Chapters C.4 A-F or C.29 of this Annex.

2. Overall, the respiration inhibition test seems to have been applied successfully since it was first published, but on some occasions spurious results were reported, e.g. (2) (4) (5). Concentration related respiration curves are sometimes bi-phasic, dose-response plots have been distorted and EC_{50} values have been unexpectedly low (5). Investigations showed that such results are obtained when the activated sludge used in the test nitrifies significantly and the test chemical has a greater effect on the oxidation of ammonium than on general heterotrophic oxidation. Therefore, these spurious results may be overcome by performing additional testing using a specific inhibitor of nitrification. By measuring the oxygen uptake rates in the presence and absence of such an inhibitor, e.g. N-allylthiourea (ATU), the separate total, heterotrophic and nitrification oxygen uptake rates can be calculated (4) (7) (8). Thus, the inhibitory effects of a test chemical on the two processes may be determined and the EC_{50} values for both organic carbon oxidation (heterotrophic) and ammonium oxidation (nitrification) may be calculated in the usual way. It should be noted that in some rare cases, the inhibitory effect of N-allylthiourea may be partially or completely nullified as a result of complexation with test chemicals or medium supplements, e.g. Cu^{++} ions (6). Cu^{++} ions are essential for *Nitrosomonas*, but are toxic in higher concentration.

3. The need for nitrification in the aerobic treatment of wastewaters, as a necessary step in the process of removing nitrogen compounds from wastewaters by denitrification to gaseous products, has become urgent particularly in European countries; the EU has now set lower limits for the concentration of nitrogen in treated effluents discharged to receiving waters[1].

4. For most purposes, the method to assess the effect on organic carbon oxidation processes alone is adequate. However, in some cases an examination of the effect on nitrification alone, or on both nitrification and organic carbon oxidation separately, are needed for the interpretation of the results and understanding the effects.

PRINCIPLE OF THE TEST METHOD

5. The respiration rates of samples of activated sludge fed with synthetic sewage are measured in an enclosed cell containing an oxygen electrode after a contact time of 3 hours. Under consideration of the realistic exposure scenario, longer contact times could be appropriate. If the test chemical is rapidly degraded e.g. abiotically via hydrolysis, or is volatile and the concentration cannot be adequately maintained, additionally a shorter exposure period e.g. 30 minutes can be used. The sensitivity of each batch of activated sludge should be checked with a suitable reference chemical on the day of exposure. The test is typically used to determine the EC_x (e.g. EC_{50}) of the test chemical and/or the no-observed effect concentration (NOEC).

6. The inhibition of oxygen uptake by micro-organisms oxidising organic carbon may be separately expressed from that by micro-organisms oxidising ammonium by measurement of the rates of uptake of oxygen in the absence and presence of N-allylthiourea, a specific inhibitor of the oxidation of ammonium to nitrite by the first-stage nitrifying bacteria. In this case the percentage inhibition of the rate of oxygen uptake is calculated by comparison of the rate of oxygen uptake in the presence of a test chemical with the mean oxygen uptake rate of the corresponding controls containing no test chemical, both in the presence and absence of the specific inhibitor, N-allylthiourea.

7. Any oxygen uptake arising from abiotic processes may be detected by determining the rate in mixtures of test chemical, synthetic sewage medium and water, omitting activated sludge.

INFORMATION OF THE TEST CHEMICAL

[1] Council Directive 91/271/EEC of 21 May 1991 concerning urban waste-water treatment. OJ L 135, 30/05/1991, p. 40–52

8. The identification (preferably CAS number), name (IUPAC), purity, water solubility, vapour pressure, volatility and adsorption characteristics of the test chemical should be known to enable correct interpretation of results to be made. Normally, volatile chemicals cannot be tested adequately unless special precautions are taken (see paragraph 21).

APPLICABILITY OF THE TEST METHOD

9. The test method may be applied to water-soluble, poorly soluble and volatile chemicals. However, it may not always be possible to obtain EC_{50} values with chemicals of limited solubility and valid results with volatile chemicals may only be obtained providing that the bulk (say > 80%) of the test chemical remains in the reaction mixture at the end of the exposure period(s). Additional analytical support data should be submitted to refine the EC_x concentration when there is any uncertainty regarding the stability of the test chemical or its volatility.

REFERENCE CHEMICALS

10. Reference chemicals should be tested periodically in order to assure that the test method and test conditions are reliable, and to check the sensitivity of each batch of activated sludge used as microbial inoculum on the day of exposure. The chemical 3,5-dichlorophenol (3,5-DCP) is recommended as the reference inhibitory chemical, since it is a known inhibitor of respiration and is used in many types of test for inhibition/toxicity (4). Also copper (II) sulphate pentahydrate can be used as a reference chemical for the inhibition of total respiration (9). N-methylaniline can be used as a specific reference inhibitor of nitrification (4).

VALIDITY CRITERIA AND REPRODUCIBILITY

11. The blank controls (without the test chemical or reference chemical) oxygen uptake rate should not be less than 20 mg oxygen per one gramme of activated sludge (dry weight of suspended solids) in an hour. If the rate is lower, the test should be repeated with washed activated sludge or with the sludge from another source. The coefficient of variation of oxygen uptake rate in control replicates should not be more than 30% at the end of definitive test.

12. In a 2004 international ring test organised by ISO (4) using activated sludge derived from domestic sewage, the EC_{50} of 3,5-DCP was found to lie in the range 2 mg/l to 25 mg/l for total respiration, 5 mg/l to 40 mg/l for heterotrophic respiration and 0.1 mg/l to 10 mg/l for nitrification respiration. If the EC_{50} of 3,5-DCP does not lie in the expected range, the test should be repeated with activated sludge from another source. The EC_{50} of copper (II) sulphate pentahydrate should lie in the range of 53-155 mg/l for the total respiration (9).

DESCRIPTION OF THE TEST METHOD

Test vessels and apparatus

13. Usual laboratory equipment and the following should be used:

(a) Test vessels – for example, 1000 ml beakers to contain 500 ml of reaction mixture (see 5 in Fig.1);

(b) Cell and attachments for measuring concentration of dissolved oxygen; a suitable oxygen electrode; an enclosed cell to contain the sample with no headspace and a recorder (e.g. 7, 8, 9 in Fig.1 of Appendix 2); alternatively, a BOD bottle may be used with a suitable sleeve adaptor for sealing the oxygen electrode against the neck of the bottle (see Fig. 2 of Appendix 3). To avoid loss of displaced liquid on insertion of the oxygen electrode, it is advisable first to insert a funnel or glass tube through the sleeve, or to use vessels with flared-out rims. In both cases a magnetic stirrer or alternative stirrer method, e.g. self-stirring probe, should be used;

(c) Magnetic stirrers and followers, covered with inert material, for use in measurement chamber and/or in the test vessels;

(d) Aeration device: if necessary, compressed air should be passed through an appropriate filter to remove dust and oil and through wash bottles containing water to humidify the air. The contents of vessels should be aerated with Pasteur pipettes, or other aeration devices, which do not adsorb chemicals. An orbital shaker operated at orbiting speeds between 150 and 250 rpm with flasks of, for example, 2000 ml capacity, can be used to satisfy the oxygen demand for the sludge and overcome difficulties with chemicals that produce excessive foam, are volatile and therefore lost, or are difficult to disperse when aerated by air sparging. The test system is typically a number of beakers aerated continuously and sequentially established (e.g. at ca. 10 – 15 minute intervals), then analysed in a sequential manner. Validated instrumentation that allows the simultaneous aeration and measurement of the oxygen consumption rate in the mixtures may also be used;

(e) pH-meter;

(f) Centrifuge, general bench-top centrifuge for sludge capable of 10,000 m/s^2.

Reagents

14. Analytical grade reagents should be used throughout.

Water

15. Distilled or deionised water, containing less than 1mg/l DOC, should be used except where chlorine free tap water is specified.

Synthetic sewage feed

16. The medium should be prepared to contain the following constituents at the stated amounts:

- peptone 16 g
- meat extract (or a comparable vegetable extract) 11 g
- urea 3 g
- sodium chloride (NaCl) 0.7 g
- calcium chloride dihydrate ($CaC1_2$, $2H_2O$) 0.4 g
- magnesium sulphate heptahydrate ($MgSO_4$, $7H_2O$) 0.2 g
- anhydrous potassium monohydrogen phosphate (K_2HPO_4) 2. 8g
- distilled or deionised water to 1 litre

17. The pH of this solution should be 7.5 ± 0.5. If the prepared medium is not used immediately, it should be stored in the dark at 0^oC to 4^oC, for no longer than 1 week or under conditions, which do not change its composition. It should be noted that this synthetic sewage is a 100 fold concentrate of that described in the OECD Technical Report "Proposed method for the determination of the biodegradability of surfactants used in synthetic detergents" June 11, 1976, with moreover dipotassium hydrogen phosphate added.

18. Alternatively, components of the medium can be sterilised individually prior to storage, or the peptone and meat extract can be added shortly before carrying out the test. Prior to use, the medium should be thoroughly mixed and the pH adjusted if necessary to pH 7.5 ± 0.5.

Test chemical

19. A stock solution should be prepared for readily water soluble test substances up to the maximum water solubility only (precipitations are not acceptable). Poorly water soluble substances, mixtures with components of different water solubility and adsorptive substances should be directly weighed into the test vessels. In these cases, use of stock solutions may be an alternative if dissolved concentrations of the test chemicals are analytically determined in the test vessels (prior to adding activated sludge). If water accommodated fractions (WAFs) are prepared, an analytical determination of the dissolved concentrations of the test chemicals in the test vessels is also essential. Using organic solvents, dispersants/emulsifiers to improve solubility should be avoided. Ultrasonication of stock solutions and pre-stirring suspensions, e.g. overnight, is possible when there is adequate information available concerning the stability of the test chemical under such conditions.

20. The test chemical may adversely affect pH within the test system. The pH of the test chemical-treated mixtures should be determined prior to the test set up, in a preliminary trial, to ascertain whether pH adjustment will be necessary prior the main test and again on the day of the main test. Solutions/suspensions of test chemical in water should be neutralised prior to inoculum addition, if necessary. However, since neutralisation may change the chemical properties of the chemical, further testing, depending on the purposes of the study, could be performed to assess the effect of the test chemical on the sludge without pH adjustment.

21. The toxic effects of volatile chemicals, especially in tests in which air is bubbled

through the system, can result in variable effect levels occurring owing to losses of the substance during the exposure period. Caution should be exercised with such substances by performing substance specific analysis of control mixtures containing the substance and modifying the aeration regime.

Reference chemical

22. If 3,5-dichlorophenol is used as reference chemical, a solution of 1.00 g of 3,5-dichlorophenol in 1000 ml of water should be prepared (15). Warm water and/or ultrasonication should be used to accelerate the dissolution and make the solution up to volume when it has cooled to room temperature. However, it should be ensured that the reference chemical is not structurally changed. The pH of the solution should be checked and adjusted, if necessary, with NaOH or H_2SO_4 to pH 7 – 8.

23. If copper(II)sulphate pentahydrate is used as a reference chemical, concentrations of 58 mg/l, 100 mg/l and 180 mg/l (a factor of 1.8) are used. The substance is weighed in directly into the test vessels (29 – 50 – 90 mg for 500 ml total volume). It is then dissolved with 234 ml of autoclaved tap water. Copper(II)sulphate pentahydrate is easily soluble. When the test is started, 16 ml of synthetic sewage and 250 ml of activated sludge are added.

Specific inhibitor of nitrification

24. A 2.32 g/l stock solution of N-allylthiourea (ATU) should be prepared. The addition of 2.5 ml of this stock solution to an incubation mixture of final volume of 500 ml results in a final concentration of 11.6 mg ATU/l (10^{-4}mol/l) which is known to be sufficient (4) to cause 100% inhibition of nitrification in a nitrifying activated sludge containing 1.5g/l suspended solids.

Abiotic control

25. Under some rare conditions, a test chemical with strong reducing properties may cause measurable abiotic oxygen consumption. In such cases, abiotic controls are necessary to discriminate between abiotic oxygen uptake by the test chemical and microbial respiration. Abiotic controls may be prepared by omitting the inoculum from the test mixtures. Similarly, abiotic controls without inoculum may be included when supporting analytical measurements are performed to determine the achieved concentration during the exposure phase of the test, e.g. when using stock solutions of poorly water soluble chemicals with components with different water solubility. In specific cases it may be necessary to prepare an abiotic control with sterilised inoculum (e.g. by autoclaving or adding sterilising toxicants). Some chemicals may produce or consume oxygen only if the surface area is big enough for reaction, even if they normally need a much higher temperature or pressure to do so. In this respect special attention should be given to peroxy substances. A sterilised inoculum provides a big surface area.

Inoculum

26. For general use, activated sludge should be collected from the exit of the aeration tank, or near the exit from the tank, of a well-operated wastewater treatment plant receiving predominantly domestic sewage. Depending on the purpose of the test, other adequate types or sources of activated sludge, e.g. sludge grown in the laboratory, may also be used at suitable suspended solids concentrations of 2 g/l to 4 g/l. However, sludges from different treatment plants are likely to exhibit different characteristics and sensitivities.

27. The sludge may be used as collected but coarse particles should be removed by settling for a short period, e.g. 5 to 15 minutes, and decanting the upper layer of finer solids or sieving (e.g. 1 mm^2 mesh). Alternatively, the sludge may be homogenised in a blender for a *ca*. 15 seconds or longer, but caution is needed regarding the shear forces and the temperature change which might occur for long periods of blending.

28. Washing the sludge is often necessary, e.g. if the endogenous respiration rate is low. The sludge should first be centrifuged for a period to produce a clear supernatant and pellet of sewage solids e.g. 10 minutes at ca.10,000 m/s^2. The supernatant liquid should be discarded and the sludge re-suspended in chlorine-free tap water, with shaking, and the wash-water should then be removed by re-centrifuging and discarding again. The washing and centrifuging process should be repeated, if necessary. The dry mass of a known volume of the re-suspended sludge should be determined and the sludge concentrated by removing liquor or diluted further in chlorine-free tap water to obtain the required sludge solids concentration of 3 g/l. The activated sludge should be continuously aerated (e.g. 2 l/minute) at the test temperature and, where possible used on day of collection. If this is not possible, the sludge should be fed daily with the synthetic sewage feed (50 ml synthetic sewage feed/l activated sludge) for two additional days. The sludge is then used for the test and the results are accepted as valid, provided that no significant change in its activity, assessed by its endogenous heterotrophic and nitrification respiration rate, has occurred.

29. Difficulties can arise if foaming occurs during the incubation to the extent that the foam and the sludge solids carried on it, are expelled from the aeration vessels. Occasionally, foaming may simply result from the presence of the synthetic sewage, but foaming should be anticipated if the test chemical is, or contains, a surfactant. Loss of sludge solids from the test mixtures will result in artificially lowered respiration rates that could mistakenly be interpreted as a result of inhibition. In addition, aeration of surfactant solution concentrates the surfactant in the foam layer; loss of foam from the test system will lower the exposure concentrations. The foaming can be controlled by simple mechanical methods (e.g. occasional manual stirring using a glass rod) or by adding a surfactant-free silicone emulsion antifoam agent and/or use the shake flask aeration method. If the problem is associated with the presence of the synthetic sewage, the sewage composition should be modified by including an antifoam reagent at a rate of e.g. 50 µl/l. If foaming is caused by the test chemical, the quantity needed for abatement should be determined at the maximum test concentration, and then all individual aeration vessels should be identically treated (including those, e.g. blank controls and reference vessels where foam is absent). If antifoam agents are used, there should be no interaction with inoculum and/or test chemical.

TEST PROCEDURE

30. The inhibition of three different oxygen uptakes may be determined, total, heterotrophic only and that due to nitrification. Normally, the measurement of total oxygen uptake inhibition should be adequate. The effects on heterotrophic oxygen uptake from the oxidation of organic carbon, and due to the oxidation of ammonium are needed when there is a specific requirement for such two separate end-points for a particular chemical or (optionally) to explain atypical dose-response curves from inhibition of total oxygen uptake.

Test conditions

31. The test should be performed at a temperature within the range 20±2°C.

Test mixtures

32. Test mixtures (F_T as in Table 1) containing water, synthetic sewage feed and the test chemical should be prepared to obtain different nominal concentrations of the test chemical (See Table 1 for example of volumes of constituents). The pH should be adjusted to 7.5 ± 0.5, if necessary; mixtures should be diluted with water and the inoculum added to obtain equal final volumes in the vessels and to begin the aeration.

Reference mixtures

33. Mixtures (F_R) should be prepared with the reference chemical, e.g. 3,5-dichlorophenol, in place of the test chemical in the same way as the test mixtures.

Blank controls

34. Blank controls (F_B) should be prepared at the beginning and end of the exposure period in tests in which the test beakers are set up sequentially at intervals. In tests performed using equipment which allows simultaneous measurements of oxygen consumption to be made, at least two blank controls should be included in each batch of simultaneous analysis. Blank controls contain an equal volume of activated sludge and synthetic medium but not test or reference chemical. They should be diluted with water to the same volume as the test and reference mixtures.

Abiotic control

35. If necessary, for example if a test chemical is known or suspected to have strong reducing properties, a mixture F_A should be prepared to measure the abiotic oxygen consumption. The mixture should have the same amounts of test chemical, synthetic sewage feed and the same volume as the test mixtures, but no activated sludge.

General procedure and measurements

36. Test mixtures, reference mixtures and the blank and abiotic controls are incubated at

the test temperature under conditions of forced aeration (0.5 to 1 l/min) to keep the dissolved oxygen concentration above 60 – 70% saturation and to maintain the sludge flocs in suspension. Stirring the cultures is also necessary to maintain sludge flocs in suspension. The incubation is considered to begin with the initial contact of the activated sludge inoculum with the other constituents of the final mixture. At the end of incubation, after the specified exposure times of usually 3 hours, samples are withdrawn to measure the rate of decrease of the concentration of dissolved oxygen in the cell designed for the purpose (Fig.2 of Appendix 3) or in a completely filled BOD bottle. The manner in which the incubations begin also depends on the capacity of the equipment used to measure oxygen consumption rates. For example, if it comprises a single oxygen probe, the measurements are made individually. In this case, the various mixtures needed for the test in synthetic sewage should be prepared but the inoculum should be withheld, and the requisite portions of sludge should be added to each vessel of the series. Each incubation should be started in turn, at conveniently timed intervals of e.g. 10 to 15 minutes. Alternatively, the measuring system may comprise multiple probes that facilitate multiple simultaneous measurements; in this case, inoculum may be added at the same time to appropriate groups of vessels.

37. The activated sludge concentration in all test, reference and blank (but not abiotic control) mixtures is nominally 1.5 g/l of suspended solids. The oxygen consumption should be measured after 3 hours of exposure. Additional 30-minute exposure measurements should be performed as appropriate and previously described in paragraph 5.

Nitrification potential of sludge

38. In order to decide whether sludge nitrifies and, if so, at what rate, mixtures (F_B) as in the blank control and additional 'control' mixtures (F_N) but which also contain N-allylthiourea at 11.6 mg/l should be prepared. The mixtures should be aerated and incubated at 20° ± 2°C for 3 hours. Then the rates of oxygen uptake should be measured and the rate of oxygen uptake due to nitrification calculated.

Test designs

Range-finding test

39. A preliminary test is used, when necessary, to estimate the range of concentrations of the test chemical needed in a definitive test for determining the inhibition of oxygen consumption. Alternatively, the absence of inhibition of oxygen consumption by the test chemical in a preliminary test may demonstrate that a definitive test is unnecessary, but triplicates at the highest tested concentration of the preliminary test (typically 1000 mg/l, but dependent on the data requirement) should be included.

Table 1: Examples of mixtures for the preliminary test

Reagent	Original Concentration

Test chemical stock solution Synthetic medium stock solution Activated sludge stock suspension	10 g/l See paragraph 16 3 g/l of suspended solids				
Components of mixtures	Dosing into test vessels (a)				
	F_{T1}	F_{T2}	F_{T3-5}	F_{B1-2}	F_A
Test chemical stock solution (ml) (paragraphs 19 to 21)	0.5	5	50	0	50
Synthetic sewage feed stock solution (ml) (paragraph 16)	16	16	16	16	16
Activated sludge suspension (ml) (paragraphs 26 to 29)	250	250	250	250	0
Water (paragraph 15)	233.5	229	184	234	434
Total volume of mixtures (ml)	500	500	500	500	500
Concentrations in the mixture					
Test suspension (mg/l)	10	100	1000	0	1000
Activated sludge (suspended solids) (mg/l)	1500	1500	1500	1500	0
(a) The same procedure should be followed with the reference chemical, to give flasks F_{R1-3}					

40. The test should be performed using at least three concentrations of the test chemical, for example, 10 mg/l, 100 mg/l and 1000 mg/l with a blank control and, if necessary, at least three abiotic controls with the highest concentrations of the test chemical (see as example Table 1). Ideally the lowest concentration should have no effect on oxygen consumption. The rates of oxygen uptake and the rate of nitrification, if relevant, should be calculated; then the percentage inhibition should be calculated. Depending on the purpose of the test, it is also possible to simply determine the toxicity of a limit concentration, e.g. 1000 mg/l. If no statistically significant toxic effect occurs at this concentration, further testing at higher or lower concentrations is not necessary. It should be noted that poorly water soluble substances, mixtures with components of different water solubility and adsorptive substances should be directly weighed into the test vessels. In this case, the volume reserved for the test substance stock solution should be replaced with dilution water.

Definitive test

Inhibition of total oxygen uptake

41. The test should be carried out using a range of concentrations deduced from the preliminary test. In order to obtain both a NOEC and an EC_x (e.g. EC_{50}), six controls and five treatment concentrations in a geometric series with five replicates are in most cases recommended. The abiotic control does not need to be repeated if there was no oxygen uptake in the preliminary test, but if significant uptake occurs abiotic controls should be included for each concentration of test chemical. The sensitivity of the sludge should be checked using the reference chemical 3.5-dichlorophenol. The sludge sensitivity should be checked for each test series, since the sensitivity is known to

fluctuate. In all cases, samples are withdrawn from the test vessels after 3 hours, and additionally 30 minutes if necessary, for measurement of the rate of oxygen uptake in the oxygen electrode cell. From the data collected, the specific respiration rates of the control and test mixtures are calculated; the percentage inhibition is then calculated from equation 7, below.

Differentiation between inhibition of heterotrophic respiration and nitrification

42. The use of the specific nitrification inhibitor, ATU, enables the direct assessment of the inhibitory effects of test chemicals on heterotrophic oxidation, and by subtracting the oxygen uptake rate in the presence of ATU from the total uptake rate (no ATU present), the effects on the rate of nitrification may be calculated. Two sets of reaction mixtures should be prepared according to the test designs for EC_x or NOEC described in paragraph 41, but additionally, ATU should be added to each mixture of one set at a final concentration of 11.6 mg/l, which has been shown to inhibit nitrification completely in sludge with suspended solids concentrations of up to 3000 mg/l (4). The oxygen uptake rates should be measured after the exposure period; these direct values represent heterotrophic respiration only, and the differences between these and the corresponding total respiration rates represent nitrification. The various degrees of inhibition are then calculated.

Measurements

43. After the exposure period(s) a sample from the first aeration vessel should be transferred to the oxygen electrode cell (Fig. 1 of Appendix 2) and the concentration of dissolved oxygen should immediately be measured. If a multiple electrode system is available, then the measurements may be made simultaneously. Stirring (by means of a covered magnet) is essential at the same rate as when the electrode is calibrated to ensure that the probe responds with minimal delay to changing oxygen concentrations, and to allow regular and reproducible oxygen measurements in the measuring vessel. Usually, the self-stirring probe system of some oxygen electrodes is adequate. The cell should be rinsed with water between measurements. Alternatively, the sample can be used to fill a BOD bottle (Fig. 2 of Appendix 3) fitted with a magnetic stirrer. An oxygen probe with a sleeve adaptor should then be inserted into the neck of the bottle and the magnetic stirrer should be started. In both cases the concentration of dissolved oxygen should continuously be measured and recorded for a period, usually 5 to 10 minutes or until the oxygen concentration falls below 2 mg/l. The electrode should be removed, the mixture returned to the aeration vessel and aerating and stirring should be continued, if measurement after longer exposure periods is necessary.

Verification of the test chemical concentration

44. For some purposes, it may be necessary to measure the concentration of the test chemical in the test vessels. It should be noted that if stock solutions of:

- poorly water soluble substances,
- mixtures with components with different water solubility, or

- substances with good water solubility, but where the concentration of the stock solution is near the maximum water solubility,

are used, the dissolved fraction is unknown, and the true concentration of the test chemical that is transferred into the test vessels is not known. In order to characterise the exposure, an analytical estimation of the test chemical concentrations in the test vessels is necessary. To simplify matters, analytical estimation should be performed before the addition of the inoculum. Due to the fact that only dissolved fractions will be transferred into test vessels, measured concentrations may be very low.

45. To avoid time-consuming and expensive analytics, it is recommended to simply weigh the test chemical directly into the test vessels and to refer to the initial weighed nominal concentration for subsequent calculations. A differentiation between dissolved, undissolved or adsorbed fractions of the test chemical is not necessary because all these fractions appear under real conditions in a waste water treatment plant likewise, and these fractions may vary depending on the composition of the sewage. The aim of the test method is to estimate a non inhibitory concentration realistically and it is not suitable to investigate in detail which fractions make a contribution to the inhibition of the activated sludge organisms. Finally, adsorptive substances should be also weighed directly into the test vessels; and the vessels should be silanised in order to minimise losses through adsorption.

DATA AND REPORTING

Calculation of oxygen uptake rates

46. The oxygen uptake rates should be calculated from the mean of the measured values, e.g. from the linear part of the graphs of oxygen concentration versus time, limiting the calculations to oxygen concentrations between 2.0 mg/l and 7.0 mg/l, since higher and lower concentrations may themselves influence rates of consumption. Excursion into concentration bands below or above these values is occasionally unavoidable and necessary, for example, when respiration is heavily suppressed and consequently very slow or if a particular activated sludge respires very quickly. This is acceptable provided the extended sections of the uptake graph are straight and their gradients do not change as they pass through the 2.0 mg/l or 7.0 mg/l O_2 boundaries. Any curved sections of the graph indicate that the measurement system is stabilising or the uptake rate is changing and should not be used for the calculation of respiration rates. The oxygen uptake rate should be expressed in milligrammes per litre per hour (mg/lh) or milligrammes per gramme dry sludge per hour (mg/gh). The oxygen consumption rate, R, in mg/lh, may be calculated or interpolated from the linear part of the recorded oxygen decrease graph according to Equation 1:

$$R = (Q_1 - Q_2)/\Delta_t \times 60 \qquad (1)$$

where:
Q_1 is the oxygen concentration at the beginning of the selected section of the linear phase (mg/l);

Q_2 is the oxygen concentration at the end of the selected section of the linear phase (mg/l);
Δ_t is the time interval between these two measurements (min.).

47. The specific respiration rate (R_s) is expressed as the amount of oxygen consumed per g dry weight of sludge per hour (mg/gh) according to Equation 2:

$$R_s = R/SS \qquad (2)$$

where SS is the concentration of suspended solids in the test mixture (g/l).

48. The different indices of R which may be combined are:

S	specific rate
T	total respiration rate
N	rate due to nitrification respiration
H	rate due to heterotrophic respiration
A	rate due to abiotic processes
B	rate based on blank assays (mean)

Calculation of oxygen uptake rate due to nitrification

49. The relationship between total respiration (R_T), nitrification respiration (R_N) and heterotrophic respiration (R_H) is given by Equation 3:

$$R_N = R_T - R_H \qquad (3)$$

where:
R_N is the rate of oxygen uptake due to nitrification (mg/lh);
R_T is the measured rate of oxygen uptake by the blank control (no ATU; F_B) (mg/lh).
R_H is the measured rate of oxygen uptake of the blank control with added ATU (F_N) (mg/lh).

50. This relationship is valid for blank values (R_{NB}, R_{TB}, R_{HB}), abiotic controls (R_{NA}, R_{TA}, R_{HA}) and assays with test chemicals (R_{NS}, R_{TS}, R_{HS}) (mg/gh). Specific respiration rates are calculated from:

$$R_{NS} = R_N/SS \qquad (4)$$
$$R_{TS} = R_T/SS \qquad (5)$$
$$R_{HS} = R_H/SS \qquad (6)$$

51. If R_N is insignificant (e.g. < 5% of R_T in blank controls) in a preliminary test, it may be assumed that the heterotrophic oxygen uptake equals the total uptake and that no nitrification is occurring. An alternative source of activated sludge would be needed if

the tests were to consider effects on heterotrophic and nitrifying micro-organisms. A definitive test is performed if there is evidence of suppressed oxygen uptake rates with different test chemical concentrations.

Calculation of percentage of inhibition

52. The percentage inhibition, I_T, of <u>total</u> oxygen consumption at each concentration of test chemical, is given by Equation 7:

$$I_T = [1 - (R_T - R_{TA})/R_{TB}] \times 100\% \qquad (7)$$

53. Similarly, the percentage inhibition of <u>heterotrophic</u> oxygen uptake, I_H, at each concentration of test chemical, is given by Equation 8:

$$I_H = [1 - (R_H - R_{HA})/R_{HB}] \times 100\% \qquad (8)$$

54. Finally, the inhibition of oxygen uptake due to nitrification, I_N, at each concentration, is given by Equation 9:

$$I_N = [1 - (R_T - R_H)/(R_{TB} - R_{HB})] \times 100\% \qquad (9)$$

55. The percentage inhibition of oxygen uptake should be plotted against logarithm of the test chemical concentration (inhibition curve, see Fig.3 of Appendix 4). Inhibition curves are plotted for each aeration period of 3 h or additionally after 30 min. The concentration of test chemical which inhibits the oxygen uptake by 50% (EC_{50}) should be calculated or interpolated from the graph. If suitable data are available, the 95% confidence limits of the EC_{50}, the slope of the curve, and suitable values to mark the beginning of inhibition (for example, EC_{10} or EC_{20}) and the end of the inhibition range (for example, EC_{80} or EC_{90}) may be calculated or interpolated.

56. It should be noted that in view of the variability often observed in the results, it may in many cases be sufficient to express the results additionally in order of magnitude, for example:

EC_{50} <1 mg/l
EC_{50} 1 mg/l to 10 mg/l
EC_{50} 10 mg/l to 100 mg/l
EC_{50} > 100mg/l

Interpretation of results

EC_x

57. EC_x-values including their associated lower and upper 95% confidence limits for the parameter are calculated using appropriate statistical methods (e.g. probit analysis, logistic or Weibull function, trimmed Spearman-Karber method or simple interpolation (11)). 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 (x) should be subjected to regression analysis.

NOEC estimation

58. If a statistical analysis is intended to determine the NOEC, per-vessel statistics (individual vessels are considered as replicates) are necessary. Appropriate statistical methods should be used according to the OECD Document on Current Approaches in the Statistical Analysis of Ecotoxicity Data: a Guidance to Application (11). In general, adverse effects of the test chemical compared to the control are investigated using one-tailed (smaller) hypothesis testing at $p \leq 0.05$.

Test report

59. The test report should include the following information:

Test chemical

- common name, chemical name, CAS number, purity;
- physico-chemical properties of the test chemical (e.g. log K_{ow}, water solubility, vapour pressure, Henry's constant (H) and possible information on the fate of the test chemical e.g. adsorption to activated sludge);

Test system

- source, conditions of operation of the wastewater treatment plant and influent it receives, concentration, pre-treatment and maintenance of the activated sludge;

Test conditions

- test temperature, pH during the test and duration of the exposure phase(s);

Results

- specific oxygen consumption of the controls (mg O2/(g sludge × h);
- all measured data, inhibition curve(s) and method for calculation of EC50;
- EC50 and, if possible, 95 per cent confidence limits, possibly EC20, EC80; possibly NOEC and the used statistical methods, if the EC50 cannot be determined;
- results for total, and if appropriate, heterotrophic and nitrification inhibition;
- abiotic oxygen uptake in the physico-chemical control (if used);
- name of the reference chemical and results with this chemical;
- all observations and deviations from the standard procedure, which could have influenced the result.

LITERATURE

(1) Brown, D., Hitz, H.R. and Schäfer, L. (1981). The assessment of the possible inhibitory effect of dyestuffs on aerobic waste-water bacteria, Experience with a screening test. *Chemosphere* 10 (3): 245-261.

(2) King, E. F. and Painter H. A. (1986). Inhibition of respiration of activated sludge; variability and reproducibility of results. *Toxicity Assessment* 1(1): 27-39.

(3) OECD (1984), Activated sludge, Respiration inhibition test, Test Guideline No. 209, Guidelines for the testing of chemicals, OECD, Paris.

(4) ISO (2007). ISO 8192 Water Quality- Test for inhibition of oxygen consumption by activated sludge for carbonaceous and ammonium oxidation, International Organization for Standardization.

(5) Bealing, D. J. (2003). Document ISO/TC147/WGI/N.183, International Organization for Standardization.

(6) Painter, H A, Jones K (1963). The use of the wide-bore dropping-mercury electrode for the determination of the rates of oxygen uptake and oxidation of ammonia by micro-orgranisms. *Journal of Applied Bacteriology* 26 (3): 471-483.

(7) Painter, H. A. (1986). Testing the toxicity of chemicals by the inhibition of respiration of activated sludge. *Toxicity Assessment* 1:515-524.

(8) Robra, B. (1976). Wasser/Abwasser 117, 80.

(9) Fiebig S. and Noack, U. (2004). The use of copper(II)sulphate pentahydrate as reference substance in the activated sludge respiration inhibition test - acc. to the OECD guideline 209. *Fresenius Environmental Bulletin* 13 No. 12b: 1556-1557.

(10) ISO (1995). ISO 10634 Water Quality – Guidance for the preparation and treatment of poorly water-soluble organic compounds for the subsequent evaluation of their biodegradability in aqueous medium, International Organization for Standardization.

(11) OECD (2006). Current approaches in the statistical analysis of ecotoxicity data: a guidance to application, Series on testing and assessment No. 54, ENV/JM/MONO(2006)18, OECD, Paris.

Appendix 1

DEFINITIONS

The following definitions are applicable to this test method.

Chemical means a substance or a mixture.

ECx (Effect concentration for x% effect) is the concentration that causes an x% of an effect on test organisms within a given exposure period when compared with a control. For example, an EC_{50} is a concentration estimated to cause an effect on a test end point in 50% of an exposed population over a defined exposure period.

NOEC (no observed effect concentration) is the test chemical concentration at which no effect is observed. In this test, the concentration corresponding to the NOEC, has no statistically significant effect ($p < 0.05$) within a given exposure period when compared with the control.

Test chemical means any substance or mixture tested using this test method.

Appendix 2

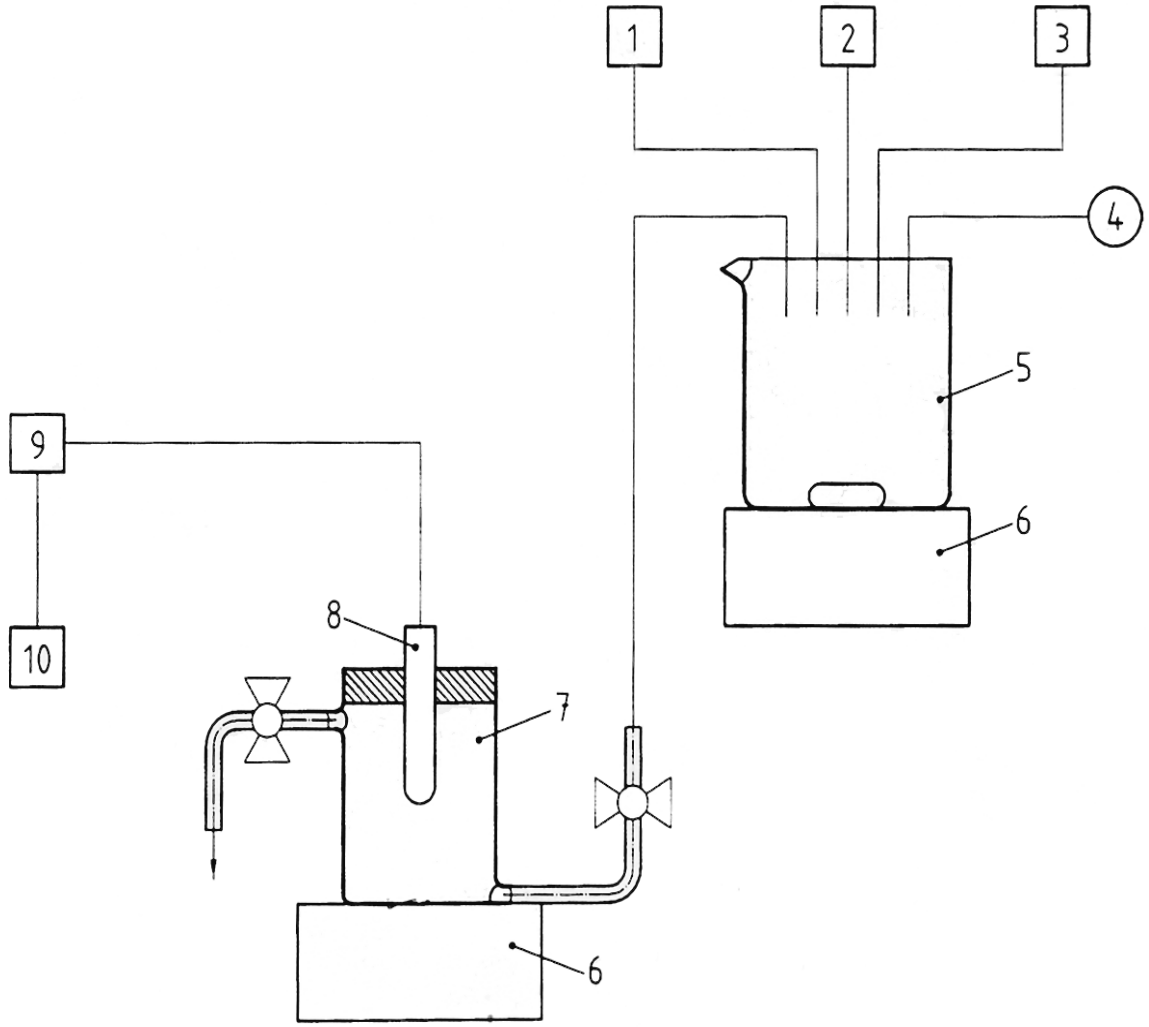

Fig. 1: Examples for measuring unit

Key

1 activated sludge	6 magnetic stirrer
2 synthetic medium	7 oxygen measuring cell
3 test chemical	8 oxygen electrode
4 air	9 oxygen measuring instrument
5 mixing vessel	10 recorder

Appendix 3

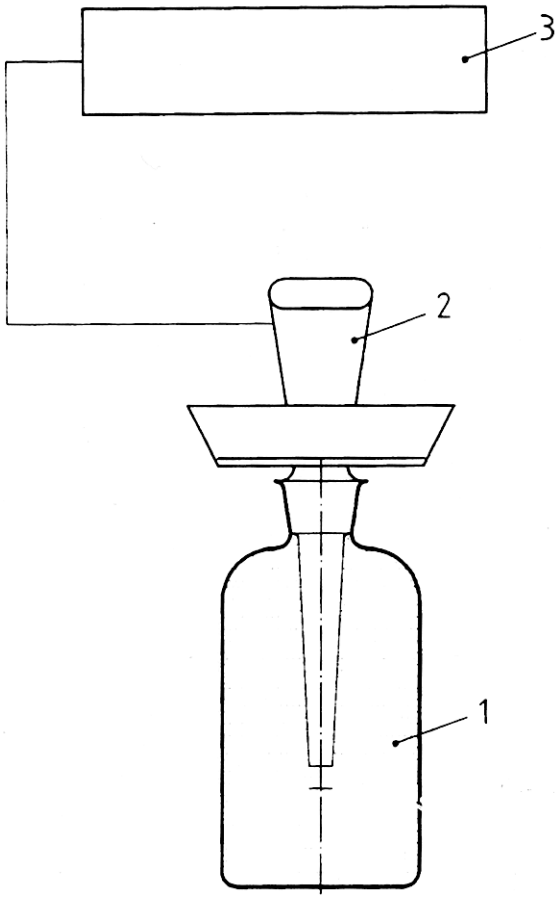

Fig. 2: Example of measuring unit, using a BOD bottle

Key

1 Test vessel

2 oxygen electrode

3 oxygen measuring instrument

Appendix 4

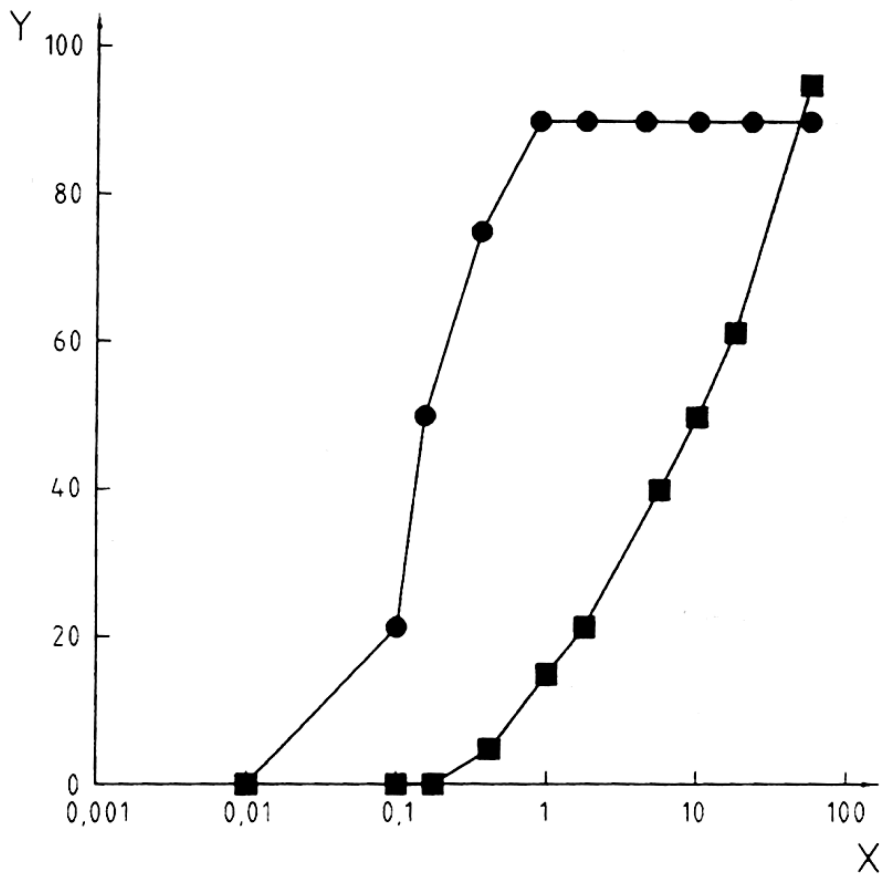

Fig. 3: Example of inhibition curves

Key

X concentration of 3,5-dichlorophenol (mg/l)

Y inhibition (%)

■ inhibition heterotrophic respiration using a nitrifying sludge

● inhibition nitrification using a nitrifying sludge"

(5) Chapter C.26 is replaced by the following:

"C.26 Lemna species Growth Inhibition Test

INTRODUCTION

1. This test method is equivalent to OECD Test Guideline (TG) 221 (2006). It is designed to assess the toxicity of chemicals to freshwater aquatic plants of the genus *Lemna* (duckweed). It is based on existing methods (1)(2)(3)(4)(5)(6) but includes modifications of those methods to reflect recent research and consultation on a number of key issues. This Test Method has been validated by an international ring-test (7).

2. This test method describes toxicity testing using *Lemna gibba* and *Lemna minor*, both of which have been extensively studied and are the subject of the standards referred to above. The taxonomy of *Lemna* spp. is difficult, being complicated by the existence of a wide range of phenotypes. Although genetic variability in the response to toxicants can occur with *Lemna*, there are currently insufficient data on this source of variability to recommend a specific clone for use with this test method. It should be noted that the test is not conducted axenically but steps are taken at stages during the test procedure to keep contamination by other organisms to a minimum.

3. Details of testing with renewal (semi-static and flow-through) and without renewal (static) of the test solution are described. Depending on the objectives of the test and the regulatory requirements, it is recommended to consider the application of semi-static and flow through methods, e.g. for chemicals that are rapidly lost from solution as a result of volatilisation, photodegradation, precipitation or biodegradation. Further guidance is given in (8).

4. Definitions used are given in Appendix 1.

PRINCIPLE OF THE TEST

5. Exponentially growing plant cultures of the genus *Lemna* are allowed to grow as monocultures in different concentrations of the test chemical over a period of seven days. The objective of the test is to quantify chemical-related effects on vegetative growth over this period based on assessments of selected measurement variables. Frond number is the primary measurement variable. At least one other measurement variable (total frond area, dry weight or fresh weight) is also measured, since some chemicals may affect other measurement variables much more than frond numbers. To quantify chemical-related effects, growth in the test solutions is compared with that of the controls and the concentration bringing about a specified x % inhibition of growth (e.g. 50 %) is determined and expressed as the EC_x (e.g. EC_{50})

6. The test endpoint is inhibition of growth, expressed as logarithmic increase in measurement variable (average specific growth rate) during the exposure period. From the average specific growth rates recorded in a series of test solutions, the concentration bringing about a specified x % inhibition of growth rate (e.g. 50%) is determined and expressed as the E_rC_x (e.g. E_rC_{50}).

7. An additional response variable used in this Test Method is yield, which may be needed to fulfil specific regulatory requirements in some countries. It is defined as measurement variables at the end of the exposure period minus the measurement variables at the start of the exposure period. From the yield recorded in a series of test solutions, the concentration bringing about a specified x % inhibition of yield (e.g., 50%) is calculated and expressed as the E_yC_x (e.g. E_yC_{50}).

8. In addition, the lowest observed effect concentration (LOEC) and the no observed effect concentration (NOEC) may be statistically determined.

INFORMATION ON THE TEST CHEMICAL

9. An analytical method, with adequate sensitivity for quantification of the chemical in the test medium, should be available.

10. Information on the test chemical which may be useful in establishing the test conditions includes the structural formula, purity, water solubility, stability in water and light, pK_a , K_{ow}, vapour pressure and biodegradability. Water solubility and vapour pressure can be used to calculate Henry´s Law constant, which will indicate if significant losses of the test chemical during the test period are likely. This will help indicate whether particular steps to control such losses should be taken. Where information on the solubility and stability of the test chemical is uncertain, it is recommended that these be assessed under the conditions of the test, i.e. growth medium, temperature, lighting regime to be used in the test.

11. When pH control of the test medium is particularly important, e.g. when testing metals or chemicals which are hydrolytically unstable, the addition of a buffer to the growth medium is recommended (see paragraph 21). Further guidance for testing chemicals with physical-chemical properties that make them difficult to test is provided in (8).

VALIDITY OF THE TEST

12. For the test to be valid, the doubling time of frond number in the control must be less than 2.5 days (60 h), corresponding to approximately a seven-fold increase in seven days and an average specific growth rate of 0.275 d^{-1}. Using the media and test conditions described in this Test Method, this criterion can be attained using a static test regime (5). It is also anticipated that this criterion will be achievable under semi-static and flow-through test conditions. Calculation of the doubling time is shown in

paragraph 49.

REFERENCE CHEMICAL

13. Reference chemical(s), such as 3,5-dichlorophenol used in the international ring test (7), may be tested as a means of checking the test procedure. It is advisable to test a reference chemical at least twice a year or, where testing is carried out at a lower frequency, in parallel to the determination of the toxicity of a test chemical.

DESCRIPTION OF THE METHOD

Apparatus

14. All equipment in contact with the test media should be made of glass or other chemically inert material. Glassware used for culturing and testing purposes should be cleaned of chemical contaminants that might leach into the test medium and should be sterile. The test vessels should be wide enough for the fronds of different colonies in the control vessels to grow without overlapping at the end of the test. It does not matter if the roots touch the bottoms of the test vessels, but a minimum depth of 20 mm and minimum volume of 100 mL in each test vessel is advised. The choice of test vessels is not critical as long as these requirements are met. Glass beakers, crystallising dishes or glass petri dishes of appropriate dimensions have all proved suitable. Test vessels must be covered to minimise evaporation and accidental contamination, while allowing necessary air exchange. Suitable test vessels, and particularly covers, must avoid shadowing or changes in the spectral characteristics of light.

15. The cultures and test vessels should not be kept together. This is best achieved using separate environmental growth chambers, incubators, or rooms. Illumination and temperature must be controllable and maintained at a constant level (see paragraphs 35-36).

Test organism

16. The organism used for this test is either *Lemna gibba* or *Lemna minor*. Short descriptions of duckweed species that have been used for toxicity testing are given in Appendix 2. Plant material may be obtained from a culture collection, another laboratory or from the field. If collected from the field, plants should be maintained in culture in the same medium as used for testing for a minimum of eight weeks prior to use. Field sites used for collecting starting cultures must be free of obvious sources of contamination. If obtained from another laboratory or a culture collection they should be similarly maintained for a minimum of three weeks. The source of plant material and the species and clone (if known) used for testing should always be reported.

17. Monocultures, that are visibly free from contamination by other organisms such as

algae and protozoa, should be used. Healthy plants of *L. minor* will consist of colonies comprising between two and five fronds whilst healthy colonies of *L. gibba* may contain up to seven fronds.

18. The quality and uniformity of the plants used for the test will have a significant influence on the outcome of the test and should therefore be selected with care. Young, rapidly growing plants without visible lesions or discoloration (chlorosis) should be used. Good quality cultures are indicated by a high incidence of colonies comprising at least two fronds. A large number of single fronds are indicative of environmental stress, e.g. nutrient limitation, and plant material from such cultures should not be used for testing.

Cultivation

19. To reduce the frequency of culture maintenance (e.g. when no *Lemna* tests are planned for a period), cultures can be held under reduced illumination and temperature (4 - 10°C). Details of culturing are given in Appenxix 3. Obvious signs of contamination by algae or other organisms may require surface sterilisation of a sub-sample of *Lemna* fronds, followed by transfer to fresh medium (see Appendix 3). In this eventuality the remaining contaminated culture should be discarded.

20. At least seven days before testing, sufficient colonies are transferred aseptically into fresh sterile medium and cultured for 7 - 10 days under the conditions of the test.

Test medium

21. Different media are recommended for *Lemna minor* and *Lemna gibba*, as described below. Careful consideration should be given to the inclusion of a pH buffer in the test medium (MOPS (4-morpholinepropane sulphonic acid, CAS No: 1132-61-2) in *L. minor* medium and $NaHCO_3$ in *L. gibba* medium) when it is suspected that it might react with the test chemical and influence the expression of its toxicity. Steinberg Medium (9) is also acceptable as long as the validity criteria are met.

22. A modification of the Swedish standard (SIS) Lemna growth medium is recommended for culturing and testing with L. minor. The composition of this medium is given in Appendix 4.

23. The growth medium, 20X - AAP, as described in Appendix 4, is recommended for culturing and testing with *L. gibba*.

24. Steinberg medium, as described in Appendix 4, is also suitable for *L. minor*, but may also be used for *L. gibba* as long as the validity criteria are met.

Test solutions

25. Test solutions are usually prepared by dilution of a stock solution. Stock solutions of

the test chemical are normally prepared by dissolving the chemical in growth medium.

26. The highest tested concentration of the test chemical should not normally exceed the water solubility of the chemical under the test conditions. It should be noted however that *Lemna* spp. float on the surface and may be exposed to chemicals that collects at the water-air interface (e.g. poorly water-soluble or hydrophobic chemicals or surface-active chemicals). Under such circumstances exposure will result from material other than in solution and test concentrations may, depending on the characteristics of the test chemical, exceed water solubility. For test chemicals of low water solubility it may be necessary–to prepare a concentrated stock solution or dispersion of the chemical using an organic solvent or dispersant in order to facilitate the addition of accurate quantities of the test chemical to the test medium and aid in its dispersion and dissolution. Every effort should be made to avoid the use of such materials. There should be no phytotoxicity resulting from the use of auxiliary solvents or dispersants. For example, commonly used solvents which do not cause phytotoxicity at concentrations up to 100 µl/l include acetone and dimethylformamide. If a solvent or dispersant is used, its final concentration should be reported and kept to a minimum (≤ 100 µl/l), and all treatments and controls should contain the same concentration of solvent or dispersant. Further guidance on the use of dispersants is given in (8).

Test and control groups

27. Prior knowledge of the toxicity of the test chemical to *Lemna*, e.g. from a range-finding test, will help in selecting suitable test concentrations. In the definitive toxicity test, there should normally be at least five test concentrations arranged in a geometric series. Preferably the separation factor between test concentrations should not exceed 3.2, but a larger value may be used where the concentration-response curve is flat. Justification should be provided if fewer than five concentrations are used. At least three replicates should be used at each test concentration.

28. In setting the range of test concentrations (for range-finding and/or for the definitive toxicity test), the following should be considered:

- To determine an EC_x, test concentrations should bracket the EC_x value to ensure an appropriate level of confidence. For example, if estimating the EC_{50}, the highest test concentration should be greater than the EC_{50} value. If the EC_{50} value lies outside of the range of test concentrations, associated confidence intervals will be large and a proper assessment of the statistical fit of the model may not be possible.
- If the aim is to estimate the LOEC/NOEC, the lowest test concentration should be low enough so that growth is not significantly less than that of the control. In addition, the highest test concentration should be high enough so that growth is significantly lower than that in the control. If this is not the case, the test will have to be repeated using a different concentration range (unless the highest concentration is at the limit of solubility or the maximum required limit concentration, e.g. 100 mg/l).

29. Every test should include controls consisting of the same nutrient medium, number of fronds and colonies, environmental conditions and procedures as the test vessels but without the test chemical. If an auxiliary solvent or dispersant is used, an additional

control treatment with the solvent/dispersant present at the same concentration as that in the vessels with the test chemical should be included. The number of replicate control vessels (and solvent vessels, if applicable) should be at least equal to, and ideally twice, the number of vessels used for each test concentration.

30. If determination of NOEC is not required, the test design may be altered to increase the number of concentrations and reduce the number of replicates per concentration. However, the number of control replicates must be at least three.

Exposure

31. Colonies consisting of 2 to 4 visible fronds are transferred from the inoculum culture and randomly assigned to the test vessels under aseptic conditions. Each test vessel should contain a total of 9 to 12 fronds. The number of fronds and colonies should be the same in each test vessel. Experience gained with this method and ring-test data have indicated that using three replicates per treatment, with each replicate containing 9 to 12 fronds initially, is sufficient to detect differences in growth of approximately 4 to 7% of inhibition calculated by growth rate (10 to 15% calculated by yield) between treatments (7).

32. A randomised design for location of the test vessels in the incubator is required to minimise the influence of spatial differences in light intensity or temperature. A blocked design or random repositioning of the vessels when observations are made (or repositioning more frequently) is also required.

33. If a preliminary stability test shows that the test chemical concentration cannot be maintained (i.e. the measured concentration falls below 80 % of the measured initial concentration) over the test duration (7 days), a semi-static test regime is recommended. In this case, the colonies should be exposed to freshly prepared test and control solutions on at least two occasions during the test (e.g. days 3 and 5). The frequency of exposure to fresh medium will depend on the stability of the test chemical; a higher frequency may be needed to maintain near-constant concentrations of highly unstable or volatile chemicals. In some circumstances, a flow-through procedure may be required (8)(10).

34. The exposure scenario through a foliar application (spray) is not covered in this test method; instead, see (11).

Incubation conditions

35. Continuous warm or cool white fluorescent lighting should be used to provide a light intensity selected from the range of 85-135 $\mu E \cdot m^{-2} s^{-1}$ when measured in a photosynthetically active radiation (400-700 nm) at points the same distance from the light source as the *Lemna* fronds (equivalent to 6500-10000 lux). Any differences from the selected light intensity over the test area should not exceed the range of ± 15 %. The method of light detection and measurement, in particular the type of sensor,

will affect the measured value. Spherical sensors (which respond to light from all angles above and below the plane of measurement) and "cosine" sensors (which respond to light from all angles above the plane of measurement) are preferred to unidirectional sensors, and will give higher readings for a multi-point light source of the type described here.

36. The temperature in the test vessels should be 24 ± 2 °C. The pH of the control medium should not increase by more than 1.5 units during the test. However, deviation of more than 1.5 units would not invalidate the test when it can be shown that validity criteria are met. Additional care is needed on pH drift in special cases such as when testing unstable chemicals or metals. See (8) for further guidance.

Duration

37. The test is terminated 7 days after the plants are transferred into the test vessels.

Measurements and analytical determinations

38. At the start of the test, frond number in the test vessels is counted and recorded, taking care to ensure that protruding, distinctly visible fronds are accounted for. Frond numbers appearing normal or abnormal, need to be determined at the beginning of the test, at least once every 3 days during the exposure period (i.e. on at least 2 occasions during the 7 day period), and at test termination. Changes in plant development, e.g. in frond size, appearance, indication of necrosis, chlorosis or gibbosity, colony break-up or loss of buoyancy, and in root length and appearance, should be noted. Significant features of the test medium (e.g. presence of undissolved material, growth of algae in the test vessel) should also be noted.

39. In addition to determinations of frond number during the test, effects of the test chemical on one (or more) of the following measurement variables are also assessed:

(i) total frond area,

(ii) dry weight,

(iii) fresh weight.

40. Total frond area has an advantage, in that it can be determined for each test and control vessel at the start, during, and at the end of the test. Dry or fresh weight should be determined at the start of the test from a sample of the inoculum culture representative of what is used to begin the test, and at the end of the test with the plant material from each test and control vessel. If frond area is not measured, dry weight is preferred over fresh weight.

41. Total frond area, dry weight and fresh weight may be determined as follows:

(i) Total frond area: The total frond area of all colonies may be determined by image analysis. A silhouette of the test vessel and plants can be captured using a video camera (i.e. by placing the vessel on a light box) and the resulting image digitised. By calibration with flat shapes of known area, the total frond area in a test vessel may then be determined. Care should be taken to exclude interference caused by the rim of the test vessel. An alternative but more laborious approach is to photocopy test vessels and plants, cut out the resulting silhouette of colonies and determine their area using a leaf area analyser or graph paper. Other techniques (e.g. paper weight ratio between silhouette area of colonies and unit area) may also be appropriate.

(ii) Dry weight: All colonies are collected from each of the test vessels and rinsed with distilled or deionised water. They are blotted to remove excess water and then dried at 60 °C to a constant weight. Any root fragments should be included. The dry weight should be expressed to an accuracy of at least 0.1 mg.

(iii) Fresh weight: All colonies are transferred to pre-weighed polystyrene (or other inert material) tubes with small (1 mm) holes in the rounded bottoms. The tubes are then centrifuged at 3000 rpm for 10 minutes at room temperature. Tubes, containing the now dried colonies, are re-weighed and the fresh weight is calculated by subtracting the weight of the empty tube.

Frequency of measurements and analytical determinations

42. If a static test design is used, the pH of each treatment should be measured at the beginning and at the end of the test. If a semi-static test design is used, the pH should be measured in each batch of 'fresh' test solution prior to each renewal and also in the corresponding 'spent' solutions.

43. Light intensity should be measured in the growth chamber, incubator or room at points the same distance from the light source as the *Lemna* fronds. Measurements should be made at least once during the test. The temperature of the medium in a surrogate vessel held under the same conditions in the growth chamber, incubator or room should be recorded at least daily.

44. During the test, the concentrations of the test chemical are determined at appropriate intervals. In static tests, the minimum requirement is to determine the concentrations at the beginning and at the end of the test.

45. In semi-static tests where the concentration of the test chemical is not expected to remain within ±20% of the nominal concentration, it is necessary to analyse all freshly prepared test solutions and the same solutions at each renewal (see paragraph 33). However, for those tests where the measured initial concentration of the test chemical is not within ±20% of nominal but where sufficient evidence can be provided to show that the initial concentrations are repeatable and stable (i.e. within the range 80 - 120 % of the initial concentration), chemical determinations may be carried out on only the highest and lowest test concentrations. In all cases, determination of test chemical concentrations prior to renewal need only be performed on one replicate vessel at each

test concentration (or the contents of the vessels pooled by replicate).

46. If a flow-through test is used, a similar sampling regime to that described for semi-static tests, including analysis at the start, mid-way through and at the end of the test, is appropriate, but measurement of 'spent' solutions is not appropriate in this case. In this type of test, the flow-rate of diluent and test chemical or test chemical stock solution should be checked daily.

47. If there is evidence that the concentration of the chemical being tested has been satisfactorily maintained within ± 20 % of the nominal or measured initial concentration throughout the test, analysis of the results can be based on nominal or measured initial values. If the deviation from the nominal or measured initial concentration is not within ± 20 %, analysis of the results should be based on the geometric mean concentration during exposure or models describing the decline of the concentration of the test chemical (8).

Limit test

48. Under some circumstances, e.g. when a preliminary test indicates that the test chemical has no toxic effects at concentrations up to 100 mg/l or up to its limit of solubility in the test medium (whichever is the lower), a limit test involving a comparison of responses in a control group and one treatment group (100 mg/l or a concentration equal to the limit of solubility), may be undertaken. It is strongly recommended that this be supported by analysis of the exposure concentration. All previously described test conditions and validity criteria apply to a limit test, with the exception that the number of treatment replicates should be doubled. Growth in the control and treatment group may be analysed using a statistical test to compare means, e.g. a Student's t-test.

DATA AND REPORTING

Doubling time

49. To determine the doubling time *(T_d)* of frond number and adherence to this validity criterion by the study (paragraph 12), the following formula is used with data obtained from the control vessels:

$$T_d = \ln 2 / \mu$$

where μ is the average specific growth rate determined as described in paragraphs 54-55.

Response variables

50. The purpose of the test is to determine the effects of the test chemical on the vegetative growth of *Lemna*. This Test Method describes two response variables, as different

jusidictions have different preferences and regulatory needs. In order for the test results to be acceptable in all jurisdictions, the effects should be evaluated using both response variables (a) and (b) described below.

(a) Average specific growth rate: this response variable is calculated on the basis of changes in the logarithms of frond numbers, and in addition, on the basis of changes in the logarithms of another measurement parameter (total frond area, dry weight or fresh weight) over time (expressed per day) in the controls and each treatment group. It is sometimes referred to as relative growth rate (12).

(b) Yield: this response variable is calculated on the basis of changes in frond number, and in addition, on the basis of changes in another measurement parameter (total frond area, dry weight or fresh weight) in the controls and in each treatment group until the end of the test.

51. It should be noted that toxicity values calculated by using these two response variables are not comparable and this difference must be recognised when using the results of the test. EC_x values based upon average specific growth rate (E_rC_x) will generally be higher than results based upon yield (E_yC_x) if the test conditions of this Test Method are adhered to, due to the mathematical basis of the respective approaches. This should not be interpreted as a difference in sensitivity between the two response variables, simply that the values are different mathematically. The concept of average specific growth rate is based on the general exponential growth pattern of duckweed in non-limited cultures, where toxicity is estimated on the basis of the effects on the growth rate, without being dependent on the absolute level of the specific growth rate of the control, slope of the concentration-response curve or on test duration. In contrast, results based upon the yield response variable are dependent upon all these other variables. E_yC_x is dependent on the specific growth rate of the duckweed species used in each test and on the maximum specific growth rate that can vary between species and even different clones. This response variable should not be used for comparing the sensitivity to toxicants among duckweed species or even different clones. While the use of average specific growth rate for estimating toxicity is scientifically preferred, toxicity estimates based on yield are also included in this Test Method to satisfy current regulatory requirements in some jurisdictions.

52. Toxicity estimates should be based on frond number and one additional measurement variable (total frond area, dry weight or fresh weight), because some chemicals may affect other measurement variables much more than the frond number. This effect would not be detected by calculating frond number only.

53. The number of fronds as well as any other recorded measurement variable, i.e. total frond area, dry weight or fresh weight, are tabulated together with the concentrations of the test chemical for each measurement occasion. Subsequent data analysis e.g. to estimate a LOEC, NOEC or EC_x should be based on the values for the individual replicates and not calculated means for each treatment group.

Average specific growth rate

54. The average specific growth rate for a specific period is calculated as the logarithmic increase in the growth variables -frond numbers and one other measurement variable (total frond area, dry weight or fresh weight) - using the formula below for each replicate of control and treatments:

$$\mu_{i-j} = \frac{\ln(N_j) - \ln(N_i)}{t}$$

where:

- μ_{i-j} : average specific growth rate from time i to j
- N_i : measurement variable in the test or control vessel at time i
- N_j : measurement variable in the test or control vessel at time j
- t : time period from i to j

For each treatment group and control group, calculate a mean value for growth rate along with variance estimates.

55. The average specific growth rate should be calculated for the entire test period (time "i" in the above formula is the beginning of the test and time "j" is the end of the test). For each test concentration and control, calculate a mean value for average specific growth rate along with the variance estimates. In addition, the section-by-section growth rate should be assessed in order to evaluate effects of the test chemical occurring during the exposure period (e.g. by inspecting log-transformed growth curves). Substantial differences between the section-by-section growth rate and the average growth rate indicate deviation from constant exponential growth and that close examination of the growth curves is warranted. In this case, a conservative approach would be to compare specific growth rates from treated cultures during the time period of maximum inhibition to those for controls during the same time period.

56. Percent inhibition of growth rate (I_r) may then be calculated for each test concentration (treatment group) according to the following formula:

$$\%\, I_r = \frac{(\mu_C - \mu_T)}{\mu_C} \times 100$$

where:
- % I_r: percent inhibition in average specific growth rate
- μ_C : mean value for μ in the control
- μ_T : mean value for μ in the treatment group

Yield

57. Effects on yield are determined on the basis of two measurement variables, frond number and one other measurement variable (total frond area, dry weight or fresh

weight) present in each test vessel at the start and at the end of the test. For dry weight or fresh weight, the starting biomass is determined on the basis of a sample of fronds taken from the same batch used to inoculate the test vessels (see paragraph 20). For each test concentration and control, calculate a mean value for yield along with variance estimates. The mean percent inhibition in yield (%I_y) may be calculated for each treatment group as follows:

$$\% \, I_y = \frac{(b_c - b_T)}{b_c} \times 100$$

where:

- % I_y : percent reduction in yield
- b_C : final biomass minus starting biomass for the control group
- b_T : final biomass minus starting biomass in the treatment group

Plotting concentration-response curves

58. Concentration-response curves relating mean percentage inhibition of the response variable (I_r, or I_y calculated as shown in paragraph 56 or 57) and the log concentration of the test chemical should be plotted.

EC_x estimation

59. Estimates of the EC_x (e.g., EC_{50}) should be based upon both average specific growth rate (E_rC_x) and yield (E_yC_x), each of which should in turn be based upon frond number and one additional measurement variable (total frond area, dry weight, or fresh weight). This is because there are test chemicals that impact frond number and other measurement variables differently. The desired toxicity parameters are therefore four EC_x values for each inhibition level x calculated: E_rC_x (frond number); E_rC_x (total frond area, dry weight, or fresh weight); E_yC_x (frond number); and E_yC_x (total frond area, dry weight, or fresh weight).

Statistical procedures

60. The aim is to obtain a quantitative concentration-response relationship by regression analysis. It is possible to use a weighted linear regression after having performed a linearising transformation of the response data, for instance into probit or logit or Weibull units (13), but non-linear regression procedures are preferred techniques that better handle unavoidable data irregularities and deviations from smooth distributions. Approaching either zero or total inhibition such irregularities may be magnified by the transformation, interfering with the analysis (13). It should be noted that standard methods of analysis using probit, logit, or Weibull transforms are intended for use on quantal (e.g. mortality or survival) data, and must be modified to accommodate growth rate or yield data. Specific procedures for determination of EC_x values from continuous data can be found in (14), (15), and (16).

61. For each response variable to be analysed, use the concentration-response relationship

to calculate point estimates of EC_x values. When possible, the 95% confidence limits for each estimate should be determined. Goodness of fit of the response data to the regression model should be assessed either graphically or statistically. Regression analysis should be performed using individual replicate responses, not treatment group means.

62. EC_{50} estimates and confidence limits may also be obtained using linear interpolation with bootstrapping (17), if available regression models/methods are unsuitable for the data.

63. For estimation of the LOEC and hence the NOEC, it is necessary to compare treatment means using analysis of variance (ANOVA) techniques. The mean for each concentration must then be compared with the control mean using an appropriate multiple comparison or trend test method. Dunnett's or Williams'test may be useful (18)(19)(20)(21). It is necessary to assess whether the ANOVA assumption of homogeneity of variance holds. This assessment may be performed graphically or by a formal test (22). Suitable tests are Levene's or Bartlett's. Failure to meet the assumption of homogeneity of variances can sometimes be corrected by logarithmic transformation of the data. If heterogeneity of variance is extreme and cannot be corrected by transformation, analysis by methods such as step-down Jonkheere trend tests should be considered. Additional guidance on determining the NOEC can be found in (16).

64. Recent scientific developments have led to a recommendation of abandoning the concept of NOEC and replacing it with regression based point estimates EC_x. An appropriate value for x has not been established for this *Lemna* test. However, a range of 10 to 20 % appears to be appropriate (depending on the response variable chosen), and preferably both the EC_{10} and EC_{20} should be reported.

Reporting

65. The test report must include the following:

Test chemical:

- physical nature and physical-chemical properties, including water solubility limit;
- chemical identification data (e.g., CAS Number), including purity (impurities).

Test species:

- scientific name, clone (if known) and source.

Test conditions:

- test procedure used (static, semi-static or flow-through);
- date of start of the test and its duration;
- test medium;

- description of the experimental design: test vessels and covers, solution volumes, number of colonies and fronds per test vessel at the beginning of the test;
- test concentrations (nominal and measured as appropriate) and number of replicates per concentration;
- methods of preparation of stock and test solutions including the use of any solvents or dispersants;
- temperature during the test;
- light source, light intensity and homogeneity;
- pH values of the test and control media;
- test chemical concentrations and the method of analysis with appropriate quality assessment data (validation studies, standard deviations or confidence limits of analyses);
- methods for determination of frond number and other measurement variables, e.g. dry weight, fresh weight or frond area;
- all deviations from this Test Method.

Results:

- raw data: number of fronds and other measurement variables in each test and control vessel at each observation and occasion of analysis;
- means and standard deviations for each measurement variable;
- growth curves for each concentration (recommended with log transformed measurement variable, see paragraph 55);
- doubling time/growth rate in the control based on the frond number;
- calculated response variables for each treatment replicate, with mean values and coefficient of variation for replicates;
- graphical representation of the concentration/effect relationship;
- estimates of toxic endpoints for response variables e.g. EC_{50}, EC_{10}, EC_{20}, and associated confidence intervals. If calculated, LOEC and/or NOEC and the statistical methods used for their determination;
- if ANOVA has been used, the size of the effect which can be detected (e.g. the least significant difference);
- any stimulation of growth found in any treatment;
- any visual signs of phytotoxicity as well as observations of test solutions;
- discussion of the results, including any influence on the outcome of the test resulting from deviations from this Test Method.

LITERATURE

(1) ASTM International. (2003). Standard Guide for Conducting Static Toxicity Test With *Lemna gibba* G3. E 1415-91 (Reapproved 1998). pp. 733-742. In, Annual Book of ASTM Standards, Vol. 11.05 Biological Effects and Environmental Fate; Biotechnology; Pesticides, ASTM, West Conshohocken, PA.

(2) US EPA - United States Environmental Protection Agency. (1996). OPPTS 850.4400 Aquatic Plant Toxicity Test Using *Lemna* spp., "Public draft". EPA 712-C-96-156. 8pp.

(3) AFNOR - Association Française de Normalisation. (1996). XP T 90-337: Détermination de l´inhibition de la croissance de *Lemna minor*. 10pp.

(4) SSI - Swedish Standards Institute. (1995). Water quality - Determination of growth inhibition (7-d) *Lemna minor*, duckweed. SS 02 82 13. 15pp. (in Swedish).

(5) Environment Canada. (1999). Biological Test Method: Test for Measuring the Inhibition of Growth Using the Freshwater Macrophyte, *Lemna minor*. EPS 1/RM/37 - 120 pp.

(6) Environment Canada. (1993) Proposed Guidelines for Registration of Chemical Pesticides: Non-Target Plant Testing and Evaluation. Canadian Wildlife Service, Technical Report Series No. 145.

(7) Sims I., Whitehouse P. and Lacey R. (1999) The OECD *Lemna* Growth Inhibition Test. Development and Ring-testing of draft OECD Test Guideline. R&D Technical Report EMA 003. WRc plc - Environment Agency.

(8) OECD (2000). Guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixtures. OECD Environmental Health and Safety Publications, Series on Testing and Assessment No.23. Organisation for Economic Co-operation and Development, Paris.

(9) International Organisation for Standardisation. ISO DIS 20079. Water Quality - Determination of the Toxic Effect of Water Constituents and Waste Water to Duckweed (*Lemna minor*) - Duckweed Growth Inhibition Test.

(10) Walbridge C. T. (1977). A flow-through testing procedure with duckweed (*Lemna minor* L.). Environmental Research Laboratory - Duluth, Minnesota 55804. US EPA Report No. EPA-600/3-77 108. September 1977.

(11) Lockhart W. L., Billeck B. N. and Baron C. L. (1989). Bioassays with a floating plant (*Lemna minor*) for effects of sprayed and dissolved glyphosate. *Hydrobiologia*, 118/119, 353 - 359.

(12) Huebert, D.B. and Shay J.M. (1993) Considerations in the assessment of toxicity using duckweeds. *Environmental Toxicology and Chemistry*, 12, 481-483.

(13) Christensen, E.R.,, Nyholm, N. (1984): Ecotoxicological Assays with Algae: Weibull Dose-Response Curves. *Env. Sci. Technol*. 19, 713-718.

(14) Nyholm, N. Sørensen, P.S., Kusk, K.O. and Christensen, E.R. (1992): Statistical treatment of data from microbial toxicity tests. *Environ. Toxicol. Chem*. 11, 157-167.

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

(16) OECD. (2006). Current Approaches in the Statistical Analysis of Ecotoxicity Data: A Guidance to Application. Organisation for Economic Co-operation and Development, Paris.

(17) Norberg-King T.J. (1988) An interpolation estimate for chronic toxicity: The ICp approach. National Effluent Toxicity Assessment Center Technical Report 05-88. US EPA, Duluth, MN.

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

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

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

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

(22) Brain P. and Cousens R. (1989). An equation to describe dose-responses where there is stimulation of growth at low doses. *Weed Research*, 29, 93-96.

Appendix 1

DEFINITIONS

The following definitions and abbreviations are used for the purposes of this Test Method:

Biomass is the dry weight of living matter present in a population. In this test, surrogates for biomass, such as frond counts or frond area are typically measured and the use of the term "biomass" thus refers to these surrogate measures as well.

Chemical means a substance or a mixture.

Chlorosis is yellowing of frond tissue.

Clone is an organism or cell arisen from a single individual by asexual reproduction. Individuals from the same clone are, therefore, genetically identical.

Colony means an aggregate of mother and daughter fronds (usually 2 to 4) attached to each other. Sometimes referred to as a plant.

EC_x is the concentration of the test chemical dissolved in test medium that results in a x % (e.g. 50%) reduction in growth of *Lemna* within a stated exposure period (to be mentioned explicitly if deviating from full or normal test duration) . To unambiguously denote an EC value deriving from growth rate or yield the symbol "E_rC" is used for growth rate and "E_yC" is used for yield, followed by the measurement variable used, e.g. E_rC (frond number).

Flow-through is a test in which the test solutions are replaced continuously.

Frond is an individual/single "leaf-like" structure of a duckweed plant. It is the smallest unit, i.e. individual, capable of reproduction.

Gibbosity means fronds exhibiting a humped or swollen appearance.

Growth is an increase in the measurement variable, e.g. frond number, dry weight, wet weight or frond area, over the test period.

Growth rate (average specific growth rate) is the logarithmic increase in biomass during the exposure period.

Lowest Observed Effect Concentration (LOEC) is the lowest tested concentration at which the chemical is observed to have a statistically significant reducing effect on growth (at $p < 0.05$) when compared with the control, within a given exposure time. However, all test concentrations above the LOEC must have a harmful effect equal to or greater than those observed at the LOEC. When these two conditions cannot be satisfied, a full explanation must be given for how the LOEC (and hence the NOEC) has been selected.

Measurement variables are any type of variables which are measured to express the test endpoint using one ore more different response variables. In this method frond number, frond area, fresh weight and dry weight are measurement variables.

Monoculture is a culture with one plant species.

Necrosis is dead (i.e. white or water-soaked) frond tissue.

No Observed Effect Concentration (NOEC) is the test concentration immediately below the LOEC.

Phenotype is the observable characteristics of an organism determined by the interaction of its genes with its environment.

Response variable are variables for the estimation of toxicity derived from any measured variables describing biomass by different methods of calculation. For this Test Method growth rates and yield are response variables derived from measurement variables like frond number, frond area, fresh weight or dry weight.

Semi-static (renewal) test is a test in which the test solution is periodically replaced at specific intervals during the test.

Static test is a test method without renewal of the test solution during the test.

Test chemical is any substance or mixture tested using this test method.

Test endpoint describes the general factor that will be changed relative to control by the test chemical as aim of the test. In this test method the test endpoint is inhibition of growth which may be expressed by different response variables which are based on one or more measurement variables.

Test medium is the complete synthetic growth medium on which test plants grow when exposed to the test chemical. The test chemical will normally be dissolved in the test medium.

Yield is value of a measurement variable to express biomass at the end of the exposure period minus the measurement variable at the start of the exposure period.

Appendix 2

Description of *Lemna* spp.

The aquatic plant commonly referred to as duckweed, *Lemna* spp., belongs to the family Lemnaceae which has a number of world-wide species in four genera. Their different appearance and taxonomy have been exhaustively described (1)(2). *Lemna gibba* and *L. minor* are species representative of temperate areas and are commonly used for toxicity tests. Both species have a floating or submerged discoid stem (frond) and a very thin root emanates from the centre of the lower surface of each frond. *Lemna* spp. rarely produce flowers and the plants reproduce by vegetatively producing new fronds (3). In comparison with older plants the younger ones tend to be paler, have shorter roots and consist of two to three fronds of different sizes. The small size of *Lemna*, its simple structure, asexual reproduction and short generation time makes plants of this genus very suitable for laboratory testing (4)(5).

Because of probable interspecies variation in sensitivity, only comparisons of sensitivity within a species are valid.

Examples of *Lemna* species which have been used for testing: Species Reference

Lemna aequinoctialis: Eklund, B. (1996). The use of the red alga *Ceramium strictum* and the duckweed *Lemna aequinoctialis* in aquatic ecotoxicological bioassays. Licentiate in Philosophy Thesis 1996:2. Dep. of Systems Ecology, Stockholm University.

Lemna major : Clark, N. A. (1925). The rate of reproduction of *Lemna major* as a function of intensity and duration of light. J. phys. Chem., 29: 935-941.

Lemna minor: United States Environmental Protection Agency (US EPA). (1996). OPPTS 850.4400 Aquatic Plant Toxicity Test Using *Lemna* spp., "Public draft". EPA 712-C-96-156. 8pp.

Association Française de Normalisation (AFNOR). (1996). XP T 90-337: Détermination de l´inhibition de la croissance de *Lemna minor*. 10pp.

Swedish Standards Institute (SIS). (1995). Water quality - Determination of growth inhibition (7-d) *Lemna minor*, duckweed. SS 02 82 13. 15pp. (in Swedish).

Lemna gibba : ASTM International. (2003). Standard Guide for Conducting Static Toxicity Test With *Lemna gibba* G3. E 1415-91 (Reapproved 1998). pp. 733-742.

United States Environmental Protection Agency (US EPA). (1996). OPPTS 850.4400 Aquatic Plant Toxicity Test Using *Lemna* spp., "Public draft". EPA 712-C-96-156. 8pp.

Lemna paucicostata: Nasu, Y., Kugimoto, M. (1981). *Lemna* (duckweed) as an indicator of water pollution. I. The sensitivity of *Lemna paucicostata* to heavy metals. Arch. Environ. Contam. Toxicol., 10:1959-1969.

Lemna perpusilla: Clark, J. R. et al. (1981). Accumulation and depuration of metals by duckweed (*Lemna perpusilla)*. Ecotoxicol. Environ. Saf., 5:87-96.

Lemna trisulca: Huebert, D. B., Shay, J. M. (1993). Considerations in the assessment of toxicity using duckweeds. Environ. Toxicol. and Chem., 12:481- 483.

Lemna valdiviana: Hutchinson, T.C., Czyrska, H. (1975). Heavy metal toxicity and synergism to floating aquatic weeds. Verh.-Int. Ver. Limnol., 19:2102-2111.

Sources of *Lemna* species

University of Toronto Culture Collection of Algae and Cyanobacteria
Department of Botany, University of Toronto
Toronto, Ontario, Canada, M5S 3 B2
Tel: +1-416-978-3641
Fax:+1-416-978-5878
e-mail: jacreman@botany.utoronto.ca

North Carolina State University
Forestry Dept
Duckweed Culture Collection
Campus Box 8002
Raleigh, NC 27695-8002
United States
phone 001 (919) 515-7572
astomp@unity.ncsu.edu

Institute of Applied Environmental Research (ITM) Stockholm University
SE-106 91
STOCKHOLM
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Federal Environmental Agency (UBA)
FG III 3.4
Schichauweg 58
12307 Berlin
Germany
e-mail: lemna@uba.de

LITERATURE

(1) Hillman, W.S. (1961). The Lemnaceae or duckweeds: A review of the descriptive and experimental literature. The Botanical Review, 27:221-287.

(2) Landolt, E. (1986). Biosystematic investigations in the family of duckweed (*Lemnaceae*). Vol. 2. Geobotanischen Inst. ETH, Stiftung Rubel, Zürich, Switzerland.

(3) Björndahl, G. (1982). Growth performance, nutrient uptake and human utilization of duckweeds *(Lemnaceae* family). ISBN 82-991150-0-0. The Agricultural Research Council of Norway, University of Oslo.

(4) Wang, W. (1986). Toxicity tests of aquatic pollutants by using common duckweed. Environmental Pollution, Ser B, 11:1-14.

(5) Wang, W. (1990). Literature review on duckweed toxicity testing. Environmental Research, 52:7-22.

Appendix 3

Maintenance of stock culture

Stock cultures can be maintained under lower temperatures (4-10^{o}C) for longer times without needing to be re-established. The *Lemna* growth medium may be the same as that used for testing but other nutrient rich media can be used for stock cultures.

Periodically, a number of young, light-green plants are removed to new culture vessels containing fresh medium using an aseptic technique. Under the cooler conditions suggested here, sub-culturing may be conducted at intervals of up to three months.

Chemically clean (acid-washed) and sterile glass culture vessels should be used and aseptic handling techniques employed. In the event of contamination of the stock culture e.g. by algae or funghi, steps are necessary to eliminate the contaminating organisms. In the case of algae and most other contaminating organisms, this can be achieved by surface sterilisation. A sample of the contaminated plant material is taken and the roots cut off. The material is then shaken vigorously in clean water, followed by immersion in a 0.5% (v/v) sodium hypochlorite solution for between 30 seconds and 5 minutes. The plant material is then rinsed with sterile water and transferred, as a number of batches, into culture vessels containing fresh growth medium. Many fronds will die as a result of this treatment, especially if longer exposure periods are used, but some of those surviving will usually be free of contamination. These can then be used to re-inoculate new cultures.

Appendix 4

Media

Different growth media are recommended for *L. minor* and *L. gibba*. For *L. minor*, a modified Swedish Standard (SIS) medium is recommended whilst for *L. gibba*, 20X AAP medium is recommended. Compositions of both media are given below. When preparing these media, reagent or analytical-grade chemicals should be used and deionised water.

Swedish Standard (SIS) *Lemna* growth medium

- Stock solutions I - V are sterilised by autoclaving (120 ^{o}C, 15 minutes) or by membrane filtration (approximately 0.2 μm pore size).
- Stock VI (and optional VII) are sterilised by membrane filtration only; these should not be autoclaved.
- Sterile stock solutions should be stored under cool and dark conditions. Stocks I - V should be discarded after six months whilst stocks VI (and optional VII) have a shelf life of one month.

Stock solution No.	Substance	Concentration in stock solution (g/l)	Concentration in prepared medium (mg/•l)	Prepared medium	
				Element	Concentration (mg/•l)
I	$NaNO_3$ KH_2PO_4	8.50 1.34	85 13.4	Na ; N K ; P	32 ; 14 6.0 ; 2.4
II	$MgSO_4 . 7H_2O$	15	75	Mg ; S	7.4 ; 9.8
III	$CaCl_2 . 2H_2O$	7.2	36	Ca ; Cl	9.8 ; 17.5
IV	Na_2CO_3	4.0	20	C	2.3
V	H_3BO_3 $MnCl_2 . 4H_2O$ $Na_2MoO_4 . 2H_2O$ $ZnSO_4 . 7H_2O$ $CuSO_4 . 5H_2O$ $Co(NO_3)_2 . 6H_2O$	1.0 0.20 0.010 0.050 0.0050 0.010	1.00 0.20 0.010 0.050 0.0050 0.010	B Mn Mo Zn Cu Co	0.17 0.056 0.0040 0.011 0.0013 0.0020
VI	$FeCl_3 . 6H_2O$ Na_2-EDTA $2H_2O$	0.17 0.28	0.84 1.4	Fe -	0.17 -
VII	MOPS (buffer)	490	490	-	-

To prepare one litre of SIS medium, the following are added to 900 ml of deionised

water:

- 10 ml of stock solution I
- 5 ml of stock solution II
- 5 ml of stock solution III
- 5 ml of stock solution IV
- 1 ml of stock solution V
- 5 ml of stock solution VI
- 1 ml of stock solution VII (optional)

Note: A further stock solution VII (MOPS buffer) may be needed for certain test chemicals (see paragraph 11).

The pH is adjusted to 6.5 ± 0.2 with either 0.1 or 1 mol HCl or NaOH, and the volume adjusted to one litre with deionised water.

20X AAP growth medium

Stock solutions are prepared in sterile distilled or deionised water.

Sterile stock solutions should be stored under cool and dark conditions. Under these conditions the stock solutions will have a shelf life of at least 6 – 8 weeks.

Five nutrient stock solutions (A1, A2, A3, B and C) are prepared for 20X - AAP medium, using reagent-grade chemicals. The 20 ml of each nutrient stock solution is added to approximately 850 ml deionised water to produce the growth medium. The pH is adjusted to 7.5 ± 0.1 with either 0.1 or 1 mol HCl or NaOH, and the volume adjusted to one litre with deionised water. The medium is then filtered through a 0.2 µm (approximate) membrane filter into a sterile container.

Growth medium intended for testing should be prepared 1-2 days before use to allow the pH to stabilise. The pH of the growth medium should be checked prior to use and readjusted if necessary by the addition of 0.1 or 1 mol NaOH or HCl as described above.

Stock solution No.	Sustance	Concentration in stock solution (g/•l)*	Concentration in prepared medium (mg/•l)*	Prepared medium	
				Element	Concentration (mg/•l)*
A1	$NaNO_3$ $MgCl_2.6H_2O$ $CaCl_2.2H_2O$	26 12 4.4	510 240 90	Na;N Mg Ca	190;84 58.08 24.04
A2	$MgSO_4.7H_2O$	15	290	S	38.22
A3	$K_2HPO_4.3H_2.O$	1.4	30	K;P	9.4;3.7
B	H_3BO_3 $MnCl_2.4H_2O$ $FeCl_3.6H_2O$ $Na_2EDTA.2H_2O$	0.19 0.42 0.16 0.30	3.7 8.3 3.2 6.0	B Mn Fe -	0.65 2.3 0.66 -

	$ZnCl_2$ $CoCl_2.6H_2O$ $Na_2MoO_4.2H_2O$ $CuCl_2.2H_2O$	3.3 mg/l 1.4 mg/l 7.3 mg/l 0.012 mg/l	66 µg/l 29 µg/l 145 µg/l 0.24 µg/l	Zn Co Mo Cu	31 µg/l 7.1 µg/l 58 µg/l 0.080 µg/l
C	$NaHCO_3$	15	300	Na;C	220; 43

*Unless noted

Note: The theoretically appropriate final bicarbonate concentration (which will avoid appreciable pH adjustment) is 15 mg/L, not 300 mg/L. However, the historical use of 20X-AAP medium, including the ring test for this guideline, is based upon 300 mg/L. (I. Sims, P. Whitehouse and R. Lacey. (1999) The OECD *Lemna* Growth Inhibition Test. Development and Ring-testing of draft OECD Test Guideline. R&D Technical Report EMA 003. WRc plc - Environment Agency.)

STEINBERG medium (After ISO 20079)

Concentrations and stock solutions

The modified Steinberg medium is used in ISO 20079 for *Lemna* minor alone (as only *Lemna minor* is allowed there) but tests showed good results could be reached with *Lemna gibba* too.

When preparing the medium, reagent- or analytical grade chemicals and deionised water should be used.

Prepare the nutrient medium from stock solutions or the 10 fold concentrated medium which allows maximum concentration of the medium without precipitation.

Table 1: pH-stabilised STEINBERG medium (modified acc. to Altenburger)

Component		**Nutrient medium**	
Macroelements	mol weight	mg/l	mmol/l
KNO_3	101.12	350.00	3.46
$Ca(NO_3)_2 \cdot 4H_2O$	236.15	295.00	1.25
KH_2PO_4	136.09	90.00	0.66
K_2HPO_4	174.18	12.60	0.072
$MgSO_4 \cdot 7H_2O$	246.37	100.00	0.41
Microelements	mol weight	µg/l	µmol/l
H_3BO_3	61.83	120.00	1.94
$ZnSO_4 \cdot 7H_2O$	287.43	180.00	0.63
$Na_2MoO_4 \cdot 2H_2O$	241.92	44.00	0.18
$MnCl_2 \cdot 4H_2O$	197.84	180.00	0.91
$FeCl_3 \cdot 6H_2O$	270.21	760.00	2.81

EDTA Disodium-dihydrate	372.24	1 500.00	4.03

Table 2: Stock solutions (Macroelements)

1. Macroelements (50-fold concentrated)	**g/l**
Stock solution 1: KNO_3 KH_2PO_4 K_2HPO_4	 17.50 4.5 0.63
Stock solution 2: $MgSO_4 \cdot 7H_2O$	 5.00
Stock solution 3: $Ca(NO_3)_2 \cdot 4H_2O$	 14.75

Table 3: Stock solutions (Microelements)

2. Microelements (1 000-fold concentrated)	**mg/l**
Stock solution 4: H_3BO_3	 120.0
Stock solution 5: $ZnSO_4 \cdot 7H_2O$	 180.0
Stock solution 6: $Na_2MoO_4 \cdot 2H_2O$	 44.0
Stock solution 7: $MnCl_2 \cdot 4H_2O$	 180.0
Stock solution 8: $FeCl_3 \cdot 6H_2O$ EDTA Disodium-dihydrate	 760.00 1 500.00

- Stock solutions 2 and 3 and separately 4 to 7 may be pooled (taking into account the required concentrations).
- For longer shelf life treat stock solutions in an autoclave at 121 °C for 20 min or alternatively carry out a sterile filtration (0.2 µm). For stock solution 8 sterile filtration (0.2 µm) is strongly recommended.

Preparation of the final concentration of STEINBERG medium (modified)

- Add 20 ml of stock solutions 1, 2 and 3 (see table 2) to about 900 ml deionised water to avoid precipitation.
- Add 1.0 ml of stock solutions 4, 5, 6, 7 and 8 (see table 3).

- The pH should be to 5.5 +/- 0.2 (adjust by addition of a minimised volume of NaOH solution or HCl).
- Adjust with water to 1000 ml.
- If stock solutions are sterilised and appropriate water is used no further sterilisation is necessary. If sterilisation is done with the final medium stock solution 8 should be added after autoclaving (at 121 °C for 20 min).

Preparation of 10-fold-concentrated STEINBERG medium (modified) for intermediate storage

- Add to 20 ml of stock solutions 1, 2 and 3 (see table 2) to about 30 ml water to avoid precipitation.
- Add 1.0 ml of stock solutions 4, 5, 6, 7 and 8 (see table 3). Adjust with water to 100 ml.
- If stock solutions are sterilised and appropriate water is used no further sterilisation is necessary. If sterilisation is done with the final medium stock solution 8 should be added after autoclaving (at 121 °C for 20 min).
- The pH of the medium (final concentration) should be 5.5±0.2."

(6) the following Chapters C.31 to C.46 are added:

"C.31. Terrestrial Plant Test: Seedling Emergence and Seedling Growth Test

INTRODUCTION

1. This test method is equivalent to OECD Test Guideline (TG) 208 (2006). Test methods are periodically reviewed in the light of scientific progress and applicability to regulatory use. This updated test method is designed to assess potential effects of chemicals on seedling emergence and growth. As such it does not cover chronic effects or effects on reproduction (i.e. seed set, flower formation, fruit maturation). Conditions of exposure and properties of the chemical to be tested must be considered to ensure that appropriate test methods are used (e.g. when testing metals/metal compounds the effects of pH and associated counter ions should be considered) (1). This test method does not address plants exposed to vapours of chemicals. The test method is applicable to the testing of general chemicals, biocides and crop protection products (also known as plant protection products or pesticides). It has been developed on the basis of existing methods (2) (3) (4) (5) (6) (7). Other references pertinent to plant testing were also considered (8) (9) (10). Definitions used are given in Appendix 1.

PRINCIPLE OF THE TEST

2. The test assesses effects on seedling emergence and early growth of higher plants following exposure to the test chemical in the soil (or other suitable soil matrix). Seeds are placed in contact with soil treated with the test chemical and evaluated for effects following usually 14 to 21 days after 50 % emergence of the seedlings in the control group. Endpoints measured are visual assessment of seedling emergence, dry shoot weight (alternatively fresh shoot weight) and in certain cases shoot height, as well as an assessment of visible detrimental effects on different parts of the plant. These measurements and observations are compared to those of untreated control plants.

3. Depending on the expected route of exposure, the test chemical is either incorporated into the soil (or possibly into artificial soil matrix) or applied to the soil surface, which properly represents the potential route of exposure to the chemical. Soil incorporation is done by treating bulk soil. After the application the soil is transferred into pots, and then seeds of the given plant species are planted in the soil. Surface applications are made to potted soil in which the seeds have already been planted. The test units (controls and treated soils plus seeds) are then placed under appropriate conditions to support germination/growth of plants.

4. The test can be conducted in order to determine the dose-response curve, or at a single concentration/rate as a limit test according to the aim of the study. If results from the single concentration/rate test exceed a certain toxicity level (e.g. whether effects greater than x% are observed), a range-finding test is carried out to determine upper and lower limits for toxicity followed by a multiple concentration/rate test to generate a dose-response curve. An appropriate statistical analysis is used to obtain effective concentration EC_x or effective application rate ER_x (e.g. EC_{25}, ER_{25}, EC_{50}, ER_{50}) for the most sensitive parameter(s) of interest. Also, the no observed effect concentration (NOEC) and lowest observed effect concentration (LOEC) can be calculated in this test.

INFORMATION ON THE TEST CHEMICAL

5. The following information is useful for the identification of the expected route of exposure to the chemical and in designing the test: structural formula, purity, water solubility, solubility in organic solvents, 1-octanol/water partition coefficient, soil sorption behaviour, vapour pressure, chemical stability in water and light, and biodegradability.

VALIDITY OF THE TEST

6. In order for the test to be considered valid, the following performance criteria must be met in the controls:

- the seedling emergence is at least 70%;
- the seedlings do not exhibit visible phytotoxic effects (e.g. chlorosis, necrosis, wilting, leaf and stem deformations) and the plants exhibit only normal variation in growth and morphology for that particular species;
- the mean survival of emerged control seedlings is at least 90% for the duration of the study;
- environmental conditions for a particular species are identical and growing media contain the same amount of soil matrix, support media, or substrate from the same source.

REFERENCE CHEMICAL

7. A reference chemical may be tested at regular intervals, to verify that performance of the test and the response of the particular test plants and the test conditions have not changed significantly over time. Alternatively, historical biomass or growth measurement of controls could be used to evaluate the performance of the test system in particular laboratories, and can serve as an intra-laboratory quality control measure.

DESCRIPTION OF THE METHOD

Natural soil - Artificial substrate

8. Plants may be grown in pots using a sandy loam, loamy sand, or sandy clay loam that contains up to 1.5 percent organic carbon (approx. 3 percent organic matter). Commercial potting soil or synthetic soil mix that contains up to 1.5 percent organic carbon may also be used. Clay soils should not be used if the test chemical is known to have a high affinity for clays. Field soil should be sieved to 2 mm particle size in order to homogenise it and remove coarse particles. The type and texture, % organic carbon, pH and salt content as electronic conductivity of the final prepared soil should be reported. The soil should be classified according to a standard classification scheme (11). The soil could be pasteurised or heat treated in order to reduce the effect of soil pathogens.

9. Natural soil may complicate interpretation of results and increase variability due to varying physical/chemical properties and microbial populations. These variables in turn alter moisture-holding capacity, chemical-binding capacity, aeration, and nutrient and trace element content. In addition to the variations in these physical factors, there will also be variation in chemical properties such as pH and redox potential, which may affect the bioavailability of the test chemical (12) (13) (14).

10. Artificial substrates are typically not used for testing of crop protection products, but they may be of use for the testing of general chemicals or where it is desired to minimize the variability of the natural soils and increase the comparability of the test results. Substrates used should be composed of inert materials that minimize interaction with the test chemical, the solvent carrier, or both. Acid washed quartz sand, mineral wool and glass beads (e.g. 0.35 to 0.85 mm in diameter) have been found to be suitable inert materials that minimally absorb the test chemical (15), ensuring that the chemical will be maximally available to the seedling via root uptake. Unsuitable substrates would include vermiculite, perlite or other highly absorptive materials. Nutrients for plant growth should be provided to ensure that plants are not stressed through nutrient deficiencies, and where possible this should be assessed via chemical analysis or by visual assessment of control plants.

Criteria for selection of test species

11. The species selected should be reasonably broad, e.g., considering their taxonomic diversity in the plant kingdom, their distribution, abundance, species specific life-cycle characteristics and region of natural occurrence, to develop a range of responses (8) (10) (16) (17) (18) (19) (20). The following characteristics of the possible test species should be considered in the selection:

- the species have uniform seeds that are readily available from reliable standard seed source(s) and that produce consistent, reliable and even germination, as well as uniform seedling growth;
- plant is amenable to testing in the laboratory, and can give reliable and reproducible results within and across testing facilities;
- the sensitivity of the species tested should be consistent with the responses of plants found in the environment exposed to the chemical;
- they have been used to some extent in previous toxicity tests and their use in, for example, herbicide bioassays, heavy metal screening, salinity or mineral stress tests or allelopathy studies indicates sensitivity to a wide variety of stressors;

- they are compatible with the growth conditions of the test method;
- they meet the validity criteria of the test.

Some of the historically most used test species are listed in Appendix 2 and potential non-crop species in Appendix 3.

12. The number of species to be tested is dependent on relevant regulatory requirements, therefore it is not specified in this test method.

Application of the test chemical

13. The chemical should be applied in an appropriate carrier (e.g. water, acetone, ethanol, polyethylene glycol, gum Arabic, sand). Mixtures (formulated products or formulations) containing active ingredients and various adjuvants can be tested as well.

Incorporation into soil/artificial substrate

14. Chemicals which are water soluble or suspended in water can be added to water, and then the solution is mixed with soil with an appropriate mixing device. This type of test may be appropriate if exposure to the chemical is through soil or soil pore-water and that there is concern for root uptake. The water-holding capacity of the soil should not be exceeded by the addition of the test chemical. The volume of water added should be the same for each test concentration, but should be limited to prevent soil agglomerate clumping.

15. Chemicals with low water solubility should be dissolved in a suitable volatile solvent (e.g. acetone, ethanol) and mixed with sand. The solvent can then be removed from the sand using a stream of air while continuously mixing the sand. The treated sand is mixed with the experimental soil. A second control is established which receives only sand and solvent. Equal amounts of sand, with solvent mixed and removed, are added to all treatment levels and the second control. For solid, insoluble test chemicals, dry soil and the chemical are mixed in a suitable mixing device. Hereafter, the soil is added to the pots and seeds are sown immediately.

16. When an artificial substrate is used instead of soil, chemicals that are soluble in water can be dissolved in the nutrient solution just prior to the beginning of the test. Chemicals that are insoluble in water, but which can be suspended in water by using a solvent carrier, should be added with the carrier, to the nutrient solution. Water-insoluble chemicals, for which there is no non-toxic water-soluble carrier available, should be dissolved in an appropriate volatile solvent. The solution is mixed with sand or glass beads, placed in a rotary vacuum apparatus, and evaporated, leaving a uniform coating of chemical on sand or beads. A weighed portion of beads should be extracted with the same organic solvent and the chemical assayed before the potting containers are filled.

Surface application

17. For crop protection products, spraying the soil surface with the test solution is often

used for application of the test chemical. All equipment used in conducting the tests, including equipment used to prepare and administer the test chemical, should be of such design and capacity that the tests involving this equipment can be conducted in an accurate way and it will give a reproducible coverage. The coverage should be uniform across the soil surfaces. Care should be taken to avoid the possibilities of chemicals being adsorbed to or reacting with the equipment (e.g. plastic tubing and lipophilic chemicals or steel parts and elements). The test chemical is sprayed onto the soil surface simulating typical spray tank applications. Generally, spray volumes should be in the range of normal agricultural practice and the volumes (amount of water etc. should be reported). Nozzle type should be selected to provide uniform coverage of the soil surface. If solvents and carriers are applied, a second group of control plants should be established receiving only the solvent/carrier. This is not necessary for crop protection products tested as formulations.

Verification of test chemical concentration/rate

18. The concentrations/rates of application must be confirmed by an appropriate analytical verification. For soluble chemicals, verification of all test concentrations/rates can be confirmed by analysis of the highest concentration test solution used for the test with documentation on subsequent dilution and use of calibrated application equipment (e.g., calibrated analytical glassware, calibration of sprayer application equipment). For insoluble chemicals, verification of compound material must be provided with weights of the test chemical added to the soil. If demonstration of homogeneity is required, analysis of the soil may be necessary.

PROCEDURE

Test design

19. Seeds of the same species are planted in pots. The number of seeds planted per pot will depend upon the species, pot size and test duration. The number of plants per pot should provide adequate growth conditions and avoid overcrowding for the duration of the test. The maximum plant density would be around 3 - 10 seeds per 100 cm^2 depending to the size of the seeds. As an example, one to two corn, soybean, tomato, cucumber, or sugar beet plants per 15cm container; three rape or pea plants per 15 cm container; and 5 to 10 onion, wheat, or other small seeds per 15 cm container are recommended. The number of seeds and replicate pots (the replicate is defined as a pot, therefore plants within the same pot do not constitute a replicate) should be adequate for optimal statistical analysis (21). It should be noted that variability will be greater for test species using fewer large seeds per pot (replicate), when compared to test species where it is possible to use greater numbers of small seeds per pot. By planting equal seed numbers in each pot this variability may be minimized.

20. Control groups are used to assure that effects observed are associated with or attributed only to the test chemical exposure. The appropriate control group should be identical in every respect to the test group except for exposure to the test chemical. Within a given test, all test plants including the controls should be from the same source. To prevent bias, random assignment of test and control pots is required.

21. Seeds coated with an insecticide or fungicide (i.e. "dressed" seeds) should be avoided. However, the use of certain non-systemic contact fungicides (e.g. captan, thiram) is permitted by some regulatory authorities (22). If seed-borne pathogens are a concern, the seeds may be soaked briefly in a weak 5 % hypochlorite solution, then rinsed extensively in running water and dried. No remedial treatment with other crop protection product is allowed.

Test conditions

22. The test conditions should approximate those conditions necessary for normal growth for the species and varieties tested (Appendix 4 provides examples of test condition). The emerging plants should be maintained under good horticultural practices in controlled environment chambers, phytotrons, or greenhouses. When using growth facilities these practices usually include control and adequately frequent (e.g. daily) recording of temperature, humidity, carbon dioxide concentration, light (intensity, wave length, photosynthetically active radiation) and light period, means of watering, etc., to assure good plant growth as judged by the control plants of the selected species. Greenhouse temperatures should be controlled through venting, heating and/or cooling systems. The following conditions are generally recommended for greenhouse testing:

- temperature: 22 ^{o}C ± 10 ^{o}C;
- humidity: 70 % ± 25 %;
- photoperiod: minimum 16 hour light;
- light intensity: 350 ± 50 μE/m2/s. Additional lighting may be necessary if intensity decreases below 200 μE/m2/s, wavelength 400 - 700 nm except for certain species whose light requirements are less.

Environmental conditions should be monitored and reported during the course of the study. The plants should be grown in non-porous plastic or glazed pots with a tray or saucer under the pot. The pots may be repositioned periodically to minimize variability in growth of the plants (due to differences in test conditions within the growth facilities). The pots must be large enough to allow normal growth.

23. Soil nutrients may be supplemented as needed to maintain good plant vigour. The need and timing of additional nutrients can be judged by observation of the control plants. Bottom watering of test containers (e.g. by using glass fiber wicks) is recommended. However, initial top watering can be used to stimulate seed germination and, for soil surface application it facilitates movement of the chemical into the soil.

24. The specific growing conditions should be appropriate for the species tested and the test chemical under investigation. Control and treated plants must be kept under the same environmental conditions, however, adequate measures should be taken to prevent cross exposure (e.g. of volatile chemicals) among different treatments and of the controls to the test chemical.

Testing at a single concentration/rate

25. In order to determine the appropriate concentration/rate of a chemical for conducting a single-concentration or rate (challenge/limit) test, a number of factors must be considered. For general chemicals, these include the physical/chemical properties of the chemical. For crop protection products, the physical/chemical properties and use pattern of the test chemical, its maximum concentration or application rate, the number of applications per season and/or the persistence of the test chemical need to be considered. To determine whether a general chemical possesses phytotoxic properties, it may be appropriate to test at a maximum level of 1000 mg/kg dry soil.

Range-finding test

26. When necessary a range-finding test could be performed to provide guidance on concentrations/rates to be tested in definitive dose-response study. For the range-finding test, the test concentrations/rates should be widely spaced (e.g. 0.1, 1.0, 10, 100 and 1000 mg/kg dry soil). For crop protection products concentrations/rates could be based on the recommended or maximum concentration or application rate, e.g. 1/100, 1/10, 1/1 of the recommended/maximum concentration or application rate.

Testing at multiple concentrations/rates

27. The purpose of the multiple concentration/rate test is to establish a dose-response relationship and to determine an EC_x or ER_x value for emergence, biomass and/or visual effects compared to un-exposed controls, as required by regulatory authorities.

28. The number and spacing of the concentrations or rates should be sufficient to generate a reliable dose-response relationship and regression equation and give an estimate of the $EC_{x.}$ or $ER_{x.}$. The selected concentrations/rates should encompass the EC_x or ER_x values that are to be determined. For example, if an EC_{50} value is required it would be desirable to test at rates that produce a 20 to 80 % effect. The recommended number of test concentrations/rates to achieve this is at least five in a geometric series plus untreated control, and spaced by a factor not exceeding three. For each treatment and control group, the number of replicates should be at least four and the total number of seeds should be at least 20. More replicates of certain plants with low a germination rate or variable growth habits may be needed to increase the statistical power of the test. If a larger number of test concentrations/rates are used, the number of replicates may be reduced. If the NOEC is to be estimated, more replicates may be needed to obtain the desired statistical power (23).

Observations

29. During the observation period, i.e. 14 to 21 days after 50 % of the control plants (also solvent controls if applicable) have emerged, the plants are observed frequently (at least weekly and if possible daily) for emergence and visual phytotoxicity and mortality. At the end of the test, measurement of percent emergence and biomass of surviving plants should be recorded, as well as visible detrimental effects on different parts of the plant. The latter include abnormalities in appearance of the emerged seedlings, stunted growth, chlorosis, discoloration, mortality, and effects on plant development. The final biomass can be measured using final average dry shoot weight

of surviving plants, by harvesting the shoot at the soil surface and drying them to constant weight at 60° C. Alternatively, the final biomass can be measured using fresh shoot weight. The height of the shoot may be another endpoint, if required by regulatory authorities. A uniform scoring system for visual injury should be used to evaluate the observable toxic responses. Examples for performing qualitative and quantitative visual ratings are provided in references (23) (24).

DATA AND REPORTING

Statistical analysis

Single concentration/rate test

30. Data for each plant species should be analyzed using an appropriate statistical method (21). The level of effect at the test concentration/rate should be reported, or the lack of reaching a given effect at the test concentration/rate (e.g., <*x* % effect observed at *y* concentration or rate)

Multiple concentration/rate test

31. A dose-response relationship is established in terms of a regression equation. Different models can be used: for example, for estimating EC_x or ER_x (e.g. EC_{25}, ER_{25}, EC_{50}, ER_{50}) and its confidence limits for emergence as quantal data, logit, probit, Weibull, Spearman-Karber, trimmed Spearman-Karber methods, etc. could be appropriate. For the growth of the seedlings (weight and height) as continuous endpoints EC_x or ER_x and its confidence limits can be estimated by using appropriate regression analysis (e.g. Bruce-Versteeg non-linear regression analysis (25)). Wherever possible, the R^2 should be 0.7 or higher for the most sensitive species and the test concentrations/rates used encompass 20% to 80% effects. If the NOEC is to be estimated, application of powerful statistical tests should be preferred and these should be selected on the basis of data distribution (21) (26).

Test report

32. The test report should present results of the studies as well as a detailed description of test conditions, a thorough discussion of results, analysis of the data, and the conclusions drawn from the analysis. A tabular summary and abstract of results should be provided. The report must include the following:

Test chemical:

- chemical identification data, relevant properties of the chemical tested (e.g. log P_{ow}, water solubility, vapour pressure and information on environmental fate and behaviour, if available);
- details on preparation of the test solution and verification of test concentrations as specified in paragraph 18.

Test species:

- details of the test organism: species/variety, plant families, scientific and common

names, source and history of the seed as detailed as possible (i.e. name of the supplier, percentage germination, seed size class, batch or lot number, seed year or growing season collected, date of germination rating), viability, etc.;
- number of mono- and di-cotyledon species tested;
- rationale for selecting the species;
- description of seed storage, treatment and maintenance.

Test conditions:
- testing facility (e.g. growth chamber, phytotron and greenhouse);
- description of test system (e.g., pot dimensions, pot material and amounts of soil);
- soil characteristics (texture or type of soil: soil particle distribution and classification, physical and chemical properties including % organic matter, % organic carbon and pH);
- soil/substrate (e.g. soil, artificial soil, sand and others) preparation prior to test;
- description of nutrient medium if used;
- application of the test chemical: description of method of application, description of equipment, exposure rates and volumes including chemical verification, description of calibration method and description of environmental conditions during application;
- growth conditions: light intensity (e.g. PAR, photosynthetically active radiation), photoperiod, max/min temperatures, watering schedule and method, fertilization;
- number of seeds per pot, number of plants per dose, number of replicates (pots) per exposure rate;
- type and number of controls (negative and/or positive controls, solvent control if used);
- duration of the test.

Results:
- table of all endpoints for each replicate, test concentration/rate and species;
- the number and percent emergence as compared to controls;
- biomass measurements (shoot dry weight or fresh weight) of the plants as percentage of the controls;
- shoot height of the plants as percentage of the controls, if measured;
- percent visual injury and qualitative and quantitative description of visual injury (chlorosis, necrosis, wilting, leaf and stem deformation, as well as, any lack of effects) by the test chemical as compared to control plants;
- description of the rating scale used to judge visual injury, if visual rating is provided;
- for single rate studies, the percent injury should be reported;
- EC_x or ER_x (e.g. EC_{50}, ER_{50}, EC_{25}, ER_{25}) values and related confidence limits. Where regression analysis is performed, provide the standard error for the regression equation, and the standard error for individual parameter estimate (e.g. slope, intercept);
- NOEC (and LOEC) values if calculated;
- description of the statistical procedures and assumptions used;
- graphical display of these data and dose-response relationship of the species tested.

Deviations from the procedures described in this test method and any unusual

occurrences during the test.

LITERATURE

(1) Schrader G., Metge K., and Bahadir M. (1998). Importance of salt ions in ecotoxicological tests with soil arthropods. Applied Soil Ecology, 7, 189-193.

(2) International Organisation of Standards. (1993). ISO 11269-1. Soil Quality -- Determination of the Effects of Pollutants on Soil Flora – Part 1: Method for the Measurement of Inhibition of Root Growth.

(3) International Organisation of Standards. (1995). ISO 11269-2. Soil Quality -- Determination of the Effects of Pollutants on Soil Flora – Part 2: Effects of Chemicals on the Emergence and Growth of Higher Plants.

(4) American Standard for Testing Material (ASTM). (2002). E 1963-98. Standard Guide for Conducting Terrestrial Plant Toxicity Tests.

(5) U.S. EPA. (1982). FIFRA, 40CFR, Part 158.540. Subdivision J, Parts 122-1 and 123-1.

(6) US EPA. (1996). OPPTS Harmonized Test Guidelines, Series 850. Ecological Effects Test Guidelines:
- 850.4000: Background - Non-target Plant Testing;
- 850.4025: Target Area Phytotoxicity;
- 850.4100: Terrestrial Plant Toxicity, Tier I (Seedling Emergence);
- 850.4200: Seed Germination/Root Elongation Toxicity Test;
- 850.4225: Seedling Emergence, Tier II;
- 850.4230: Early Seedling Growth Toxicity Test.

(7) AFNOR, X31-201. (1982). Essai d'inhibition de la germination de semences par une substance. AFNOR X31-203/ISO 11269-1. (1993) Determination des effets des polluants sur la flore du sol: Méthode de mesurage de l'inhibition de la croissance des racines.

(8) Boutin, C., Freemark, K.E. and Keddy, C.J. (1993). Proposed guidelines for registration of chemical pesticides: Non-target plant testing and evaluation. Technical Report Series No.145. Canadian Wildlife Service (Headquarters), Environment Canada, Hull, Québec, Canada.

(9) Forster, R., Heimbach, U., Kula, C., and Zwerger, P. (1997). Effects of Plant Protection Products on Non-Target Organisms - A contribution to the Discussion of Risk Assessment and Risk Mitigation for Terrestrial Non-Target Organisms (Flora and Fauna). Nachrichtenbl. Deut. Pflanzenschutzd. No 48.

(10) Hale, B., Hall, J.C., Solomon, K., and Stephenson, G. (1994). A Critical Review of the Proposed Guidelines for Registration of Chemical Pesticides; Non-Target Plant Testing and Evaluation, Centre for Toxicology, University of Guelph, Ontario Canada.

(11) Soil Texture Classification (US and FAO systems): Weed Science, 33, Suppl. 1 (1985) and Soil Sc. Soc. Amer. Proc. 26:305 (1962).

(12) Audus, L.J. (1964). Herbicide behaviour in the soil. In: Audus, L.J. ed. *The Physiology and biochemistry of Herbicides*, London, New York, Academic Press, NY, Chapter 5, pp. 163-206.

(13) Beall, M.L., Jr. and Nash, R.G. (1969). Crop seedling uptake of DDT, dieldrin, endrin, and heptachlor from soil, J. Agro. 61:571-575.

(14) Beetsman, G.D., Kenney, D.R. and Chesters, G. (1969). Dieldrin uptake by corn as affected by soil properties, J. Agro. 61:247-250.

(15) U.S. Food and Drug Administration (FDA). (1987). Environmental Assessment Technical Handbook. Environmental Assessment Technical Assistance Document 4.07, Seedling Growth, 14 pp., FDA, Washington, DC.

(16) McKelvey, R.A., Wright, J.P., Honegger, J.L. and Warren, L.W. (2002). A Comparison of Crop and Non-crop Plants as Sensitive Indicator Species for Regulatory Testing. Pest Management Science vol. 58:1161-1174

(17) Boutin, C.; Elmegaard, N. and Kjær, C. (2004). Toxicity testing of fifteen non-crop plant species with six herbicides in a greenhouse experiment: Implications for risk assessment. Ecotoxicology vol. 13(4): 349-369.

(18) Boutin, C., and Rogers, C.A. (2000). Patterns of sensitivity of plant species to various herbicides – An analysis with two databases. Ecotoxicology vol.9(4):255-271.

(19) Boutin, C. and Harper, J.L. (1991). A comparative study of the population dynamics of five species of *Veronica* in natural habitats. J. Ecol. 9:155-271.

(20) Boutin, C., Lee, H.-B., Peart, T.E., Batchelor, S.P. and Maguire, R.J. . (2000). Effects of the sulfonylurea herbicide metsulfuron methyl on growth and reproduction of five wetland and terrestrial plant species. Envir. Toxicol. Chem. 19 (10): 2532-2541.

(21) OECD (2006). Guidance Document, Current Approaches in the Statistical Analysis of Ecotoxicity Data: A Guidance to Application. Series on Testing

and Assessment No 54, Organisation for Economic Co-operation and Development, Paris.

(22) Hatzios, K.K. and Penner, D. (1985). Interactions of herbicides with other agrochemicals in higher plants. Rev. Weed Sci. 1:1-63.

(23) Hamill, P.B., Marriage, P.B. and G. Friesen. (1977). A method for assessing herbicide performance in small plot experiments. Weed Science 25:386-389.

(24) Frans, R.E. and Talbert, R.E. (1992). Design of field experiments and the measurement and analysis of plant response. In: B. Truelove (Ed.) Research Methods in Weed Science, 2nd ed. Southern weed Science Society, Auburn, 15-23.

(25) Bruce, R.D. and Versteeg, D. J.(1992). A Statistical Procedure for Modelling Continuous Toxicity Data. Environmental Toxicology and Chemistry 11, 1485-1492.

(26) Chapter C.33 of this Annex: Earthworm Reproduction Test (*Eisenia fetida/Eisenia andrei*).

Appendix 1

DEFINITIONS

Active ingredient (a.i.) (or active substance (a.s.)) is a material designed to provide a specific biological effect (e.g., insect control, plant disease control, weed control in the treatment area), also known as technical grade active ingredient, active substance.

Chemical means a substance or a mixture.

Crop Protection Products (CPPs) or plant protection product (PPPs) or pesticides are materials with a specific biological activity used intentionally to protect crops from pests (e.g., fungal diseases, insects and competitive plants).

EC_x. x% Effect Concentration or ER_x. x% Effect Rate is the concentration or the rate that results in an undesirable change or alteration of x% in the test endpoint being measured relative to the control (e.g., 25% or 50% reduction in seedling emergence, shoot weight, final number of plants present, or increase in visual injury would constitute an EC_{25}/ER_{25} or EC_{50}/ER_{50} respectively).

Emergence is the appearance of the coleoptile or cotyledon above the soil surface.

Formulation is the commercial formulated product containing the active substance (active ingredient), also known as final preparation[1] or typical end-use product (TEP).

LOEC (Lowest Observed Effect Concentration) is the lowest concentration of the test chemical at which effect was observed. In this test, the concentration corresponding to the LOEC, has a statistically significant effect ($p < 0.05$) within a given exposure period when compared to the control, and is higher than the NOEC value.

Non-target plants: Those plants that are outside the target plant area. For crop protection products, this usually refers to plants outside the treatment area.

NOEC (No Observed Effect Concentration) is the highest concentration of the test chemical at which no effect was observed. In this test, the concentration corresponding to the NOEC, has no statistically significant effect ($p < 0.05$) within a given exposure period when compared with the control.

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[1] Final Preparation: The formulated product containing the active chemical (active ingredient) sold in commerce.

Phytotoxicity: Detrimental deviations (by measured and visual assessments) from the normal pattern of appearance and growth of plants in response to a given chemical.

Replicate is the experimental unit which represents the control group and/or treatment group. In these studies, the pot is defined as the replicate.

Visual assessment: Rating of visual damage based on observations of plant stand, vigour, malformation, chlorosis, necrosis, and overall appearance compared with a control.

Test Chemical : Any substance or mixture tested using this test method.

Appendix 2

LIST OF SPECIES HISTORICALLY USED IN PLANT TESTING

Family	Species	Common names
DICOTYLEDONAE		
Apiaceae (Umbelliferae)	*Daucus carota*	Carrot
Asteraceae (Compositae)	*Helianthus annuus*	Sunflower
Asteraceae (Compositae)	*Lactuca sativa*	Lettuce
Brassicaceae (Cruciferae)	*Sinapis alba*	White Mustard
Brassicaceae (Cruciferae)	*Brassica campestris var. chinensis*	Chinese cabbage
Brassicaceae (Cruciferae)	*Brassica napus*	Oilseed rape
Brassicaceae (Cruciferae)	*Brassica oleracea var. capitata*	Cabbage
Brassicaceae (Cruciferae)	*Brassica rapa*	Turnip
Brassicaceae (Cruciferae)	*Lepidium sativum*	Garden cress
Brassicaceae (Cruciferae)	*Raphanus sativus*	Radish
Chenopodiaceae	*Beta vulgaris*	Sugar beet
Cucurbitaceae	*Cucumis sativus*	Cucumber
Fabaceae (Leguminosae)	*Glycine max (G. soja)*	Soybean
Fabaceae (Leguminosae)	*Phaseolus aureus*	Mung bean

Fabaceae (Leguminosae)	*Phaseolus vulgaris*	Dwarf bean, French bean, Garden bean
Fabaceae (Leguminosae)	*Pisum sativum*	Pea
Fabaceae (Leguminosae)	*Trigonella foenum-graecum*	Fenugreek
Fabaceae (Leguminosae)	*Lotus corniculatus*	Birdsfoot trefoil
Fabaceae (Leguminosae)	*Trifolium pratense*	Red Clover
Fabaceae (Leguminosae)	*Vicia sativa*	Vetch
Linaceae	*Linum usitatissimum*	Flax
Polygonaceae	*Fagopyrum esculentum*	Buckwheat
Solanaceae	*Solanum lycopersicon*	Tomato
MONOCOTYLEDONAE		
Liliaceae (Amarylladaceae)	*Allium cepa*	Onion
Poaceae (Gramineae)	*Avena sativa*	Oats
Poaceae (Gramineae)	*Hordeum vulgare*	Barley
Poaceae (Gramineae)	*Lolium perenne*	Perennial ryegrass
Poaceae (Gramineae)	*Oryza sativa*	Rice
Poaceae (Gramineae)	*Secale cereale*	Rye
Poaceae (Gramineae)	*Sorghum bicolor*	Grain sorghum, Shattercane
Poaceae (Gramineae)	*Triticum aestivum*	Wheat
Poaceae (Gramineae)	*Zea mays*	Corn

Appendix 3

LIST OF POTENATIAL NON-CROP SPECIES

OECD Potential Species for Plant Toxicity Testing.

NOTE: The following table provides information for 52 non-crop species (references are given in brackets for each entry). Emergence rates provided are from published literature and are for general guidance only. Individual experience may vary depending upon seed source and other factors.

FAMILY Species Botanical Name (English Common Name)	Lifespan[1] & Habitat	Seed Weight (mg)	Photoperiod for germination or growth[2]	Planting Depth (mm)[3]	Time to Germinate (days)[4]	Special Treatments[5]	Toxicity Test[6]	Seed Suppliers[7]	Other References[8]
APIACEAE *Torilis japónica* (Japanese Hedge-parsley)	A, B disturbed areas, hedgerows, pastures (16, 19)	1.7 - 1.9 (14, 19)	L = D (14)	0 (1, 19)	5 (50%) (19)	cold stratification (7, 14, 18, 19) maturation may be necessary (19) germination inhibited by darkness (1, 19) no special treatments (5)	POST (5)		
ASTERACEAE *Bellis perennis* (English Daisy)	P grassland, arable fields, turf (16, 19)	0.09-0.17 (4, 19)	L = D (14)	0 (4)	3 (50%) (19) 11 (100%) (18)	germination not affected by irradiance (18, 19) no special treatments (4, 14)	POST (4)	A, D, F	7
Centaurea cyanus (Cornflower)	A fields, roadsides, open habitats (16)	4.1 -4.9 (4, 14)	L = D (14)	0-3 (2, 4, 14)	14-21 (100%) (14)	no special treatments (2, 4)	POST (2,4)	A, D, E, F	7

Centaurea nigra (Black Knapweed)	P fields, roadsides, open habitats (16, 19)	2.4-2.6 (14, 19)	L = D (14)	0 (19)	3 (50%) (19) 4 (97%) (18)	maturation may be necessary (18, 19) germination inhibited by darkness (19) no special treatments (5, 14, 26)	POST (5, 22, 26)	A	
Inula h elenium Elecampane	P moist, disturbed sites (16)	1 - 1.3 (4, 14, 29)		0 (4, 29)		no special treatments (4)	POST (4)	A, F	
Leontodon hispidus (Big Hawkbit)	P fields, roadsides, disturbed areas (16, 19)	0.85 -1.2 (14, 19)	L = D (14)	0 (19)	4 (50%) (19) 7 (80%) (18)	germination inhibited by darkness (17, 18, 19) no special treatments (5, 23)	POST (5, 22, 23)		
Rudbeckia hirta (Black-eyed Susan)	B, P disturbed (16)	0.3 (4, 14)	L = D (14)	0 (4, 33)	< 10 (100%) (33)	no special treatments (4, 14, 33)	POST (4, 33)	C, D, E, F	
Solidago canadensis Canada Goldenrod	P pasture, open areas (16)	0.06-0.08 (4, 14)	L = D (11)	0 (4)	14-21 (11)	mix with equal part sand and soak in 500ppm GA for 24 hrs (11) no special treatments (4)	POST (4)	E, F	
Xanthium pensylvanicum (Common Cocklebur)	A fields, open habitats (16)	25-61 (14, 29)		0(1) 5(29)		germination may be inhibited by darkness (1) soak in warm water for 12 hrs (29)	PRE & POST (31)	A	
Xanthium spinosum (Spiny Cocklebur)	A open habitats (16)	200 (14)	L = D (14) L > D (6)	10 (6)		scarification (14) no special treatments (6)	PRE & POST (6)	A	
Xanthium strumarium (Italian Cocklebur)	A fields, open habitats (16)	67.4 (14)	L = D (14)	10-20 (6, 21)		no special treatments (6, 14, 21)	PRE & POST (6, 21, 28, 31)	A	
BRASSICACEAE *Cardamine pratensis* (Cuckoo Flower)	P fields, roadsides, turf (16, 19)	0.6 (14, 19)	L = D (14)	0 (19)	5 (50%) (19) 15 (98%) (18)	germination inhibited by darkness (18, 19) no special treatments (5, 14, 22)	POST (5, 22)	F	

CARYOPHYLLACEAE *Lychnis flos-cuculi* (Ragged Robin)	P (16)	0.21 (14)	L = D (14)		< 14 (100%) (14, 25)	maturation may be necessary (18) no special treatments (5, 14, 15, 22-26)	POST (5, 15, 22-26)	F	
CHENOPODIACEAE *Chenopodium album* (Lamb's Quarters)	A field margins, disturbed areas (16, 19)	0.7 - 1.5 (14, 19, 34)	L = D (14)	0 (1, 19)	2 (50%) (19)	treatment differs depending on seed colour (19) dry storage dormancy (19) germination inhibited by darkness (1, 18, 19) cold stratification (18) no special treatments (14, 34)	PRE & POST (28, 31, 34)	A	32
CLUSIACEAE *Hypericum perforatum* **(**Common St. John's Wort)	P fields, arable land, open habitats (16, 19)	0.1 -0.23 (14, 19)	L= D (14)	0 (1, 19)	3 (19) 11 (90%) (18)	germination inhibited by darkness (1, 18, 19) no special treatments (5, 14, 15, 25, 27)	POST (5, 15, 25, 27)	A, E, F	
CONVOLVULACEAE *Ipomoea hederacea* (Purple Morning Glory)	A roadsides, open habitats, cornfields (16)	28.2 (14)	L > D (6, 10)	10-20 (6, 10, 21)	4 (100%) (10)	germination not affected by irradiance (1) no special treatments (6, 21)	PRE & POST (6, 12, 21, 28)	A	
CYPERACEAE *Cyperus rotundus* (Purple Nutsedge)	P arable land, pastures, roadsides (16, 30)	0.2 (14)	L= D (14)	0 (1) 10-20 (6, 10)	12 (91%) (10)	germination inhibited by darkness (1) no special treatments (6, 10, 14)	PRE & POST (6, 28, 31)	B	7

FABACEAE *Lotus corniculatus* (Bird's-foot Trefoil)	P grassy areas, roadsides, open habitats (16, 19)	1-1.67 (14, 19)	L = D (14)		1 (50%) (19)	scarification (14, 19) germination not affected by irradiance (18, 19) no special treatments (23, 25)	POST (5, 23, 25)	A, D, E, F	
Senna obtusifolia (Cassia, Sicklepod)	A moist woods (16)	23-28 (9)	L = D (14) L > D (9)	10-20 (6,9)		soak seeds in water for 24 hours (9) scarification (14) seed viability differs depending on colour (1) no special treatments (6)	POST (6,9)	A	
Sesbania exaltata (Hemp)	A alluvial soil (16)	11 - 13 (9, 14)	L > D (9)	10-20 (9, 21)		soak seeds in water for 24 hours (9) germination not affected by irradiance (1) no special treatments (21)	PRE & POST (9, 21, 28, 31)	A	
Trifolium pratense (Red Clover)	P fields, roadsides, arable land (16, 19)	1.4 - 1.7 (14, 19)	L= D (14)		1 (50%) (19)	scarification (14, 18) may need maturation (19) germination not affected by irradiance (1, 19) no special treatments (5)	POST (5)	A, E, F	
LAM IAC E AE *Leonurus cardiaca* (Motherwort)	P open areas (16)	0.75 -1.0 (4, 14)	L= D (14)	0 (4)		no special treatments (4, 14)	POST (4)	F	

Mentha spicata (Spearmint)	P moist areas (16)	2.21 (4)		0 (4)		no special treatments (4)	POST (4)	F	
Nepeta cataria (Catnip)	P disturbed areas (16)	0.54 (4, 14)	L= D (14)	0 (4)		no special treatments (2, 4, 14)	POST (2,4)	F	
Prunella vulgaris (Self-heal)	P arable fields, grassy areas, disturbed sites (16, 19)	0.58 -1.2 (4, 14, 19)	L= D (14)	0 (4, 19)	5 (50%) (19) 7 (91%) (18)	germination inhibited by darkness (18, 19) greater germination with larger seeds (1) no special treatments (4, 14, 22)	POST (4, 22)	A, F	
Stachys officinalis (Hedge-nettle)	P grasslands, field margins (19)	14-18 (14, 19)	L= D (14)		7 (50%) (19)	no special treatments (5, 14, 22)	POST (5, 22)	F	
MALVACEAE *Abutilón theophrasti* (Velvetleaf)	A fields, open habitats (16)	8.8 (14)	L= D (14)	10-20 (6, 10, 21)	4 (84%) (10)	scarification (14) no special treatments (5, 10, 21)	PRE & POST (6, 22, 28, 31)	A, F	
Sida spinosa (Prickly Sida)	A fields, roadsides (16)	3.8 (14)	L= D (14)	10-20 (6, 21)		scarification (14) germination not affected by irradiance (1) no special treatments (6, 21)	PRE & POST (6, 21, 28, 31)	A, F	

PAPAVERACEAE *Papaver rhoeas* (Poppy)	A fields, arable land, disturbed sites (16, 19)	0.1 -0.3 (4, 14, 19, 29)	L= D (14)	0 (4, 29)	4 (50%) (19)	cold stratification & scarification (1, 19, 32) no special treatments (4, 14, 29)	POST (4)	A, D, E, F, G	
POACEAE *Agrostis tenuis* (Common Bentgrass)	lawns, pastures (16)	0.07 (14)	L > D (Ю)	20 (10)	10 (62%) (10)	germination inhibited by darkness (1, 17-19) no special treatments (10)	POST (10)	A, E	
Alopecurus myosuroides (Foxtail)	A fields, open habitats (16)	0.9-1.6 (29, 34)	L = D (14)	2 (29)	< 24 (30%) (34)	scarification (14) treat with 101 mg/L KNO3 (14) warm stratification (1) germination inhibited by darkness (1) no special treatments (34)	PRE & POST (28, 34)	A	32
Avena fatua (Wild Oats)	A cultivated areas, open habitats (16)	7-37.5 (14, 30)	L = D (14) L > D (6)	10-20 (6, 10)	3 (70%) (18)	scarification (7, 32) darkness inhibits germination (1) cold stratification (1, 18) no special treatments (6, 10, 14)	PRE & POST (6, 10, 28, 31)	A	
Bromus tectorum (Downy Brome)	A fields, roadsides, arable land (16)	0.45-2.28 (14, 29)	L = D (14)	3 (29)		maturation period (1, 7, 32) germination inhibited by light (1) no special treatments (14)	PRE & POST (28, 31)	A	
Cynosurus cristatus (Dog's-tail Grass)	P fields, roadsides, open habitats (16, 19)	0.5-0.7 (14, 19, 29)	L = D (14)	0 (29)	3 (50%) (19)	germination not affected by irradiance (19) no special treatments (14, 29)	POST (5)	A	
Digitaria sanguinalis (Crabgrass)	A fields, turf, open habitats (16)	0.52-0.6 (14, 30)	L = D (14)	10-20 (21)	7 (75%) 14 (94%) (7)	scarification, cold stratification, & maturation (1, 7, 14, 32) treat with 101 mg/L KNO3 (14) germination inhibited by darkness (1) no special treatments (21)	PRE & POST (18, 25, 31)	A	

Echinochloa crusgalli (Barnyard Grass)	A (16)	1.5 (14)	L = D (14) L > D (3)	10-20 (7, 21)		scarification (7, 32) germination not affected by irradiance (1) no special treatments (3, 14, 21)	PRE & POST (3, 21, 28, 31)	A	
Elymus canadensis (Canada Wild Rye)	P riparian, disturbed sites (16)	4-5 (14, 30)	L = D (11)	1 (11)	14-28 (11)	no special treatments (2, 11)	POST (2)	C, D, E	
Festuca pratensis (Fescue)	P fields, moist areas (16, 19)	1.53-2.2 (16, 19)	L = D (14) L > D (10)	20 (10)	9 (74%) (10) 2 (50%) (19)	no special treatments (10, 19)	POST (10)	A	7
Hordeum pusillum (Little Barley)	A pastures, roadsides, open habitats (16)	3.28 (14)				warm stratification (1) germination not affected by irradiance (1)	PRE (31)		7
Phieum pratense (Timothy)	P pastures, arable fields, disturbed sites (16, 19)	0.45 (14, 19)	L > D (10, 14)	0-10 (10, 19)	2 (74%) (10) 8 (50%) (19)	germination inhibited by darkness (19) germination not affected by irradiance (17) no special treatments (10, 14, 17, 19)	POST (10)	A, E	
POLYGONACEAE *Polygonum convolvulus* (Black Bindweed)	A open habitats, roadsides (16)	5-8 (4, 14, 29)	L = D (20)	0-2 (4, 29)		cold stratification for 4 - 8 weeks (1, 2, 4, 20, 29) germination not affected by irradiance (1)	PRE & POST 1, 2, 20, 28, 31	A	32
Polygonum lapathifolium (Pale Persicaria)	A moist soil (16)	1.8-2.5 (14)	L > D (6)		5 (94%) (18)	germination not affected by irradiance (1) germination inhibited by darkness (18) cold stratification (1) no special treatments (5)	PRE & POST (6)	A, E	
Polygonum pennsylvanicum (Pennsylvania Smartweed)	A fields, open habitats (16)	3.6-7 (14, 29)		2 (29)		cold stratification for 4 wks at 0 - 5oC (1, 29) germination inhibited by darkness (1)	PRE (31)	A, E	
Polygonum periscaria (Smartweed)	A disturbed areas, arable	2.1 -2.3 (14, 19)	L > D (13)	0 (19)	< 14 (13) 2 (50%) (19)	scarification, cold stratification, GA treatment (14) cold stratification, maturation (17-19)	POST (13)	A	32

	land (16, 19)					germination inhibited by darkness (19) no special treatments (13)			
Rumex crispus (Curly Dock)	P arable fields, roadsides open areas (16, 19)	1.3-1.5 (4, 14, 19)	L = D (14, 33)	0 (4, 19, 33)	3 (50%) (19) 6 (100%) (33)	germination inhibited by darkness (18, 19) maturation may be necessary (18) no special treatments (4, 14, 33)	POST (4, 33)	A, E	32
PRIMULACEAE *Anagallis arvensis* (Scarlett Pimpernel)	A arable fields, open areas, disturbed sites (16, 19)	0.4-0.5 (4, 14, 19)	L = D (14)		1 (50%) (19)	cold stratification, GA treatment (1,14, 18, 19, 32) light required for germination (1) no special treatments (2, 4)	POST (2,4)	A, F	
RANUNCULACEAE *Ranunculus acris* (Common Buttercup)	P arable fields, roadsides, open areas (16, 19)	1.5-2 (14, 19, 29)	L = D (14)	1 (29)	41 -56 (19, 29)	no special treatments (5, 14, 22, 24 -26)	POST (5, 22, 24-26)		32
ROSACEAE *Geum urbanum* (Yellow Avens)	P hedgerows, moist areas (16, 19)	0.8 - 1.5 (14, 19)	L = D (14)	0 (19)	5 (50%) (19) 16 (79%) (18)	germination inhibited by darkness (18, 19) warm stratification (1) no special treatments (5, 14, 22, 25, 26)	POST (5, 22, 25, 26)	A	
RUBIACEAE Galium aparine (Cleavers)	A arable fields, moist areas, disturbed sites (16, 19)	7-9 (14, 19)	L = D (14)		5 (50%) (19) 6 (100%) (18)	cold stratification (1, 18, 19) germination not affected by irradiance (18, 19) light inhibits germination (1) no special treatments (6, 14)	PRE & POST (6, 28)	A	32
Galium mollugo (Hedge Bedstraw)	P hedgebanks, open areas (8)	7 (29)	L = D (14)	2 (29)		no special treatments (5, 14, 22, 24, 26, 29)	POST (5, 22, 24, 26)	A	
SCROPHULARIACEAE *Digitalis purpurea* (Foxglove)	B, P hedgerows, open areas (16, 19)	0.1 -0.6 (4, 14, 19)	L = D (14)	0 (4, 19)	6 (50%) (19) 8 (99%) (18)	germination inhibited by darkness (1, 17-19) no special treatments (4, 22-26)	POST (4, 22 - 26)	D, G, F	
Veronica persica (Speedwell)	A arable fields, open	0.5-0.6 (14, 19)	L = D (14)	0 (19)	3(19) 5 (96%) (18)	germination inhibited by darkness (18, 19) cold stratification (18) no special	PRE & POST (28)	A	32

	areas, disturbed sites (16, 19)					treatments (14)			

[1] A = Annuals, B = Biennials, P = Perennials.
[2] References 11,14 and 33 referto proportion of light (L) and darkness (D) required to induce seed germination. References 3, 6, 9, 10, 13, 20 referto growing conditions in greenhouses.
[3] 0 mm indicates seeds were sown on the soil surface or that seeds need light to germinate.
[4] The numbers provided represent the number of days in which a percent of seeds germinated according to provided reference, e.g., 3 days (50%) germination (reference 19).
[5] Duration of maturation and or stratification not always available. Except for cold treatment requirements, temperature conditions are not specified since in greenhouse testing there is limited temperature control. Most seeds will germinate under normal fluctuation of temperatures found in greenhouses.
[6] Indicates species was utilized in either a pre-emergence (PRE) and/or post-emergence (POST) plant toxicity test involving herbicides.
[7] Provides example(s) of commercial seed suppliers.
[8] Provides two alternative reference(s) that were consulted.

Seed Suppliers Cited

Supplier ID	Supplier Information
A	Herbiseed New Farm, Mire Lane, West End, Twyford RG10 0NJ ENGLAND +44 (0) 1189 349 464 www. herbiseed.com
B	Tropilab Inc. 8240 Ulmerton Road, Largo, FL 33771-3948 USA (727) 344 - 4050 www.tropilab.com
C	Pterophylla - Native Plants & Seeds #316 Regional Road 60, RR#1, Walsingham, ON N0E 1X0 CANADA (519) 586 - 3985
D	Applewood Seed Co. 5380 Vivian St., Arvada, CO 80002 USA (303) 431 - 7333 www.applewoodseed.com
E	Ernst Conservation Seeds 9006 Mercer Pike, Meadville, PA 16335 USA (800) 873 - 3321 www.ernstseed.com
F	Chiltern Seeds Bortree Stile, Ulverston, Cumbria LA12 7PB ENGLAND +44 1229 581137 www.chiltemseeds.co.uk
G	Thompson & Morgan

	P.O. Box 1051, Fort Erie, ON L2A 6C7 CANADA (800) 274 - 7333 www.thompson-morgan.com

REFERENCES CITED

(1) Baskin, C.C. & Baskin, J.M. 1998. Seeds. Academic Press, Toronto

(2) Blackburn, L.G. & Boutin, C. 2003. Subtle effects of herbicide use in the context of genetically modified crops: a case study with glyphosate (Round-Up®). Ecotoxicology, 12:271-285.

(3) Boutin, C., Lee, H-B., Peart, T., Batchelor, P.S., & Maguire, R.J. 2000. Effects of the sulfonylurea herbicide metsulfuron methyl on growth and reproduction of five wetland and terrestrial plant species. Environmental Toxicology & Chemistry, 19(10):2532-2541.

(4) Boutin, C., Elmegaard, N., & Kjaer, C. 2004. Toxicity testing of fifteen non-crop plant species with six herbicides in a greenhouse experiment: implications for risk assessment. Ecotoxicology, 13:349-369.

(5) Breeze, V., Thomas, G., & Butler, R. 1992. Use of a model and toxicity data to predict the risks to some wild plant species from drift of four herbicides. Annals of Applied Biology, 121:669-677.

(6) Brown, R.A., & Farmer, D. 1991. Track-sprayer and glasshouse techniques for terrestrial plant bioassays with pesticides. In: Plants for toxicity assessment: 2nd volume. ASTM STP 1115, J.W. Gorsuch, W.R. Lower, W.Wang, & M.A. Lewis, eds. American Society for Testing & Materials, Philadelphia. pp 197 – 208.

(7) Buhler, D.D. & Hoffman, M.L. 1999. Anderson's guide to practical methods of propagating weeds and other plants. Weed Science Society of America, Lawrence, K.

(8) Clapham, A.R., Tutin, T.G., & Warburg, E.F. 1981. Excursion flora of the British Isles, 3rd ed. Cambridge University Press, Cambridge

(9) Clay, P.A. & Griffin, J.L. 2000. Weed seed production and seedling emergence response to late-season glyphosate applications. Weed Science, 48:481-486.

(10) Cole, J.F.H. & Canning, L. 1993. Rationale for the choice of species in the regulatory testing of the effects of pesticides on terrestrial non-target plants. BCPC – Weeds. pp. 151 – 156.

(11) Fiely, M. (Ernst Conservation Seeds). 2004. Personal communication. (www.ernstseed.com)

(12) Fletcher, J.S., Johnson, F.L., & McFarlane, J.C. 1990. Influence of greenhouse versus field testing and taxonomic differences on plant sensitivity to chemical treatment. Environmental Toxicology & Chemistry, 9:769-776.

(13) Fletcher, J.S., Pfleeger, T.G., Ratsch, H.C., & Hayes, R. 1996. Potential impact of low levels of chlorsulfuron and other herbicides on growth and yield of nontarget plants. Environmental Toxicology & Chemistry, 15(7):1189-1196.

(14) Flynn, S., Turner, R.M., and Dickie, J.B. 2004. Seed Information Database (release 6.0, Oct 2004) Royal Botanic Gardens, Kew (www.rbgkew.org.uk/data/sid)

(15) Franzaring, J., Kempenaar, C., & van der Eerden, L.J.M. 2001. Effects of vapours of chlorpropham and ethofumesate on wild plant species. Environmental Pollution, 114:21-28.

(16) Gleason, H.A. & Cronquist, A. 1991. Manual of vascular plants of northeastern United States and adjacent Canada, 2nd ed. New York Botanical Garden, Bronx, NY

(17) Grime, J.P. 1981. The role of seed dormancy in vegetation dynamics. Annals of Applied Biology, 98:555-558.

(18) Grime, J.P., Mason, G., Curtis, A.V., Rodman, J., Band, S.R., Mowforth, M.A.G., Neal, A.M., & Shaw, S. 1981. A comparative study of germination characteristics in a local flora. Journal of Ecology, 69:1017-1059.

(19) Grime, J.P., Hodgson, J.G., & Hunt, R. 1988. Comparative plant ecology: a functional approach to common British species. Unwin Hyman Ltd., London

(20) Kjaer, C. 1994. Sublethal effects of chlorsulfuron on black bindweed (Polygonum convolvulus L.). Weed Research, 34:453-459.

(21) Klingaman, T.E., King, C.A., & Oliver, L.R. 1992. Effect of application rate, weed species, and weed stage of growth on imazethapyr activity. Weed Science, 40:227-232.

(22) Marrs, R.H., Williams, C.T., Frost, A.J., & Plant, R.A. 1989. Assessment of the effects of herbicide spray drift on a range of plant species of conservation interest. Environmental Pollution, 59:71-86.

(23) Marrs, R.H., Frost, A.J., & Plant, R.A. 1991. Effects of herbicide spray drift on selected species of nature conservation interest: the effects of plant age and surrounding vegetation structure. Environmental Pollution, 69:223-235.

(24) Marrs, R.H., Frost, A.J., & Plant, R.A. 1991. Effects of mecoprop drift on some plant species of conservation interest when grown in standardized mixtures in microcosms. Environmental Pollution, 73:25-42.

(25) Marrs, R.H., Frost, A.J., Plant, R.A., & Lunnis, P. 1993. Determination of buffer zones to protect seedlings of non-target plants

from the effects of glyphosate spray drift. Agriculture, Ecosystems, & Environment, 45:283-293.

(26) Marrs, R.H. & Frost, A.J. 1997. A microcosm approach to detection of the effects of herbicide spray drift in plant communities. Journal of Environmental Management, 50:369-388.

(27) Marshall, E.J.P. & Bernie, J.E. 1985. Herbicide effects on field margin flora. BCPC – Weeds. pp. 1021-1028.

(28) McKelvey, R.A., Wright, J.P., & Honegger, J.L. 2002. A comparison of crop and non-crop plants as sensitive species for regulatory testing. Pest Management Science, 58:1161-1174.

(29) Morton, S. (Herbiseed). 2004. Personal communication. (http://www.herbiseed.com)

(30) USDA, NRCS. 2004. The Plants Database, version 3.5. (http://plants.usda.gov). National Plant Data Centre, Baton Rouge, LA 70874-4490 USA

(31) USEPA. 1999. One-Liner Database. [U.S. E.P.A./Office of Pesticide Programs/Environmental Fate and Effects Division/Environmental Epidemiology Branch].

(32) Webster, R.H. 1979. Technical Report No. 56: Growing weeds from seeds and other propagules for experimental purposes. Agricultural Research Council Weed Research Organization, Oxford.

(33) White, A. L. & Boutin, C. (National Wildlife Research Centre, Environment Canada). 2004. Personal communication.

(34) Zwerger, P. & Pestemer, W. 2000. Testing the phytotoxic effects of herbicides on higher terrestrial non-target plants using a plant life-cycle test. Z. PflKrankh. PflSchutz, Sonderh., 17:711-718.

Appendix 4

EXAMPLES FOR APPROPRIATE GROWTH CONDITIONS FOR CERTAIN CROP SPECIES

The following conditions have been found suitable for 10 crop species, and can be used as a guidance for tests in growth chambers with certain other species as well:

Carbon dioxide concentration: 350 ± 50 ppm;
Relative humidity: 70 ± 5 % during light periods and 90 ± 5 % during dark periods;
Temperature: 25 ± 3 °C during the day, 20 ± 3 °C during the night;
Photoperiod: 16 hour light/8 hour darkness, assuming an average wavelength of 400 to 700 nm;
Light: luminance of 350 ± 50 $\mu E/m^2/s$, measured at the top of the canopy.

The crop species are:

- tomato (Solanum lycopersicon);
- cucumber (Cucumis sativus);
- lettuce (Lactuca sativa);
- soybean (Glycine max);
- cabbage (Brassica oleracea var. capitata);
- carrot (Daucus carota);
- oats (Avena sativa);
- perennial ryegrass (Lolium perenne);
- corn (Zea mays);
- onion (Allium cepa).

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C.32. Enchytraeid Reproduction Test

INTRODUCTION

1. This test method is equivalent to OECD test guideline (TG) 220 (2004). It is designed to be used for assessing the effects of chemicals on the reproductive output of the enchytraeid worm, *Enchytraeus albidus* Henle 1873, in soil. It is based principally on a method developed by the Umweltbundesamt, Germany (1) that has been ring-tested (2). Other methods for testing the toxicity of chemicals to Enchytraeidae and earthworms have also been considered (3)(4)(5)(6)(7)(8).

INITIAL CONSIDERATIONS

2. Soil-dwelling annelids of the genus Enchytraeus are ecologically relevant species for ecotoxicological testing. Whilst enchytraeids are often found in soils containing earthworms it is also true that they are often abundant in many soils where earthworms are absent. Enchytraeids can be used in laboratory tests as well as in semi-field and field studies. From a practical point of view, many Enchytraeus species are easy to handle and breed, and their generation time is significantly shorter than that of earthworms. The duration for a reproduction test with enchytraeids is therefore only 4-6 weeks while for earthworms (Eisenia fetida) it is 8 weeks.

3. Basic information on the ecology and ecotoxicology of enchytraeids in the terrestrial environment can be found in (9)(10)(11)(12).

PRINCIPLE OF THE TEST

4. Adult enchytraeid worms are exposed to a range of concentrations of the test chemical mixed into an artificial soil. The test can be divided into two steps: (a) a range-finding test, in case no sufficient information is available, in which mortality is the main endpoint assessed after two weeks exposure and (b) a definitive reproduction test in which the total number of juveniles produced by parent animal and the survival of parent animals are assessed. The duration of the definitive test is six weeks. After the first three weeks, the adult worms are removed and morphological changes are recorded. After an additional three weeks, the number of offspring, hatched from the cocoons produced by the adults, is counted. The reproductive output of the animals exposed to the test chemical is compared to that of the control(s) in order to determine (i) the no observed effect concentration (NOEC) and/or (ii) EC_x (e.g. EC_{10}, EC_{50}) by using a regression model to estimate the concentration that would cause a x % reduction in reproductive output. The test concentrations should bracket the EC_x (e.g. EC_{10}, EC_{50}) so that the EC_x then comes from interpolation rather than extrapolation.

INFORMATION ON THE TEST CHEMICAL

5. The water solubility, the log K_{ow}, the soil water partition coefficient (e.g. Chapter C.18 or C.19 of this Annex) and the vapour pressure of the test chemical should preferably be known. Additional information on the fate of the test chemical in soil, such as the rates of photolysis and hydrolysis is desirable.

6. This test method can be used for water soluble or insoluble chemicals. However, the mode of application of the test chemical will differ accordingly. The test method is not applicable to volatile chemicals, i.e. chemicals for which the Henry's constant or the air/water partition coefficient is greater than one, or chemicals for which the vapour pressure exceeds 0.0133 Pa at 25 °C.

VALIDITY OF THE TEST

7. For the test to be valid, the following performance criteria should be met in the controls:

- adult mortality should not exceed 20% at the end of the range-finding test and after the first three weeks of the reproduction test.
- assuming that 10 adults per vessel were used in setting up the test, an average of at least 25 juveniles per vessel should have been produced at the end of the test.
- the coefficient of variation around the mean number of juveniles should not be higher than 50% at the end of the reproduction test.

Where a test fails to meet the above validity criteria the test should be terminated unless a justification for proceeding with the test can be provided. The justification should be included in the test report.

REFERENCE CHEMICAL

8. A reference chemical should be tested either at regular intervals or possibly included in each test to verify that the response of the test organisms has not changed significantly over time. A suitable reference chemical is carbendazim, which has been shown to affect survival and reproduction of enchytraeids (13)(14), or other chemicals whose toxicity data are well known could be also used. A formulation of carbendazim known by the trade name of Derosal ™ supplied by AgrEvo Company (Frankfurt, Germany) and containing 360 g/l (32.18%) active ingredient was used in a ring-test (2). The EC_{50} for reproduction determined in the ring test was in the range of 1.2 ± 0.8 mg active ingredient (a.i) /kg dry mass (2). If a positive toxic standard is included in the test series, one concentration is used and the number of replicates should be the same as that in the controls. For carbendazim, the testing of 1.2 mg a.i./kg dry weight (tested as a liquid formulation) is recommended.

DESCRIPTION OF THE TEST

Equipment

9. The test vessels should be made of glass or other chemically inert material. Glass jars (e.g. volume: 0.20 - 0.25 litre; diameter: ≈ 6 cm) are suitable. The vessels should have transparent lids (e.g. glass or polyethylene) that are designed to reduce water evaporation whilst allowing gas exchange between the soil and the atmosphere. The lids should be transparent to allow light transmission.

10. Normal laboratory equipment is required, specifically the following:

- drying cabinet;
- stereomicroscope;
- pH-meter and photometer;
- suitable accurate balances;
- adequate equipment for temperature control;
- adequate equipment for humidity control (not essential if exposure vessels have lids);
- incubator or small room with air-conditioner;
- tweezers, hooks or loops;
- photo basin.

Preparation of the artificial soil

11. An artificial soil is used in this test (5)(7) with the following composition (based on dry weights, dried to a constant weight at 105 ^{o}C):

- 10% sphagnum peat, air-dried and finely ground (a particle size of 2 ± 1 mm is acceptable); it is recommended to check that a soil prepared with a fresh batch of peat is suitable for culturing the worms before it is used in a test;
- 20% kaolin clay (kaolinite content preferably above 30%);
- approximately 0.3 to 1.0% calcium carbonate ($CaCO_3$, pulverised, analytical grade) to obtain a pH of 6.0 ± 0.5; the amount of calcium carbonate to be added may depend principally on the quality/nature of the peat;
- approximately 70% air-dried quartz sand (depending on the amount of $CaCO_3$ needed), predominantly fine sand with more than 50% of the particles between 50 and 200 microns.

It is advisable to demonstrate the suitability of an artificial soil for culturing the worms and for achieving the test validity criteria before using the soil in a definitive test. It is especially recommended to make such a check to ensure that the performance of the test is not compromised if the organic carbon content of the artificial soil is reduced, e.g. by lowering the peat content to 4-5% and increasing the sand content accordingly. By such a reduction in organic carbon content, the possibilities of adsorption of test chemical to the soil (organic carbon) may be decreased and the availability of the test chemical to the worms may increase. It has been demonstrated that *Enchytraeus albidus* can comply with the validity criteria on reproduction when tested in field soils with lower organic carbon content than mentioned above (e.g. 2.7%) (15), and there is experience – though limited – that this can also be achieved in artificial soil with 5% peat.

Note: When using natural soil in additional (e.g. higher tier) testing, the suitability of the soil and achieving the test validity criteria should also be demonstrated.

12. The dry constituents of the soil are mixed thoroughly (e.g. in a large-scale laboratory

mixer). This should be done at least one week before starting the test. The mixed soil should be stored for two days in order to equilibrate/stabilise the acidity. For the determination of pH a mixture of soil and 1 M potassium chloride (KCl) or 0.01 M calcium chloride ($CaCl_2$) solution in a 1:5 ratio is used (see (16) and Appendix 3). If the soil is more acidic than the required range (see paragraph 11), it can be adjusted by addition of an appropriate amount of $CaCO_3$. If the soil is too alkaline it can be adjusted by the addition of more of the mixture, referred to in paragraph 11, but excluding the $CaCO_3$.

13. The maximum water holding capacity (WHC) of the artificial soil is determined in accordance with procedures described in Appendix 2. One or two days before starting the test, the dry artificial soil is pre-moistened by adding enough de-ionised water to obtain approximately half of the final water content, that being 40 to 60% of the maximum water holding capacity. At the start of the test, the pre-moistened soil is divided into portions corresponding with the number of test concentrations (and reference chemical where appropriate) and controls used for the test. The moisture content is adjusted to 40-60 % of the maximum WHC by the addition of the test chemical solution and/or by adding distilled or de-ionised water (see paragraphs 19-21). The moisture content is determined at the beginning and at the end of the test (by drying to constant weight at 105° C) and should be within the optimal range for the survival of the worms. A rough check of the soil moisture content can be obtained by gently squeezing the soil in the hand, if the moisture content is correct small drops of water should appear between the fingers.

Selection and preparation of test animals

14. The recommended test species is *Enchytraeus albidus* Henle 1837 (white potworm), a member of the family *Enchytraeidae* (order *Oligochaeta*, phylum *Annelida*). *E. albidus* is one of the largest species of enchytraeids, with specimens of up to 35 mm in length being recorded (17)(18). *E. albidus* has a world-wide distribution and is found in marine, freshwater and terrestrial habitats, mainly in decaying organic matter (seaweed, compost) and rarely in meadows (9). Its broad ecological tolerance and some morphological variations might indicate that different races exist.

15. *E. albidus* is commercially available, as a fish food. It should be checked whether the culture is contaminated by other, usually smaller, species (1) (19). If contamination occurs, all worms should be washed with water in a petri dish. Large adult specimens of *E. albidus* should then be selected (using a stereomicroscope) to start a new culture and all other worms are discarded. *E. albidus* can be bred easily in a wide range of organic materials (see Appendix 4). The life-cycle of *E. albidus* is short since maturity is reached between 33 days (at 18 °C) and 74 days (at 12 °C) (1). Only cultures that have been kept without problems in the laboratory for at least 5 weeks (one generation) will be used for the test.

16. Other species of the *Enchytraeus* genus are also suitable, e.g. *E. buchholzi* Vejdovsky 1879 or *E. crypticus* Westheide & Graefe 1992 (see Appendix 5). If other species of *Enchytraeus* are used, they must be clearly identified and the rationale for the selection of the species should be reported.

17. The animals used in the tests are adult worms. They should have eggs (white spots) in the clitellum region, and they should be approximately the same size (about 1 cm long). Synchronisation of the breeding culture is not necessary.

18. If the enchytraeids are not bred in the same soil type and under the conditions (including feeding) used for the final test they must be acclimatised for at least 24 hours and up to three days. A larger number of adults than that needed for performing the test should initially be acclimatised to allow scope for rejection of damaged or otherwise unsuitable specimens. At the end of the acclimatisation period, only worms containing eggs and exhibiting no behavioural abnormalities (e.g. trying to escape from the soil) are selected for the test. The worms are carefully removed using jeweller´s tweezers, hooks or loops and placed in a petri dish containing a small amount of fresh water. Reconstituted fresh water as proposed in Chapter C.20 of this Annex (*Daphnia magna* Reproduction Test) is preferred for this purpose since de-ionised, de-mineralised or tap water could be harmful to the worms. The worms are inspected under a stereomicroscope and any that do not contain eggs are discarded. Care is taken to remove and discard any mites or springtails that might have infected the cultures. Healthy worms not used for the test are returned to the stock culture.

Preparation of test concentrations

Test chemical soluble in water

19. A solution of the test chemical is prepared in deionised water in a quantity sufficient for all replicates of one test concentration. It is recommended to use an appropriate quantity of water to reach the required moisture content, i.e. 40 to 60% of the maximum WHC (see paragraph 13). Each solution of test chemical is mixed thoroughly with one batch of pre-moistened soil before being introduced into the test vessel.

Test chemical insoluble in water

20. For chemicals insoluble in water but soluble in organic solvents, the test chemical can be dissolved in the smallest possible volume of a suitable vehicle (e.g. acetone). Only volatile solvents should be used. The vehicle is sprayed on or mixed with a small amount, for example 2.5 g, of fine quartz sand. The vehicle is eliminated by evaporation under a fume hood for at least one hour. This mixture of quartz sand and test chemical is added to the pre-moistened soil and thoroughly mixed after adding an appropriate amount of de-ionised water to obtain the moisture required. The final mixture is introduced into the test vessels.

21. For chemicals that are poorly soluble in water and organic solvents, the equivalent of 2.5 g of finely ground quartz sand per test vessel is mixed with the quantity of test chemical to obtain the desired test concentration. This mixture of quartz sand and test chemical is added to the pre-moistened soil and thoroughly mixed after adding an appropriate amount of de-ionised water to obtain the required moisture content. The final mixture is divided between the test vessels. The procedure is repeated for each test concentration and an appropriate control is also prepared.

22. Chemicals should not normally be tested at concentrations higher than 1000 mg/kg dry mass of soil. Testing at higher concentrations may however be required in accordance with the objectives of a specific test.

PERFORMANCE OF THE TESTS

Test groups and controls

23. For each test concentration, an amount of test soil corresponding to 20 g dry weight is placed into the test vessel (see paragraphs 19-21). Controls, without the test chemical, are also prepared. Food is added to each vessel in accordance with procedures described in paragraph 29. Ten worms are randomly allocated to each test vessel. The worms are carefully transferred into each test vessel and placed on the surface of the soil using, for example, jeweller's tweezers, hooks or loops. The number of replicates for test concentrations and for controls depends on the test design used (see paragraph 34). The test vessels are positioned randomly in the test incubator and these positions are re-randomised weekly.

24. If a vehicle is used for application of the test chemical, one control series containing quartz sand sprayed or mixed with solvent should be run in addition to the test series. The solvent or dispersant concentration should be the same as that used in the test vessels containing the test chemical. A control series containing additional quartz sand (2.5 g per vessel) should be run for chemicals requiring administration in accordance with the procedures described in paragraph 21.

Test conditions

25. The test temperature is 20 ± 2 °C. To discourage worms from escaping from the soil, the test is carried out under controlled light-dark cycles (preferably 16 hours light and 8 hours dark) with illumination of 400 to 800 lux in the area of the test vessels.

26. In order to check the soil humidity, the vessels are weighed at the beginning of the test and thereafter once a week. Weight loss is replenished by the addition of an appropriate amount of deionised water. It should be noted that loss of water can be reduced by maintaining a high air-humidity (> 80%) in the test incubator.

27. The moisture content and the pH, should be measured at the beginning and the end of both the range-finding test and the definitive test. Measurements should be made in control and treated (all concentrations) soil samples prepared and maintained in the same way as the test cultures but not containing worms. Food should only be added to these soil samples at the start of the test to facilitate microbial activity. The amount of food added should be the same as that added to the test cultures. It is not necessary to add further food to these vessels during the test.

Feeding

28. A food capable of maintaining the enchytraeid population can be used. Rolled oats, preferably autoclaved before use to avoid microbial contamination (heating is also

appropriate), have been found to be a suitable feeding material.

29. Food is first provided by mixing 50 mg of ground rolled oats with the soil in each vessel before introducing the worms. Thereafter, food is supplied weekly up to Day 21. Feeding is not carried out on Day 28 since the adults have been removed at this stage and the juvenile worms need relatively little additional food beyond this point. Feeding during the test comprises 25 mg of ground rolled oats per vessel placed carefully on the surface of the soil so as to avoid injuring the worms. In order to reduce fungal growth, the oats flakes should be buried in the soil by covering with small amounts of soil. If food remains uneaten the ration should be reduced.

Design for the range-finding test

30. When necessary, a range-finding test is conducted with, for example, five test chemical concentrations of 0.1, 1.0, 10, 100, and 1000 mg/kg (dry weight of soil). One replicate for each treatment and control is sufficient.

31. The duration of the range-finding test is two weeks. At the end of the test, mortality of the worms is assessed. A worm is recorded as dead if it has no reaction to a mechanical stimulus at the anterior end. Additional information to mortality may also be useful in deciding on the range of concentrations to be used in the definitive test. Changes in adult behaviour (e.g. the inability to dig into the soil; lying motionless against the glass wall of the test vessel) and morphology (e.g. the presence of open wounds) should therefore also be recorded along with the presence of any juveniles. The latter can be determined using the staining method described in Appendix 6.

32. The LC_{50} can be approximately determined by calculating the geometrical mean of mortality data. In setting the concentration range for the definitive test, effects on the reproduction are assumed to be lower than the LC_{50} by a factor of up to 10. However, this is an empirical relation ship and in any specific case it might be different. Additional observations made in the range-finding test such as the occurrence of juveniles can help refine the test chemical concentration range to be used for the definitive test.

33. In order for an accurate determination of the LC_{50} performing the test using at least four replicates each of the test chemical concentration and an adequate number of concentrations to cause at least four statistically significantly different mean responses at these concentrations) is recommended. A similar number of the concentrations and replicates for the controls are used when they are applicable.

Design for the definitive reproduction test

34. Three designs are proposed based on recommendations arising from a ring test (2)

- For determination of the NOEC, at least five concentrations in a geometric series should be tested. Four replicates for each test concentration plus eight controls are recommended. The concentrations should be spaced by a factor not exceeding 1.8.
- For determination of the EC_x (e.g. EC_{10}, EC_{50}), at least five concentrations should be tested and the concentrations should bracket EC_x in order to enable EC_x interpolation

and not extrapolation At least four replicates for each test concentration and four control replicates are recommended. The spacing factor may vary, i.e. less than or equal to 1.8 in the expected effect range and above 1.8 at the higher and lower concentrations.

- A combined approach allows for determination of both the NOEC and EC_x. Eight treatment concentrations in a geometric series should be used. Four replicates for each treatment plus eight controls are recommended. The concentrations should be spaced by a factor not exceeding 1.8.

35. Ten adult worms per test vessel should be used (see paragraph 23). Food is added to the test vessels at the beginning of the test and then once a week (see paragraph 29) up to and including Day 21. On Day 21 the soil samples are carefully hand searched and living adult worms are observed and counted and changes in behaviour (e.g. inability to dig into the soil; lying motionless against the glass wall of the test vessel) and in morphology (e.g. open wounds) are recorded. All adult worms are then removed from the test vessels and the test soil. The test soil containing any cocoons that had been produced are incubated for three additional weeks under the same test conditions except that feeding takes place only on Day 35 (i.e. 25 mg ground rolled oats per vessel).

36. After six weeks, the newly hatched worms are counted. The method based on Bengal red staining (see Appendix 6) is recommended although other wet (but not heat) extraction and floatation techniques (see Appendix 6) have also proved suitable (4)(10)(11)(20). Bengal red staining is recommended because wet extraction from a soil substrate can be hampered by turbidity caused by suspended clay particles.

Limit test

37. If no effects are observed at the highest concentration in the range-finding test (i.e. 1000 mg/kg), the reproduction test can be performed as a limit test, using 1000 mg/kg in order to demonstrate that the NOEC for reproduction is greater than this value.

Summary and timetable for the test

38. The steps of the test can be summarised as follows:

Time	**Range-finding test**	**Definitive test**
Day −7 or earlier	- Prepare artificial soil (mixing of dry constituents)	- Prepare artificial soil (mixing of dry constituents)
Day −5	- Check pH of artificial soil - Measure max WHC of soil	- Check pH of artificial soil - Measure max WHC of soil
Day −5 to −3	- Sort worms for acclimatisation	- Sort worms for acclimatisation
Day – 3 to 0	- Acclimatise worms for at least 24 hours	- Acclimatise worms for at least 24 hours
Day −1	- Pre-moisten artificial soil and distribute into batches	- Pre-moisten artificial soil and distribute into batches
Day 0	- Prepare stock solutions	- Prepare stock solutions

	- Apply test chemical - Weigh test substrate into test vessels - Mix in food - Introduce worms - Measure soil pH and moisture content	- Apply test chemical - Weigh test substrate into test vessels - Mix in food - Introduce worms - Measure soil pH and moisture content
Day 7	- Check soil moisture content	- Check soil moisture content - Feed
Day 14	- Determine adult mortality - Estimate number of juveniles - Measure soil pH and moisture content	- Check soil moisture content - Feed
Day 21		- Observe adult behaviour - Remove adults - Determine adult mortality - Check soil moisture content - Feed
Day 28		- Check soil moisture content - No feeding
Day 35		- Check soil moisture content - Feed
Day 42		- Count juvenile worms - Measure soil pH and moisture content

DATA AND REPORTING

Treatment of results

39. Although an overview is given in Appendix 7, no definitive statistical guidance for analysing test results is given in this test method.

40. In the range finding test, the main endpoint is mortality. Changes in behaviour (e.g. inability to dig into the soil; lying motionless against the glass wall of the test vessel) and morphology (e.g. open wounds) of the adult worms should however also be recorded along with the presence of any juveniles. Probit analysis (21) or logistic regression should normally be applied to determine the LC_{50}. However, in cases where this method of analysis is unsuitable (e.g., if less then three concentrations with partial kills are available), alternative methods can be used. These methods could include moving averages (22), the trimmed Spearman-Karber method (23) or simple interpolation (e.g., geometrical mean of LC_0 and LC_{100}, as computed by the square root of LC_0 multiplied by LC_{100}).

41. In the definitive test, test endpoint is fecundity (i.e. number of juveniles produced). However, as in the range-finding test, all other harmful signs should be recorded in the final report. The statistical analysis requires the arithmetic mean and the standard deviation per treatment and per control for reproduction to be calculated.

42. If an analysis of variance has been performed, the standard deviation, s, and the degrees of freedom, df, may be replaced by the pooled variance estimate obtained from the ANOVA and by its degrees of freedom, respectively – provided variance does not depend on the concentration. In this case, use the single variances of control and treatments. Those values are usually calculated by commercial statistical software using the per-vessel results as replicates. If pooling of data for the negative and solvent controls appears reasonable rather than testing against one of those, they should be tested to see that they are not significantly different (for appropriate tests see paragraph 45 and Appendix 7).

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

NOEC Estimation

44. The application of powerful tests should be preferred. One should use information e.g. from previous experience with ring-testing or other historic data on whether data are approximately normally distributed. Variance homogeneity (homoscedasticity) is more critical. Experience tells that the variance often increases with increasing mean. In these cases, a data transformation could lead to homoscedasticity. However, such a transformation should be based on experience with historic data rather than on data under investigation. With homogeneous data, multiple t-tests such as Williams' test (α = 0.05, one-sided) (24)(25) or in certain cases Dunnett´s test (26)(27) should be performed. It should be noted that, in the case of unequal replication, the table t-values must be corrected as suggested by Dunnett and Williams. Sometimes, because of large variation, the responses do not increase/decrease regularly. In this case of strong deviation from monotonicity the Dunnett's test is more appropriate. If there are deviations from homoscedasticity, it may be reasonable to investigate possible effects on variances more closely to decide whether the t tests can be applied without losing much power (28). Alternatively, a multiple U-test, e.g. the Bonferroni-U-test according to Holm (29), or when these data exhibit heteroscedasticity but are otherwise consistent with a underlying monotone dose-response, an other non-parametric test [e.g. Jonckheere-Terpstra (30) (31) or Shirley (32) (33)] can be applied and would generally be preferred to unequal-variance t-tests. (see also the scheme in Appendix 7).

45. If a limit test has been performed and the prerequisites of parametric test procedures (normality, homogeneity) are fulfilled, the pair-wise Student t-test can be used or otherwise the Mann-Whitney-U-test procedure (29).

EC_x Estimation

46. To compute any EC_x value, the per-treatment means are used for regression analysis (linear or non-linear), after an appropriate dose-response function has been obtained. For the growth of worms as a continuous response, EC_{x-} -values can be estimated by using suitable regression analysis (35). Among suitable functions for quantal data (mortality/survival and number of offspring produced) are the normal sigmoid, logistic or Weibull functions, containing two to four parameters, some of which can also model hormetic responses. If a dose-response function was fitted by linear regression analysis a significant r^2 (coefficient of determination) and/or slope should be found

with the regression analysis before estimating the EC_x by inserting a value corresponding to x% of the control mean into the equation found by regression analysis. 95%-confidence limits are calculated according to Fieller (cited in Finney (21)) or other modern appropriate methods.

47. Alternatively, the response is modelled as a percent or proportion of model parameter which is interpreted as the control mean response. In these cases, the normal (logistic, Weibull) sigmoid curve can often be easily fitted to the results using the probit regression procedure (21). In these cases the weighting function has to be adjusted for metric responses as given by Christensen (36). However, if hormesis has been observed, probit analysis should be replaced by a four-parameter logistic or Weibull function, fitted by a non-linear regression procedure (36). If a suitable dose-response function cannot be fitted to the data, one may use alternative methods to estimate the EC_x, and its confidence limits, such as Moving Averages after Thompson (22) and the Trimmed Spearman-Karber procedure (23).

TEST REPORT

48. The test report must include the following information:

Test chemical:

- physical nature and, where relevant physical-chemical properties (e.g. water solubility, vapour pressure);
- chemical identification of the test chemical according to IUPAC nomenclature, CAS-number, batch, lot, structural formula and purity;
- expiry date of sample.

Test species:

- test animals used: species, scientific name, source of organisms and breeding conditions.

Test conditions:

- ingredients and preparation of the artificial soil;
- method of application of the test chemical;
- description of the test conditions, including temperature, moisture content, pH, etc.;
- full description of the experimental design and procedures.

Test results:

- mortality of adult worms after two weeks and the number of juveniles at the end of the range-finding test;
- mortality of adult worms after three weeks exposure and the full record of juveniles at the end of the definitive test;
- any observed physical or pathological symptoms and behavioural changes in the test organisms;
- the LC_{50}, the NOEC and/or EC_x (e.g. EC_{50}, EC_{10}) for reproduction if some of them are applicable with confidence intervals, and a graph of the fitted model used for its calculation all information and observations helpful for the interpretation of the results.

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

LITERATURE

(1) Römbke, J. (1989). Entwicklung eines Reproduktionstests an Bodenorganismen – Enchytraeen. Abschlußbericht des Battelle-Instituts e.V. Frankfurt für das Umweltbundesamt (Berlin), FE-Vorhaben 106 03 051/01.

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

(3) Westheide, W. and Bethge-Beilfuss, D. (1991). The sublethal enchytraeid test system: guidelines and some results, In: Modern Ecology: Basic and Applied Aspects. Ed. by Esser, G. and Overdieck, D. pp 497-508. Elsevier, Amsterdam,

(4) Dirven-Van Breemen, E., Baerselmann, R. and Notenboom, J. (1994). Onderzoek naar de Geschiktheid van de Potwormsoorten *Enchytraeus albidus* en *Enchytraeus crypticus* (Oligochaeta, Annelida) in Bodemecotoxicologisch Onderzoek. RIVM Rapport Nr. 719102025. 46 pp.

(5) Chapter C.8 of this Annex, Toxicity for Earthworms.

(6) ISO (International Organization for Standardization) (1993). Soil Quality - Effects of pollutants on earthworms (*Eisenia fetida*). Part 1: Determination of acute toxicity using Artificial Soil substrate, No. 11268-1. ISO, Geneve.

(7) ISO (International Organization for Standardization) (1996). Soil Quality - Effects of pollutants on earthworms (*Eisenia fetida*). Part 2: Determination of effects on reproduction, No. 11268-2. ISO, Geneve.

(8) Rundgren, S. and A.K. Augustsson (1998). Test on the enchytraeid *Cognettia sphagnetorum* (Vejdovsky 1877). In: Løkke, H. and C.A.M. Van Gestel, Handbook of soil invertebrate toxicity tests. John Wiley and Sons, Chichester, 73-94.

(9) Kasprzak, K. (1982). Review of enchytraeid community structure and function in agricultural ecosystems. Pedobiologia 23, 217-232.

(10) Römbke, J. (1995). Enchytraeen (Oligochaeta) als Bioindikator, UWSF - Z. Umweltchem. Ökotox. 7, 246-249.

(11) Dunger, W. and Fiedler, H.J. (1997). Methoden der Bodenbiologie. G. Fischer Verlag, Stuttgart, New York.

(12) Didden, W.A.M. (1993). Ecology of terrestrial Enchytraeidae. Pedobiologia 37, 2-29.

(13) Becker, H. (1991). Bodenorganismen – Prüfungskategorien der Forschung. UWSF – Z. Umweltchem. Ökotox. 3, 19-24.

(14) Römbke, J. and Federschmidt, A. (1995). Effects of the fungicide Carbendazim on Enchytraeidae in laboratory and field tests, Newsletter on Enchytraeidae 4, 79-96.

(15) Römbke, J., Riepert, F. & Achazi R. (2000): Enchytraeen als Testorganismen. In: Toxikologische Beurteilung von Böden. Heiden, S., Erb, R., Dott, W. & Eisentraeger, A. (eds.). Spektrum Verl., Heidelberg. 59-81.

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

(17) Bell, A.W. (1958). The anatomy of *Enchytraeus albidus*, with a key to the species of the genus *Enchytraeus.* Ann. Mus. Novitat. 1902, 1-13.

(18) Nielsen, C.O. and Christensen, B. (1959). The Enchytraeidae, critical revision and taxonomy of European species. Natura Jutlandica 8-9, 1-160.

(19) Bouguenec, V. and Giani, N. (1987). Deux nouvelles especes d´*Enchytraeus* (Oligochaeta, Enchytraeidae) et rediscription d´*E. bigeminus*. Remarques sur le genre *Enchytraeus*. Ann. Limnol. 23, 9-22.

(20) Korinkova, J. and Sigmund, J. (1968). The colouring of bottom-fauna samples before sorting, Vestnik Ceskoslovensko Spolecnosti Zoologicke 32, 300-305.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Appendix 1

DEFINITIONS

For the purpose of this test method the following definitions are applicable:

Chemical means a substance or a mixture.

EC_x (Effect concentration for x% effect) is the concentration that causes an x % of an effect on test organisms within a given exposure period when compared with a control. In this test the effect concentrations are expressed as a mass of test chemical per dry mass of the test soil.

LC_0 (No lethal concentration) is the concentration of a test chemical that does not kill any of exposed test organisms within a given time period. In this test the LC_0 is expressed as a mass of test chemical per dry mass of the test soil.

LC_{50} (Median lethal concentration) is the concentration of a test chemical kills 50% of exposed test organisms within a given time period. In this test the LC_{50} is expressed as a mass of test chemical per dry mass of the test soil.

LC_{100} (Totally lethal concentration) is the concentration of a test chemical kills 100% of exposed test organisms within a given time period. In this test the LC_{100} is expressed as a mass of test chemical per dry mass of the test soil.

LOEC (Lowest Observed Effect Concentration) is the lowest test chemical concentration that has a statistically significant effect ($p < 0.05$). In this test the LOEC is expressed as a mass of test chemical per dry mass of the test soil. All test concentrations above the LOEC should normally show an effect that is statistically different from the control. Any deviations from the above in identifying the LOEC must be justified in the test report.

NOEC (No Observed Effect Concentration) is the highest test chemical concentration immediately below the LOEC at which no effect is observed. In this test, the concentration corresponding to the NOEC, has no statistically significant effect ($p < 0.05$) within a given exposure period when compared with the control.

Reproduction rate is the mean number of juvenile worms produced per a number of adults over the test period.

Test chemical is any substance or mixture tested using this test method.

Appendix 2

DETERMINATION OF THE MAXIMUM WATER HOLDING CAPACITY

Determination of the water holding capacity of the artificial soil

The following method has been found appropriate. It is described in Annex C of the ISO DIS 11268-2.

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

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

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

REFERENCES:

ISO (International Organization for Standardization) (1996). Soil Quality - Effects of pollutants on earthworms (*Eisenia fetida*). Part 2: Determination of effects on reproduction, No. 11268-2. ISO, Geneve.

Appendix 3

DETERMINATION OF SOIL PH

The following method for determining the pH of a soil sample is based on the description in ISO 10390 (Soil Quality - Determination of pH).

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

REFERENCES:

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

Appendix 4

CULTURING CONDITIONS OF *ENCHYTRAEUS SP.*

Enchytraeids of the species *Enchytraeus albidus* (as well as other *Enchytraeus* species) can be cultured in large plastic boxes (e.g. 30 x 60 x 10 cm) filled with a 1:1 mixture of artificial soil and natural, uncontaminated garden soil. Compost material must be avoided since it could contain toxic chemicals such as heavy metals. Fauna should be removed from the soil before use (e.g. by deep-freezing). A substrate comprising only of artificial soil can also be used but the reproduction rate may be lower than that obtained with a mixed soil substrate. The substrate used for culturing should have a pH of 6.0 ± 0.5.

The culture is kept in the dark at a temperature of 15 to 20 °C ± 2 °C. Temperatures higher than 23 °C must be avoided. The soil must be kept moist but not wet. The correct soil moisture content is indicated when small drops of water appear between the fingers when the soil is gently squeezed. The production of anoxic conditions must be avoided by ensuring that covers to culture containers allow adequate gaseous exchange with the atmosphere. The soil should be carefully broken up each week to facilitate aeration.

The worms can be fed on rolled oats. The oats should be stored in sealed vessels and autoclaved or heated before use in order to avoid infestation with flour mites (e.g. *Glyzyphagus sp.*, *Astigmata*, *Acarina*) or predacious mites [e.g. *Hypoaspis* (*Cosmolaelaps) miles*, *Gamasida*, *Acarina*]. After a heat treatment, the food should be ground so that it can easily be strewn on the soil surface. From time to time, the rolled oats can be supplemented by the addition of vitamins, milk and cod-liver oil. Other suitable food sources are baker's yeast and the fish food "Tetramin".

Feeding takes place approximately twice a week. An appropriate quantity of rolled oats is strewn on the soil surface or carefully mixed into the substrate when breaking up the soil to facilitate aeration. The absolute amount of food provided depends on the number of worms present in the substrate. As a guide, the amount of food should be increased if it is all consumed within one day of being provided. Conversely, if food still remains on the surface at the time of the second feeding (one-week later) it should be reduced. Food contaminated with fungal growth should be removed and replaced. After three months, the worms should be transferred into a freshly prepared substrate.

Culturing conditions are deemed satisfactory if the worms: (a) do not try to leave the soil substrate, (b) move quickly through the soil, (c) exhibit a shiny outer surface without adhering soil particles, (d) are more or less whitish in colour, (e) exhibit a variety of age ranges in the cultures and (f) reproduce continuously.

Appendix 5

TEST PERFORMANCE WITH OTHER *ENCHYTRAEUS* SPECIES

Selection of species

Species other than *E. albidus* may be used but the test procedure and the validity criteria should be adapted accordingly. Since many *Enchytraeus*-species are readily available and can be satisfactorily maintained in the laboratory, the most important criterion for selecting a species other than *E. albidus* is ecological relevance and, additionally, comparable sensitivity. There may also be formal reasons for a change of species. For example, in countries where *E. albidus* does not occur and cannot be imported (e.g. due to quarantine restrictions), it will be necessary to use another *Enchytraeus* species.

Examples of suitable alternative species

- *Enchytraeus crypticus* (Westheide & Graefe 1992): In recent years, this species has often been used in ecotoxicological studies because of the simplicity of its breeding and testing. However, it is small and this makes handling more difficult compared with *E. albidus* (especially at stages prior to use of the staining method). *E. crypticus* has not been found to exist with certainty in the field, having only been described from earthworm cultures. Its ecological requirements are therefore not known.
- *Enchytraeus buchholzi* (Vejdovsky 1879): This name probably covers a group of closely related species that are morphologically difficult to distinguish. Its use for testing is not recommended until the individuals used in a test can be identified to species. *E. buchholzi* is usually found in meadows and disturbed sites such as roadsides.
- *Enchytraeus luxuriosus*: This species was originally known as *E. "minutus",* but has been recently described (1). It was first found by U. Graefe (Hamburg) in a meadow close to St. Peter-Ording (Schleswig-Holstein, Germany). *E. luxuriosus* is approximately half the size of E. albidus but larger than the other species discussed here; this could make it a good alternative to *E. albidus*.
- *Enchytraeus bulbosus* (Nielsen & Christensen 1963): This species has hitherto been reported from German and Spanish mineral soils, where it is common but not usually very abundant. In comparison to other small species of this genus, it is relatively easy to identify. Nothing is known about its behaviour in laboratory tests or its sensitivity to chemicals. It has, however, been found to be easy to culture (E. Belotti, personal communication).

Breeding conditions

All the *Enchytraeus*-species mentioned above can be cultured in the same substrates used for *E. albidus*. Their smaller size means that the culture vessels can be smaller and that, while the same food can be used, the ration size must be adjusted. The life-cycle of these species is shorter than for *E. albidus* and feeding should be carried out more frequently.

Test conditions

The test conditions are generally the same as those applying to *E. albidus,* except that:

- the size of the test vessel can (but need not) be smaller;
- the duration of the reproduction test can (but need not) be shorter, i.e. four instead of six weeks; however, the duration of the Range-Finding Test should not be changed;
- in view of the small size of the juvenile worms the use of the staining method is strongly recommended for counting;
- the validity criterion relating to "number of juveniles per test vessel in the control" should be changed to "50".

REFERENCES

(1) Schmelz, R.M. and Collado, R. (1999). Enchytraeus luxuriosus sp.nov., a new terrestrial oligochaete species (Enchytraeidae, Clitellata, Annelida). Carolinea 57, 93-100.

Appendix 6

DETAILED DESCRIPTION OF EXTRACTION TECHNIQUES

Staining with Bengal red

This method, originally developed in limnic ecology (1) was first proposed for the counting of juvenile enchytraeids in the Enchytraeidae reproduction test by W. de Coen (University of Ghent, Belgium). Independently, a modified version (Bengalred mixed with formaldehyde instead of ethanol) was developed by RIVM Bilthoven (2)(3).

At the end of the Definitive Test (i.e. after six weeks), the soil in the test vessels is transferred to a shallow container. A Bellaplast vessel or a photo basin with ribbed bottom is useful for this purpose, the latter because the "ribs" restrict movement of the worms within the field of observation. The juveniles are fixed with ethanol (approx. 5 ml per replicate). The vessels are then filled with water up to a layer of 1 to 2 cm. A few drops (200 to 300 µl) of Bengal red (1% solution in ethanol) are added (0.5% eosin is an alternative) and the two components are mixed carefully. After 12 hours, the worms should be stained a reddish colour and should be easy to count because they will be lying on the substrate surface. Alternatively, the substrate/alcohol mixture can be washed through a sieve (mesh size: 0.250 mm) before counting the worms. Using this procedure, the kaolinite, peat, and some of the sand will be washed out and the reddish coloured worms will be easier to see and count. The use of illuminated lenses (lens size at least 100 x 75 mm with a magnification factor 2 to 3x) will also facilitates counting.

The staining technique reduces counting time to a few minutes per vessel and as a guide it should be possible for one person to assess all the vessels from one test in a maximum of two days.

Wet extraction

The wet extraction should be started immediately the test finishes. The soil from each test vessel is placed into plastic sieves with a mesh size of approximately 1 mm. The sieves are then suspended in plastic bowls without touching the bottom. The bowls are carefully filled up with water until the samples in the sieves are completely under the water surface. To ensure a recovery rate of more than 90% of the worms present, an extraction period of 3 days at 20 ± 2 °C should be used. At the end of the extraction period the sieves are removed and the water (except for a small amount) is slowly decanted, taking care not to disturb the sediment at the bottom of the bowls. The plastic bowls are then shaken slightly to suspend the sediment in the overlying water. The water is transferred to a petri dish and, after the soil particles have settled), the enchytraeids can be identified, removed and counted using a stereomicroscope and soft steel forceps.

Flotation

A method based on flotation has been described in a note by R. Kuperman (4). After fixing the contents of a test vessel with ethanol, the soil is flooded with Ludox (AM-30 colloidal silica, 30 wt. % suspension in water) up to 10 to 15 mm above the soil surface.

After thoroughly mixing the soil with the flotation agent for 2 – 3 minutes, the juvenile worms floating on the surface can easily be counted.

References

(1) Korinkova, J. and Sigmund, J. (1968). The colouring of bottom-fauna samples before sorting, Vestnik Ceskoslovensko Spolecnosti Zoologicke 32, 300-305.

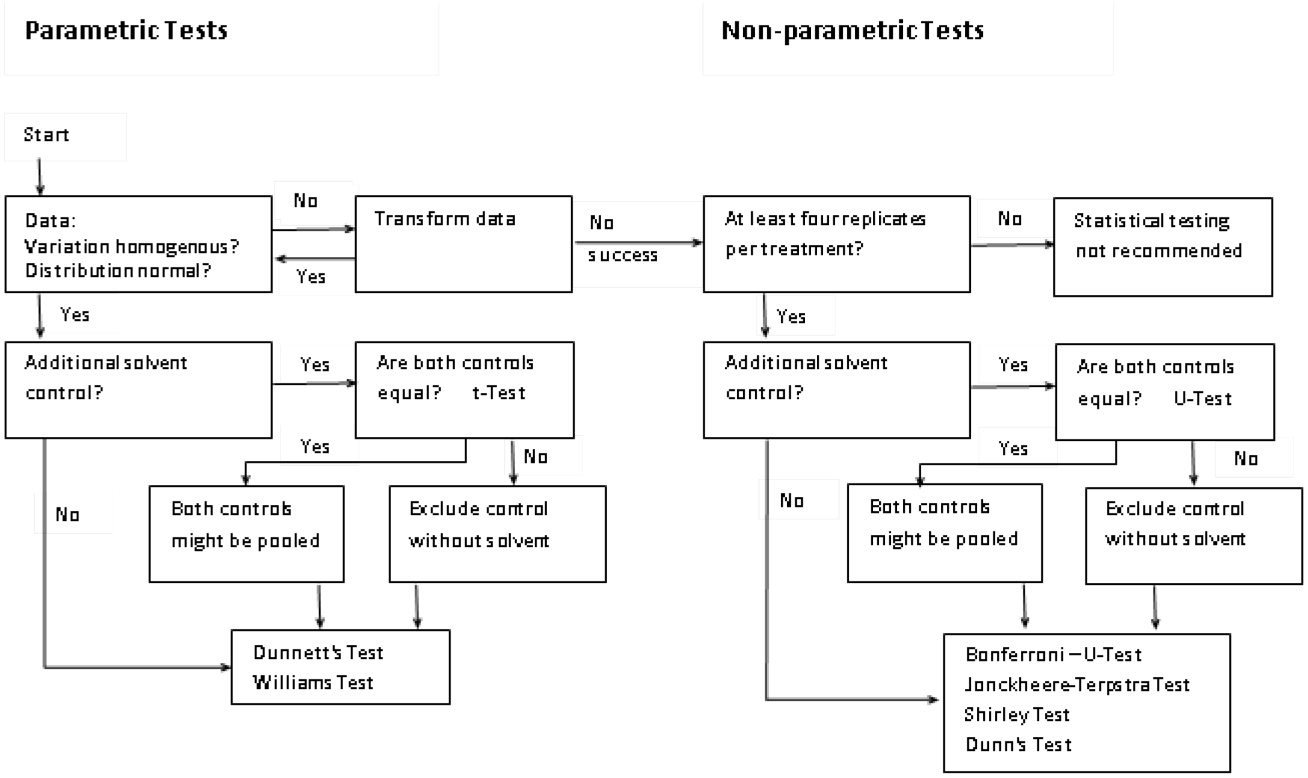
(2) Dirven-Van Breemen, E., Baerselmann, R. and Notenboom, J. (1994). Onderzoek naar de Geschiktheid van de Potwormsoorten *Enchytraeus albidus* en *Enchytraeus crypticus* (*Oligochaeta*, *Annelida*) in Bodemecotoxicologisch Onderzoek. RIVM Rapport Nr. 719102025. 46 pp.

(3) Posthuma, L., Baerselmann, R., Van Veen, R.P.M. and Dirven-Van Breemen, E.M. (1997). Single and joint toxic effects of copper and zinc on reproduction of *Enchytraeus crypticus* in relation to sorption of metals in soils. Ecotox. Envir. Safety 38, 108-121.

(4) Phillips, C.T., Checkai, R.T. and Kuperman, R.G. (1998). An alternative to the O´Connor Method for Extracting Enchytraeids from Soil. SETAC 19th Annual Meeting, Charlotte, USA. Abstract Book No. PMP069, p. 157.

Appendix 7

OVERVIEW OF THE STATISTICAL ASSESSMENT OF DATA (NOEC DETERMINATION)



C.33. Earthworm Reproduction Test (*Eisenia fetida/ Eisenia andrei*)

INTRODUCTION

1. This test method is equivalent to OECD test guideline (TG) 222 (2004). It is designed to be used for assessing the effects of chemicals in soil on the reproductive output (and other sub-lethal end points) of the earthworm species *Eisenia fetida* (Savigny 1826) or *Eisenia andrei* (Andre 1963) (1)(2). The test has been ring-tested (3). A test method for the earthworm acute toxicity test exists (4). A number of other international and national guidelines for earthworm acute and chronic tests have been published (5)(6)(7)(8).

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

PRINCIPLE OF THE TEST

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

INFORMATION ON THE TEST CHEMICAL

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

- water solubility;
- log K_{ow};

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

5. This test method is applicable to all chemicals irrespective of their water solubility. The test method is not applicable to volatile chemicals, defined here as chemicals for which Henry's constant or the air/water partition coefficient is greater than one, or to chemicals with vapour pressures exceeding 0.0133 Pa at 25 °C.

6. No allowance is made in this test method for possible degradation of the test chemical over the period of the test. Consequently it cannot be assumed that exposure concentrations will be maintained at initial values throughout the test. Chemical analysis of the test chemical at the start and the end of the test is recommended in that case.

REFERENCE CHEMICAL

7. The NOEC and/or the EC_x of a reference chemical must be determined to provide assurance that the laboratory test conditions are adequate and to verify that the response of the test organisms does not change statistically over time. It is advisable to test a reference chemical at least once a year or, when testing is carried out at a lower frequency, in parallel to the determination of the toxicity of a test chemical. Carbendazim or benomyl are suitable reference chemicals that have been shown to affect reproduction (3). Significant effects should be observed between (a) 1 and 5 mg active ingredient (a.i.)/kg dry mass or (b) 250-500 g/ha or 25-50 mg/m^2. If a positive toxic standard is included in the test series, one concentration is used and the number of replicates should be the same as that in the controls.

VALIDITY OF THE TEST

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

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

Where a test fails to meet the above validity criteria the test should be terminated unless a justification for proceeding with the test can be provided. The justification should be included in the report.

DESCRIPTION OF THE TEST

Equipment

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

10. Normal laboratory equipment is required, specifically the following:

- drying cabinet;
- stereomicroscope;
- pH-meter and photometer;
- suitable accurate balances;
- adequate equipment for temperature control;
- adequate equipment for humidity control (not essential if exposure vessels have lids);
- incubator or small room with air-conditioner;
- tweezers, hooks or loops;
- water bath.

Preparation of the artificial soil

11. An artificial soil is used in this test (5)(7) with the following composition (based on dry weights, dried to a constant weight at 105 C):

- 10 per cent sphagnum peat (as close to pH 5.5 to 6.0 as possible, no visible plant remains, finely ground, dried to measured moisture content);
- 20 per cent kaolin clay (kaolinite content preferably above 30 per cent);
- 0.3 to 1.0% calcium carbonate ($CaCO_3$, pulverised, analysis grade) to obtain an initial pH of 6.0 ± 0.5.
- 70% air-dried quartz sand (depending on the amount of $CaCO_3$ needed), predominantly fine sand with more than 50% of the particles between 50 and 200 microns.

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

Note 2: The organic carbon content of the artificial soil may be reduced, e.g. by lowering the peat content to 4-5% and increasing the sand content accordingly. By such a reduction in organic carbon content, the possibilities of adsorption of test chemical to the soil (organic carbon) may be decreased and the availability of the test chemical to the worms may increase. It has been demonstrated that Eisenia fetida can comply with the validity criteria on reproduction when tested in field soils with lower organic carbon content (e.g. 2.7%) (14), and there is experience that this can also be achieved in artificial soil with 5% peat. Therefore, it is not necessary before using such a soil in a definitive test to demonstrate the suitability of the artificial soil for allowing the test to comply with the validity criteria unless the peat content is lowered more than specified

above.

Note 3: When using natural soil in additional (e.g. higher tier) testing the suitability of the soil and achieving the test validity criteria should also be demonstrated.

12. The dry constituents of the soil are mixed thoroughly (e.g. in a large-scale laboratory mixer) in a well ventilated area. Before starting the test, the dry artificial soil is moistened by adding enough de-ionised water to obtain approximately half of the final water content, that being 40% to 60% of the maximum water holding capacity (corresponding to 50 ± 10% moisture dry mass). This will produce a substrate that has no standing or free water when it is compressed in the hand. The maximum water holding capacity (WHC) of the artificial soil is determined in accordance with procedures described in Appendix 2, ISO 11274 (15) or equivalent EU standard.

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

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

Selection and preparation of test animals

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

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

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

Preparation of test concentrations

18. Two methods of application of the test chemical can be used: mixing the test chemical into the soil (see paragraphs 19-21) or application to the soil surface (see paragraphs 22-24). The selection of the appropriate method depends on the purpose of the test. In

general, mixing of the test chemical into the soil is recommended. However application procedures that are consistent with normal agricultural practice may be required (e.g. spraying of liquid formulation or use of special pesticide formulations such as granules or seed dressings). Solvents used to aid treatment of the soil with the test chemical should be selected on the basis of their low toxicity to earthworm and appropriate solvent control must be included in the test design (see paragraph 27).

Mixing the test chemical into the soil

Test chemical soluble in water

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

Test chemical insoluble in water

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

Test chemical insoluble in water and organic solvents

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

Application of the test chemical to the soil surface

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

23. The test chemical is applied. It should not be added to the soil within half an hour of

introducing the worms (or if worms are present on the soil surface) so as to avoid any direct exposure to the test chemical by skin contact. When the test chemical is a pesticide it may be appropriate to apply it to the soil surface by spraying. The test chemical should be applied to the surface of the soil as evenly as possible using a suitable laboratory-scale spraying device to simulate spray application in the field. Before application the cover of the test container should be removed and replaced by a liner which protects the side walls of the container from spray. The liner can be made from a test container with the base removed. The application should take place at a temperature within 20 ± 2 °C of variation and for aqueous solutions, emulsions or dispersions at a water application rate of between 600 and 800 $\mu l/m^2$. The rate should be verified using an appropriate calibration technique. Special formulations like granules or seed dressings should be applied in a manner consistent with agricultural use.

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

PROCEDURE

Test groups and controls

25. A loading of 10 earthworms in 500 - 600 g dry mass of artificial soil (i.e. 50-60 g of soil per worm) is recommended. If larger quantities of soil are used, as might be the case if testing pesticides with special modes of application such as seed dressings, the loading of 50-60 g of soil per worm should be maintained by increasing the number of worms. Ten worms are prepared for each control and treatment container. The worms are washed with water and wiped and then placed on absorbent paper for a short period to allow excess water to drain.

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

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

Test conditions

28. The test temperature is 20 ± 2 °C. The test is carried out under controlled light-dark cycles (preferably 16 hours light and 8 hours dark) with illumination of 400 to 800 lux in the area of the test containers.

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

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

Feeding

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

32. Each fresh batch of food should be fed to a non-test worm culture before use in a test to ensure that it is of suitable quality. Growth and cocoon production should not be reduced compared to worms kept in a substrate that does not contain the new batch of food (conditions as described in test method C.8(4)).

33. Food is first provided one day after adding the worms and applying the test chemical to the soil. Approximately 5 g of food is spread on the soil surface of each container and moistened with de-ionised water (about 5 ml to 6 ml per container). Thereafter food is provided once a week during the 4-week test period. If food remains uneaten the ration should be reduced so as to avoid fungal growth or moulding. The adults are removed from the soil on day 28 of the test. A further 5 g of food is then administered to each test container. No further feeding takes place during the remaining 4 weeks of the test.

Selection of test concentrations

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

Experimental design

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

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

- For determination of the NOEC, at least five/twelve concentrations in a geometric series should be tested. Four replicates for each test concentration plus eight controls are recommended. The concentrations should be spaced by a factor not exceeding 2.0.
- For determination of the ECx (e.g. EC10, EC50), an adequate number of concentrations to cause at least four statistically significantly different mean responses at these concentrations is recommended. At least two replicates for each test concentration and six control replicates are recommended. The spacing factor may vary, i.e. less than or equal to 1.8 in the expected effect range and above 1.8 at the higher and lower concentrations.
- A combined approach allows for determination of both the NOEC and ECx. Eight treatment concentrations in a geometric series should be used. Four replicates for each treatment plus eight controls are recommended. The concentrations should be spaced by a factor not exceeding 1.8.

Test duration and measurements

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

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

39. At the end of the second 4-week period, the number of juveniles hatched from the cocoons in the test soil and cocoon numbers are determined using procedures described in Appendix 5. All signs of harm or damage to the worm should also be recorded throughout the test period.

Limit test

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

DATA AND REPORTING

Treatment of results

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

42. One endpoint is mortality. Changes in behaviour (e.g. inability to dig into the soil; lying motionless against the glass wall of the test vessel) and morphology (e.g. open wounds) of the adult worms should however also be recorded along with the presence of any juveniles. Probit analysis (18) or logistic regression should normally be applied to determine the LC_{50}. However, in cases where this method of analysis is unsuitable (e.g., if less than three concentrations with partial kills are available), alternative methods can be used. These methods could include moving averages (19), the trimmed Spearman-Karber method (20) or simple interpolation (e.g., geometrical mean of LC_0 and LC_{100}, as computed by the square root of LC_0 multiplied by LC_{100}).

43. The other endpoint is fecundity (e.g. number of juveniles produced). However, as in the range-finding test, all other harmful signs should be recorded in the final report. The statistical analysis requires the arithmetic mean $\bar{x}$ and the standard deviation per treatment and per control for reproduction to be calculated.

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

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

NOEC Estimation

46. The application of powerful tests should be preferred. One should use information e.g. from previous experience with ring-testing or other historic data on whether data are approximately normally distributed. Variance homogeneity (homoscedasticity) is more critical. Experience tells that the variance often increases with increasing mean. In

these cases, a data transformation could lead to homoscedasticity. However, such a transform should be based on experience with historic data rather than on data under investigation. With homogeneous data, multiple t-tests such as Williams' test (α = 0.05, one-sided) (21)(22) or in certain cases Dunnett´s test (23)(24) should be performed. It should be noted that, in the case of unequal replication, the table t-values must be corrected as suggested by Dunnett and Williams. Sometimes, because of large variation, the responses do not increase/decrease regularly. In this case of strong deviation from monotonicity the Dunnett's test is more appropriate. If there are deviations from homoscedasticity, it may be reasonable to investigate possible effects on variances more closely to decide whether the t- tests can be applied without loosing much power (25). Alternatively, a multiple U-test, e.g. the Bonferroni-U-test according to Holm (26), or when these data exhibit heteroscedasticity but are otherwise consistent with a underlying monotone dose-response, an other non-parametric test (e.g. Jonckheere-Terpstra (27)(28) or Shirley (29) (30)) can be applied and would generally be preferred to unequal-variance t-tests. (see also the scheme in Appendix 6).

47. If a limit test has been performed and the prerequisites of parametric test procedures (normality, homogeneity) are fulfilled, the pair-wise Student-t-test can be used or otherwise the Mann-Whitney-U-test procedure (31).

EC_x Estimation

48. To compute any EC_x value, the per-treatment means are used for regression analysis (linear or non-linear), after an appropriate dose-response function has been obtained. For the growth of worms as a continuous response, EC_x- -values can be estimated by using suitable regression analysis (32). Among suitable functions for quantal data (mortality/survival) and number of offspring produced are the normal sigmoid, logistic or Weibull functions, containing two to four parameters, some of which can also model hormetic responses. If a dose-response function was fitted by linear regression analysis a significant r^2 (coefficient of determination) and/or slope should be found with the regression analysis before estimating the EC_x by inserting a value corresponding to x% of the control mean into the equation found by regression analysis. 95%-confidence limits are calculated according to Fieller (cited in Finney (18)) or other modern appropriate methods.

49. Alternatively, the response is modeled as a percent or proportion of model parameter which is interpreted as the control mean response. In these cases, the normal (logistic, Weibull) sigmoid curve can often be easily fitted to the results using the probit regression procedure (18). In these cases the weighting function has to be adjusted for metric responses as given by Christensen (33). However, if hormesis has been observed, probit analysis should be replaced by a four-parameter logistic or Weibull function, fitted by a non-linear regression procedure (34). If a suitable dose-response function cannot be fitted to the data, one may use alternative methods to estimate the EC_x, and its confidence limits, such as Moving Averages after Thompson (19) and the Trimmed Spearman-Karber procedure (20).

TEST REPORT

50. The test report must include the following information:

Test chemical:

- a definitive description of the test chemical, batch, lot and CAS-number, purity;
- properties of the test chemical (e.g. log Kow, water solubility, vapour pressure, Henry's constant (H) and information on fate and behaviour).

Test organisms:

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

Test conditions

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

Test results:

- adult mortality (%) in each test container at the end of the first 4 weeks of the test;
- the total mass of adults at the beginning of the test in each test container;
- changes in body weight of live adults (% of initial weight) in each test container after the first four weeks of the test;
- the number of juveniles produced in each test container at the end of the test;
- a description of obvious or pathological symptoms or distinct changes in behaviour;
- the results obtained with the reference test chemical;
- the LC_{50}, the NOEC and/or EC_x (e.g. EC_{50}, EC_{10}) for reproduction if some of them are applicable with confidence intervals, and a graph of the fitted model used for its calculation all information and observations helpful for the interpretation of the results;
- a plot of the dose-response-relationship;
- the results applicable to each test container;

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

LITERATURE

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

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

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

(4) Chapter C.8 of this Annex, Earthworm acute toxicity test.

(5) ISO (International Organization for Standardization) (1996). Soil Quality – Effects of pollutants on earthworms (*Eisenia fetida*). Part 2: Determination of effects on reproduction, No.11268-2. ISO, Geneve.

(6) ISO (International Organization for Standardization) (1993). Soil Quality – Effects of pollutants on earthworms (*Eisenia fetida*). Part 1: Determination of acute toxicity using artificial soil substrate, No.11268-1. ISO, Geneve.

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

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

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

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

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

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

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

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

(15) ISO (International Organization for Standardization) (1992). Soil Quality –Determination of water retention characteristics –Laboratory methods, No. 11274. ISO, Geneve.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Appendix 1

DEFINITIONS

The following definitions are applicable to this test method:

Chemical means a substance or a mixture.

EC_x (Effect concentration for x% effect) is the concentration that causes an x% of an effect on test organisms within a given exposure period when compared with a control. For example, an EC_{50} is a concentration estimated to cause an effect on a test end point in 50% of an exposed population over a defined exposure period. In this test the effect concentrations are expressed as a mass of test chemical per dry mass of the test soil or as a mass of the test chemical per unit area of the soil.

LC_0 (No lethal concentration) is the concentration of a test chemical that does not kill any of exposed test organisms within a given time period. In this test the LC_0 is expressed as a mass of test chemical per dry mass of the test soil.

LC_{50} (Median lethal concentration) is the concentration of a test chemical that kills 50% of exposed test organisms within a given time period. In this test the LC_{50} is expressed as a mass of test chemical per dry mass of the test soil or as a mass of test chemical per unit area of soil.

LC_{100} (Totally lethal concentration) is the concentration of a test chemical kills 100% of exposed test organisms within a given time period. In this test the LC_{100} is expressed as a mass of test chemical per dry mass of the test soil.

LOEC (Lowest Observed Effect Concentration) is the lowest test chemical concentration that has a statistically significant effect ($p < 0.05$) In this test the LOEC is expressed as a mass of test chemical per dry mass of the test soil or as a mass of test chemical per unit area of soil. All test concentrations above the LOEC should normally show an effect that is statistically different from the control. Any deviations from the above must be justified in the test report.

NOEC (No Observed Effect Concentration) is the highest test chemical concentration immediately below the LOEC at which no effect is observed. In this test, the concentration corresponding to the NOEC, has no statistically significant effect ($p < 0.05$) within a given exposure period when compared with the control.

Reproduction rate: Mean number of juvenile worms produced per a number of adults over the test period.

Test chemical means any substance or mixture tested using this test method.

Appendix 2

DETERMINATION OF THE MAXIMUM WATER HOLDING CAPACITY OF THE SOIL

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

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

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

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

REFERENCES:

(1) ISO (International Organization for Standardisation) (1996). Soil Quality – Effects of pollutants on earthworms (*Eisenia fetida*). Part 2: Determination of effects on reproduction, No.11268-2. ISO, Geneve.

Appendix 3

DETERMINATION OF SOIL PH

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

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

REFERENCES:

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

Appendix 4

CULTURING OF EISENIA FETIDA /EISENIA ANDREI

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

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

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

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

Appendix 5

TECHNIQUES FOR COUNTING JUVENILE WORMS HATCHED FROM COCOONS

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

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

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

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

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

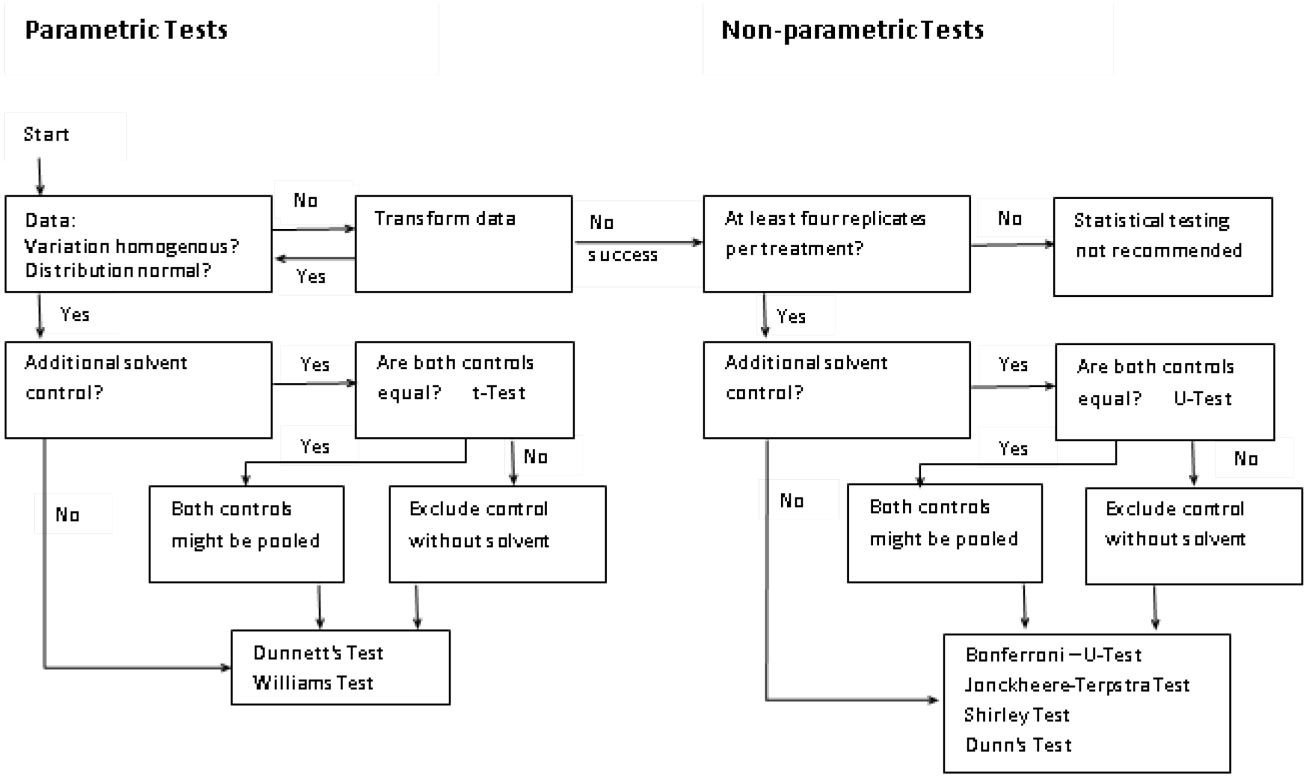
The efficiency of the method used to remove the worms (and cocoons if appropriate) from the soil substrate should always be determined. If juveniles are collected using the hand sorting technique it is advisable to carry out the operation twice on all samples.

References:

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

Appendix 6

OVERVIEW OF THE STATISTICAL ASSESSMENT OF DATA (NOEC DETERMINATION)



C.34. Determination of the inhibition of the activity of anaerobic bacteria – reduction of gas production from anaerobically digesting (sewage) sludge

INTRODUCTION

1. This test method is equivalent to the OECD test guideline (TG) 224 (2007). Chemicals discharged to the aquatic environment pass through both aerobic and anaerobic zones, where they may be degraded and/or can inhibit bacterial activity; in some cases they can remain in anaerobic zones undisturbed for decades or longer. In waste water treatment the first stage, primary settlement, is aerobic in the supernatant liquid and anaerobic in the subnatant sludge. This is followed in the secondary stage by an aerobic zone in the activated sludge aeration tank and an anaerobic zone in the subnatant sludge in the secondary settlement tank. Sludge from both of these stages is usually subjected to anaerobic treatment, producing methane and carbon dioxide which are normally used to produce electricity. In the wider environment, chemicals reaching sediments in bays, estuaries and the sea are likely to remain in these anaerobic zones indefinitely if they are not biodegradable. Larger proportions of some chemicals will preferably reach these zones because of their physical properties, such as low solubility in water, high adsorption to suspended solids, as well as inability to be biodegraded aerobically.

2. While it is desirable that chemicals discharged to the environment should be biodegradable under both aerobic and anaerobic conditions, it is essential that such chemicals do not inhibit the activity of microorganisms in either zone. In the UK there have been a few cases of complete inhibition of methane production caused by, for example, pentachlorophenol in industrial discharges, leading to very costly transportation of inhibited sludge from the digesters to 'safe' sites and importation of healthy digesting sludge from neighbouring installations. But there have been many cases of less severe disruption of digestion by several other chemicals, including aliphatic halohydrocarbons (dry-cleaning) and detergents, leading to significant impairment of digester efficiency.

3. Only one test method, C.11 (1), deals with inhibition of bacterial activity (Respiration of activated sludge), which assesses the effect of test chemicals on the rate of oxygen uptake in the presence of substrate. The method has been widely used to give early warning of possible harmful effects of chemicals on the aerobic treatment of wastewaters, as well as indicating non-inhibitory concentrations of test chemicals to be used in the various tests for biodegradability. Test method C.43 (2) offers a limited opportunity for determining the toxicity of a test chemical to gas production by anaerobic sludge, diluted to one tenth of its normal concentration of solids to allow the required precision in the assessment of percentage biodegradation. Because diluted sludge could be more sensitive to inhibitory chemicals, the ISO group decided to prepare a method using undiluted sludge. At least three texts were examined (from Denmark, Germany and the UK) and finally two ISO standards were prepared, one using undiluted sludge, ISO 13 641-1 (3) and the other using one hundredth diluted

sludge, ISO 13 641-2 (4), to represent muds and sediments having low bacterial populations. Both methods were subjected to a ring-test (5); part 1 was confirmed as an acceptable standard but there was disagreement over part 2. The UK considered that, because a significant proportion of participants reported very little or no gas production, partly because the percentage gas space was too high (at 75 %) for optimal sensitivity, the method requires further investigation.

4. Earlier work in the UK (6)(7) described a manometric method using undiluted digesting sludge, plus raw sewage sludge as the substrate, in 500 ml flasks; the apparatus was cumbersome and the stench of the raw sludge was offensive. Later the more compact and convenient apparatus of Shelton and Tiedje (8) as developed by Battersby and Wilson (9) was successfully applied by Wilson et al. (10). Kawahara et al (11) successfully prepared more standard sludges in the laboratory for use in tests for anaerobic biodegradability and inhibition on a number of chemicals. Also, raw sludge as the substrate was replaced to carry out a test either with one hundredth diluted anaerobic sludge or with muds, sediments etc. of low bacterial activity.

5. This method can provide information that is useful in predicting the likely effect of a test chemical on gas production in anaerobic digesters. However, only longer tests simulating working digesters more closely can indicate whether adaptation of the microorganisms to the test chemical can occur or whether chemicals likely to be absorbed and adsorbed onto sludge can build up to a toxic concentration over a longer period than allowed in this test.

PRINCIPLE OF THE TEST

6. Aliquots of a mixture of anaerobically digesting sludge (20 g/l to 40 g/l total solids) and a degradable substrate solution are incubated alone and simultaneously with a range of concentrations of the test chemical.in sealed vessels for up to 3 days. The amount of gas (methane plus carbon dioxide) produced is measured by the increase in pressure (Pa) in the bottles. The percentage inhibition of gas production brought about by the various concentrations of the test chemical is calculated from the amounts produced in the respective test and control bottles. The EC_{50} and other effective concentrations are calculated from plots of percentage inhibition against the concentration of the test chemicals or, more usually, its logarithm.

INFORMATION ON THE TEST CHEMICAL

7. Test chemicals should normally be used in the purest form readily available, since impurities in some chemicals, e.g. chlorophenols, can be much more toxic than the test chemical itself. However, the needs to test chemicals in the form in which they are produced/made commercially available should be considered. The use of formulated products is not routinely recommended, but for poorly soluble test chemicals the use of formulated material may be appropriate. Properties of the test chemical which should be available include solubility in water and some organic solvents, vapour pressure, adsorption coefficient, hydrolysis and biodegradability under anaerobic conditions.

APPLICABILITY OF THE METHOD

8. The test is applicable to chemicals which are soluble or insoluble in water, including volatile chemicals. But special care is necessary with materials of low water-solubility (see ref. (12)) and of high volatility. Also, inocula from other anaerobic sites, e.g. muds, saturated soils, sediments, may be used. Anaerobic bacterial systems that have previously been exposed to toxic chemicals may be adapted to maintaining their activity in the presence of xenobiotic chemicals. Inocula from adapted bacterial systems may show a higher tolerance to the test chemicals compared to inocula obtained from non-adapted systems.

REFERENCE CHEMICALS

9. To check the procedure, a reference chemical is tested by setting up appropriate vessels in parallel as part of normal test runs; 3, 5-dichlorophenol has been shown to be a consistent inhibitor of anaerobic gas production, as well as of oxygen consumption by activated sludge and other biochemical reactions. Two other chemicals have been shown to be more inhibitory to methane production than 3, 5-dichlorophenol, namely methylene bis-thiocyanate and pentachlorophenol but results with them have not been validated. Pentachlorophenol is not recommended since it is not readily available in a pure form.

REPRODUCIBILITY OF THE RESULTS

10. In an international ring test (5) there was only fair reproducibility in EC_{50} values between the 10 participating laboratories for 3, 5-dichlorophenol and 2-bromo-ethane sulphonic acid. (The range for the former was 32mg/l to 502 mg/l and for the latter 220-2190 mg/l.)

Number of laboratories	As mg/l			As mg/g sludge		
	mean	s.d.	cv(%)	mean	s.d.	cv(%)
	3, 5-Dichlorophenol					
10	153	158	103	5	4.6	92
	2-Bromo-ethane sulphonic acid					
10	1058	896	85	34	26	76

EC_{50} data from ring test – undiluted sludge

11. The high coefficients of variation between laboratories to a large extent reflect differences in the sensitivity of the sludge microorganisms due to either pre-exposure or no pre-exposure to the test chemical or other chemically related chemicals. The precision with which the EC_{50} value based on the sludge concentration was determined was barely better than the 'volumetric' value (mg/l). The three laboratories which

reported the precision of their EC_{50} values for 3.5-dichlorophenol showed much lower coefficients of variation (22, 9, and 18 % respectively for EC_{50} mg/g) than those of the means of all ten laboratories. The individual means for the three laboratories were 3.1, 3.2 and 2.8 mg/g, respectively. The lower, acceptable coefficients of variation within laboratories compared with the much higher coefficients between laboratory values, namely 9-22 % cf. 92 %, indicate that there are significant differences in the properties of the individual sludges.

DESCRIPTION OF THE METHOD

Apparatus

12. Usual laboratory equipment and the following are required:

    a) Incubator – spark-proof and controlled at 35 °C ± 2°C;

    b) Pressure-resistant glass test vessels of an appropriate nominal size[1], each fitted with a gas-tight coated septum, capable of withstanding about 2 bar or 2 x 10^5 Pa (for coating use e.g. PTFE = polytetrafluorethene). Glass serum bottles of nominal volume 125 ml, with an actual volume of around 160 ml, sealed with serum septa[2] and crimped aluminium rings are recommended; but bottles of total volume between 0.1 and 1 litre may be used successfully;

    c) Precision pressure-meter[3] and needle attachment

    Total gas production (methane plus carbon dioxide) measured by means of a pressure-meter adapted to enable measurement and venting of the gas produced. An example of a suitable instrument is a hand-held precision pressure-meter connected to a syringe needle; a three-way gas-tight valve facilities the release of excess pressure (Appendix 1). It is necessary to keep the internal volume of the pressure transducer tubing and valve as low as possible, so that errors

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[1] The recommended size is 0.1 litre to 1 litre.

[2] The use of gas-tight silicone septa is recommended. It is further recommended that the gas-tightness of caps, especially butyl rubber septa, be tested because several commercially available septa are not sufficiently gas-tight against methane and some septa do not stay tight when they are pierced with a needle under the conditions of the test.

- Gas tight coated septa are recommended and must be used for volatile chemicals (some commercial septa are relatively thin, less than 0.5 cm, and do not stay gas tight after piercing with syringe needle);
- Butyl rubber septa (about 1 cm) are recommended, if the test substances are not volatile (these normally stay gas tight after piercing.)
- Prior to the test it is recommended that the septa are carefully examined for their ability to stay gas tight after piercing.

[3] The meter should be used and calibrated at regular intervals, according to the manufacturer's instructions. If a pressure-meter of the prescribed quality is used e.g. capsulated with a steel membrane, no calibration is necessary in the laboratory. It should be calibrated by a licensed institute at the recommended intervals. The accuracy of the calibration can be checked in the laboratory with a one-point measurement at 1 x 10^5 Pa against a pressure-meter with a mechanical display. When this point is measured correctly, the linearity will also be unaltered. If other measurement devices are used (without certified calibration by the manufacturer), conversion is recommended over the total range at regular intervals (Appendix 2).

introduced by neglecting the volume of the equipment are insignificant;

d) Insulated containers, for transport of digesting sludge;
e) Three-way pressure valves;
f) Sieve, having a 1 mm square mesh;
g) Reservoir, for digesting sludge, a glass or high-density polyethylene bottle, capacity about 5 litre, fitted with a stirrer and facilities for passing a stream of nitrogen gas (see paragraph 13) through the headspace;
h) membrane filters (0.2 μm) for sterilising the substrate;
i) micro syringes, for the gas-tight connection of the pressure transducer (see paragraph 12(c)) to the headspace in the bottles (see paragraph 12(b)); also for adding insoluble liquid test materials into the bottles;
j) glove box, optional but recommended, with a slight positive pressure of nitrogen.

Reagents

13. Use analytical grade reagents throughout. Nitrogen gas, of high purity with a content of less than 5μl/l oxygen, should be used throughout.

Water

14. If dilution is necessary at any stage, use deionised water previously de-aerated. Analytical controls on this water are not necessary, but ensure that the deionising apparatus is regularly maintained. Use deionised water also for the preparation of stock solutions. Prior to the addition of the anaerobic inoculum to any solution or dilution of test material, make sure that these are oxygen-free. This is done either by blowing nitrogen gas through the dilution water (or through the dilutions) for 1 hour before adding the inoculum, or alternatively by heating the dilution water to the boiling point and cooling to room temperature in an oxygen-free atmosphere.

Digested Sludge

15. Collect actively digesting sludge from a digester at a wastewater treatment plant, or alternatively, from a laboratory digester, treating sludge from predominantly domestic sewage. Practical information regarding sludge from a laboratory digester can be found elsewhere (11). If use of an adapted inoculum is intended, digesting sludge from an industrial sewage treatment plant may be considered. Use wide-necked bottles constructed from high-density polyethylene or a similar material, which can expand, for sludge collection. Add sludge to the sample bottles to within about 1 cm from the top of the bottles, seal them tightly, preferably with a safety valve (paragraph 12(e)), and place in insulated containers (paragraph 12(d)) to minimise temperature shock, until being transferred to an incubator maintained at 35 ^{o}C ± 2^{o}C. When opening the bottles, take care to release excess gas pressure either by cautiously loosening the seal, or by means of the three-way pressure-release valve (paragraph 12(e)). It is preferable to use the sludge within a few hours of collection, otherwise store at 35 ^{o}C ± 2^{o}C under a headspace of nitrogen for up to 3 days, when little loss of activity normally occurs.

Warning – Digesting sludge produces flammable gases which present fire and explosion risks: it also contains potentially pathogenic organisms, so take appropriate precautions when handling sludge. For safety reasons, do not use glass vessels for collecting sludge.

Inoculum

16. Immediately prior to use, mix the sludge by gentle stirring and pass it through a 1 mm^2 mesh sieve (paragraph 12(f)) into a suitable bottle (paragraph 12(g)) through the headspace of which a stream of nitrogen is passed. Set aside a sample for measurement of the concentration of total dry solids (see e.g. ISO 11 923 (13) or equivalent EU standard). In general, use the sludge without dilution. The solids concentration is usually between 2% and 4% (w/v). Check the pH value of the sludge and, if necessary, adjust to 7 ± 0.5.

Test substrate

17. Dissolve 10 g nutrient broth (e.g. Oxoid), 10 g of yeast extract and 10 g of D-glucose in deionised water and dilute to 100 ml. Sterilise by filtration through a 0.2 µm membrane filter (paragraph 12(h)) and use immediately or store at 4 ^oC for not longer than 1 day.

Test chemical

18. Prepare a separate stock solution for each water-soluble test chemical to contain, for example, 10 g/l of the chemical in oxygen-free dilution water (paragraph 14). Use appropriate volumes of these stock solutions to prepare the reaction mixtures containing graded concentrations. Alternatively, prepare a dilution series of each stock solution so that the volume added to the test bottles is the same for each required final concentration. The pH of the stock solutions should be adjusted to 7 ± 0.2 if necessary.

19. For test chemicals which are insufficiently soluble in water, consult ISO 10 634 (12) or equivalent EU standard. If an organic solvent is needed to be used, avoid solvents such as chloroform and carbon tetrachloride, which are known strongly to inhibit methane production. Prepare a solution of an appropriate concentration of water-insoluble chemical in a suitable volatile solvent, for example, acetone, di-ethylether. Add the required volumes of solvent solution to the empty test bottles (paragraph 12(b)) and evaporate the solvent before the addition of sludge. For other treatments use ISO 10 634 (12) or equivalent EU standard, but be aware that any surfactants used to produce emulsions may be inhibitory to anaerobic gas production. If it is thought that the presence of organic solvents and emulsifying agents causes artefacts, the test chemical could be added directly to the test mixture as a powder or liquid. Volatile chemicals and water-insoluble liquid test chemicals may be injected into inoculated serum bottles, using micro-syringes (paragraph 12(i)).

20. Add test chemicals to the bottles to give a geometric series of concentrations, for example, 500 mg/l, 250 mg/l, 125 mg/l, 62.5 mg/l, 31.2 mg/l and 15.6 mg/l. If the range of toxicity is not known from similar chemicals, first carry out a preliminary range-finding test with concentration of 1000 mg/l, 100 mg/l and 10 mg/l to ascertain the appropriate range.

Reference chemical

21. Prepare an aqueous solution of 3,5-dichlorophenol (10 g/l) by gradually adding the

minimum amount of 5 mol/l of sodium hydroxide solution to the solid, while shaking, until it has dissolved. Then add de-oxygenated dilution water (paragraph 14) to the required volume; sonication may aid dissolution. Other reference chemicals may be used when the average range of the EC_{50} has been obtained in at least three tests with different inocula (different sources or different times of collection).

INTERFERENCE/ERRORS

22. Some constituents of sludge presumably could react with potential inhibitors making them unavailable to micro-organisms so giving lower, or no, inhibition. Also, if the sludge already contains a chemical which is inhibitory, erroneous results would be obtained when that chemical was subjected to the test. Apart from these possibilities, there are a number of identified factors which can lead to false results. These are listed in Appendix 3, together with methods of eliminating or at least reducing errors.

TEST PROCEDURE

23. The number of necessary replicates depends on the degree of precision required for the inhibition indices. If the bottle seals are sufficiently gas-tight over the duration of the test, set up just one batch (at least triplicates) of test bottles at each concentration required. Similarly, set up one batch of bottles with reference chemical and one set of controls. However, if the seals of the bottles are reliable for only one or a few piercings, set up a batch (e.g. triplicates) of the test bottles for each interval (t) for which results are required for all concentrations of a test chemical to be tested. Similarly, set up 't' batches of bottles for the reference chemical and for the controls.

24. The use of a glove box (paragraph 12(j)) is recommended. At least 30 minutes before starting the test, start a flow of nitrogen gas through the glove box containing all the necessary equipment. Ensure that the temperature of the sludge is within 35 ^{o}C ± 2^{o}C during handling and sealing of the bottles.

Preliminary Test

25. If the activity of the sludge is unknown, it is recommended to carry out a preliminary test. Set up controls to give, for example, concentrations of solids of 10 g/l, 20 g/l and 40 g/l plus substrate but use no test chemical. Also, use different volumes of reaction mixture in order to have three or four ratios of volume of headspace to volume of liquid. From the results of gas volumes produced at various time intervals, the most suitable conditions which allow two daily measurements yielding significant volumes of gas and release of pressure per day at optimal sensitivity[1] without fear of explosions.

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[1] This applies to the experimental set-up and experimental conditions whereby the volumes of gas produced – from control blanks and from vessels indicating 70 - 80 % inhibition – may be estimated with acceptable margins of error.

Addition of test chemicals

26. Add water-soluble test chemicals to empty test bottles (paragraph 12(b)) as aqueous solutions (paragraph 18). Use at least triplicate sets of bottles for each of a range of concentrations (paragraph 20). In the case of insoluble and poorly soluble test chemical, inject solutions of these in organic solvents using a micro-syringe into empty bottles to give replicate sets of each five concentrations of test chemical. Evaporate the solvent by passing a jet of nitrogen gas over the surface of the solutions in the test bottles. Alternatively, add insoluble solid chemicals as weighed amounts of the solid directly to the test bottles.

27. If insoluble and poorly water-soluble liquid test chemicals are not added using a solvent, add them directly by micro-syringe to the test bottles after addition of inoculum and test substrate (see paragraph 30). Volatile test chemicals may be added in the same way.

Addition of inoculum and substrate

28. Stir an appropriate volume of sieved digesting sludge (see paragraph 16) in a 5 litre bottle (paragraph 12(g)), while passing a stream of nitrogen gas through the headspace. Flush test bottles, containing aqueous solutions or evaporated solvent solutions of test chemicals, with a stream of nitrogen gas, for about two minutes to remove air. Dispense aliquots, e.g. 100 ml, of the well-mixed sludge into the test bottles using a large-tipped pipette or a measuring cylinder. It is essential to fill the pipette in one step to the exact volume of sludge required because of the ease of settlement of sludge solids. If more is taken up, empty the pipette and start again.

29. Then add sufficient substrate solution (paragraph 17) to give a concentration of 2 g/l of each of the nutrient broth, yeast extract and D-glucose in the mixture, while nitrogen is still flushing through. The following is an example for test batches.

Final mass concentration of test chemical in test bottles (mg/l)	Volume of test chemical (ml)		Reagents and media (ml)		
	Stock solution a) 10 g/l para. 18	Stock solution b) 1 g/l para. 18	Dilution water para. 14	Inoculum para. 16	Substrate para. 17
0	-	0	1.0	100	2
1	-	0.1	0.9	100	2
3.3	-	0.33	0.67	100	2
10	0.1	-	0.9	100	2
33	0.33	-	0.67	100	2
100	1.0	-	0	100	2

Total volume of bottle = 160 ml. Volume of liquid = 103 ml
Gas volume = 57 ml, or 35.6% of total volume.

30. Similarly flush out with nitrogen gas sufficient empty test bottles to deal with any volatile and insoluble liquid test chemical (see paragraph 27).

Controls and reference chemical

31. Set up at least triplicate sets of bottles, containing sludge and substrate only, to act as controls. Set up further replicate bottles containing sludge and substrate plus sufficient stock solution of the reference chemical, 3,5-dichlorophenol (paragraph 21) to result in a final concentration of 150 mg/l. This concentration should inhibit gas production by about 50 %. Alternatively, set up a range of concentrations of the reference chemical. In addition, set up four extra bottles for pH measurement which contain sludge, de-oxygenated water and substrate. Add the test chemical to two bottles at the highest concentration being tested and add de-oxygenated water to the remaining two bottles.

32. Ensure that all bottles – test and reference chemicals, and controls – contain the same volume (V_R) of liquid; where necessary, add de-oxygenated deionised water (paragraph 14) to make up the volume. The headspace should be between 10 % and 40 % of the bottle volume, the actual value being selected from the data obtained from the preliminary test. After adding all constituents to the bottles, remove the needle supplying the gas and seal each bottle with a rubber stopper and an aluminium cap (Paragraph 12(b)) moistening the stopper with a drop of deionised water to aid insertion. Mix the contents of each bottle by shaking.

Incubation of bottles

33. Transfer the bottles to the thermostatically controlled incubator, preferably equipped with a shaking device, and maintained at 35 ^{o}C ± 2^{o}C. The bottles are incubated in the dark. After about 1 hour, equalise the pressure in the bottles to atmosphere by inserting the syringe needle, attached to the pressure-meter (paragraph 12(c)), through the seal of each bottle in turn, open the valve until the pressure-meter reads zero and finally close the valve. The needle should be inserted at an angle of about 45^{o} to prevent gas leaking from the bottles. If the bottles are incubated without shaking facility, shake manually twice each day during the total incubation period to equilibrate the system. Incubate the bottles and invert them to prevent any loss of gas through the septum. Inversion is, however, not appropriate in cases in which insoluble test chemicals may adhere to the bottom of the flask.

Pressure measurement

34. When the bottles have reached 35 ^{o}C ± 2^{o}C, measure and record the pH of the contents of two of the four bottles set up for the purpose and discard the contents; continue incubating remaining bottles in the dark. Measure and record the pressure in the bottles twice a day over the following 48 hours to 72 hours by inserting the needle of the pressure-meter through the seal of each bottle, in turn, drying the needle between measurements. Keep all parts of the bottle at the incubation temperature during the measurement, which should be carried out as quickly as possible. Allow the pressure reading to stabilise and record it. Then open the valve for ventilation and close it when the pressure reads zero. Continue the test usually for 48 hours from the time of first equalising the pressure, designated ‘time 0’. The number of readings and ventilations

should be limited for volatile chemicals to one (at the end of incubation) or two to minimise loss of test chemical (10).

35. If the pressure reading is negative, do not open the valve. Moisture sometimes accumulates in the syringe needle and tubing, indicated by a small negative pressure reading. In this case remove the needle, shake the tubing, dry with a tissue and fit a new needle.

pH measurement

36. Measure and record the pH of the contents of each bottle after the final pressure measurement.

DATA AND REPORTING

Expression of results

37. Calculate the sum and average of the pressures recorded at each time interval for each set of replicate bottles and calculate the mean cumulative gross gas pressure at each time interval for each set of replicates. Plot curves of mean cumulative gas production (Pa) against time for control, test and reference bottles. Select a time on the linear part of the curve, usually 48 hours, and calculate the percentage inhibition (I) for each concentration from equation [1]:

$$I = (1 - P_t/P_c) \times 100 \quad [1],$$

where
I = percentage inhibition,in %;
P_t = the gas pressure produced with test material at selected time, in Pascal (Pa);
P_c = the gas pressure produced in the control at the same time, in Pascal (Pa).

It would be advisable to draw both plots, i.e. Plot I against concentration and also against logarithm of the concentration so that the curve which is nearer to linearity may be selected. Assess the EC_{50} (mg/l) value visually or by regression analysis from that curve nearer to linearity. For comparative purposes it may be more useful to express the concentration of the chemical as mg chemical/g of total dry solids. To obtain this concentration, divide the volumetric concentration (mg/l) by the volumetric concentration of dry sludge solids (g/l) (paragraph 16).

38. Calculate either the percentage inhibition achieved by the single concentration of the reference chemical used or the EC_{50} if a sufficient number of concentrations have been investigated.

39. Convert the mean pressure of the gas produced in the control P_c(Pa) to the volume by reference to the pressure-meter calibration curve (Appendix 2) and from this calculate the yield of gas, expressed as the volume produced in 48 hours from 100 ml undiluted sludge at a solids concentration of 2 % (20 g/l) to 4 % (40 g/l).

Validity criteria

40. Results from the ISO inter-laboratory trial (5) showed the reference chemical (3,5-dichlorophenol) caused 50% inhibition of gas production in a range of concentrations of 32 mg/l to 510 mg/l mean 153 mg/l (paragraph 10). This range is so wide that firm limits for inhibition cannot confidentially be set as validity criteria; this should be possible when developments have shown how to produce more consistent inocula. The volumes of gas produced in control bottles in 48 hour ranged from 21 ml/g sludge dry matter to 149 ml/g (mean 72 ml/g). There was no obvious relation between volume of gas produced and the corresponding EC_{50} value. The final pH varied between 6.1 and 7.5.

41. The test is considered to be valid when an inhibition of greater than 20% is obtained in the reference control containing 150 mg/l of 3,5-dichlorophenol, more than 50 ml of gas per g of dry matter is produced in the blank control and the pH value is within the range of 6.2 to 7.5 at the end of the test.

Test Report

42. The test report must include the following information:

Test chemical
- common name, chemical name, CAS number, structural formula and relevant physico-chemical properties;
- purity (impurities) of test chemical.

Test conditions
- volumes of liquid contents and of headspace in test vessels;
- descriptions of the test vessels and gas measurement (e.g. type of pressure-meter);
- application of test chemical and reference chemical to the test system, test concentrations used and use of any solvents;
- details of the inoculum used: name of sewage treatment plant, description of the source of waste water treated (e.g. operating temperature, sludge retention time, predominantly domestic sewage or industrial waste, etc.), concentration of solids, gas production activity of anaerobic digester, previous exposure or possible pre-adaptation to toxic chemicals or site of collection of mud, sediment etc;
- incubation temperature and range;
- number of replicates.

Results
- pH values at end of test;
- all the measured data collected in the test, blank and reference chemical control vessels, as appropriate (e.g. pressure in Pa or millibars) in tabular form;
- percentage inhibition in test and reference bottles, and the inhibition-concentration curves;
- calculation of EC_{50} values, expressed as mg/l and mg/g;
- gas production per g sludge in 48 hours;
- reasons for any rejection of the test results;
- discussion of results, including any deviations from the procedures in this test method and discuss any deviations in the test results due to interferences and errors from what would be expected;
- address also whether the purpose of the test was to measure the toxicity to either

pre-exposed or non pre-exposed microorganisms.

LITERATURE

(1) Chapter C.11 of this Annex: Activated Sludge, Respiration Inhibition Test.

(2) Chapter C.43 of this Annex: Anaerobic biodegradability of organic compounds in digested sludge: method by measurement of gas production.

(3) International Organisation for Standardisation (2003) ISO 13 641-1 Water Quality – Determination of inhibition of gas production of anaerobic bacteria – Part 1: General Test.

(4) International Organisation for Standardisation (2003) ISO 13 641-2 Water Quality – Determination of inhibition of gas production of anaerobic bacteria – Part 2: Test for low biomass concentrations.

(5) ISO (2000) Ring test of ISO 13 641-1 and ISO 13 641-2. Determination of inhibition of activity of anaerobic bacteria. BL 6958/A. Evans MR, Painter HA. Brixham Environmental Laboratory, AstraZeneca UK Ltd., Brixham, TQ5 8BA UK.

(6) Swanwick JD, Foulkes M (1971). Inhibition of anaerobic digestion of sewage sludge by chlorinated hydrocarbons. Wat. Pollut. Control, 70, 58-70.

(7) HMSO (1986) Determination of the inhibitory effects of chemicals and waste waters on the anaerobic digestion of sewage sludge. ISBN 0 117519 43 X, In: Methods for the Examination of Waters and Associated Materials UK.

(8) Shelton DR, Tiedje JM (1984). General method for determining anaerobic biodegradation potential. Appl. Env. Microbiol. 47 850-857.

(9) Battersby NS and Wilson V (1988). Evaluation of a serum bottle technique for assessing the anaerobic biodegradability of organic compounds under methanogenic conditions. Chemosphere 17, 2441-2460.

(10) Wilson V, Painter HA and Battersby NS (1992). A screening method for assessing the inhibition of the anaerobic gas production from sewage sludge. Proc. Int. Symp. on Ecotoxicology. Ecotoxicological Relevance of Test Methods, GSF Forschungzentrum, Neuherberg, Germany (1990). Eds. Steinberg C and Kettrup A, pp117-132 (1992).

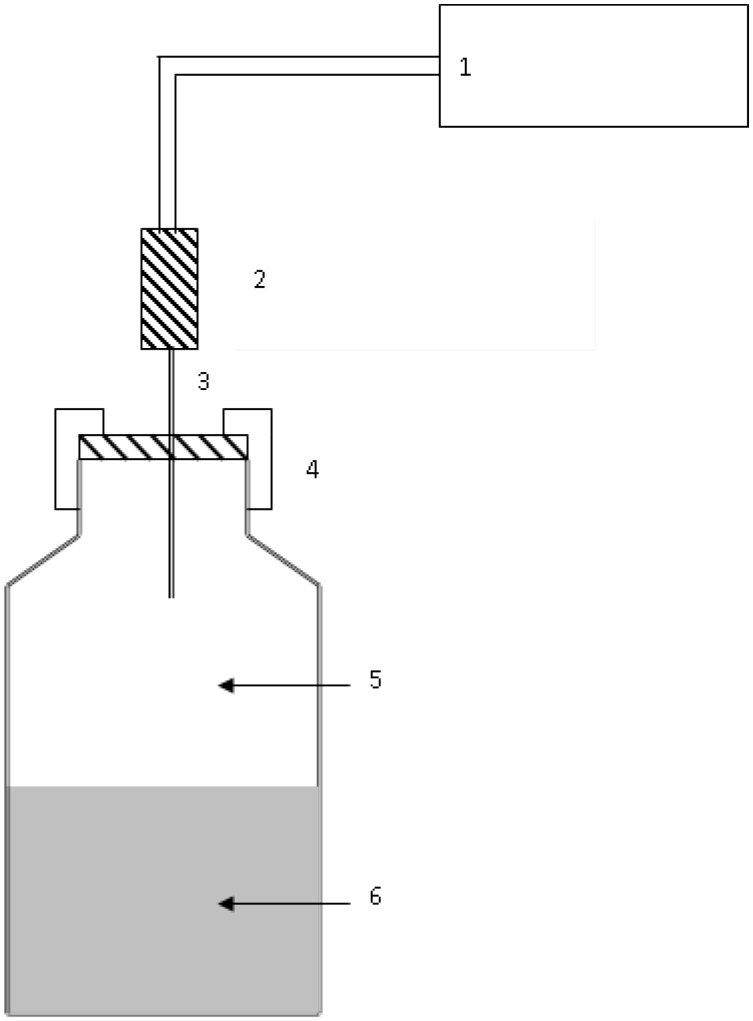
(11) Kawahara K, Yakabe Y, Chida T, and Kida K (1999). Evaluation of laboratory-made sludge for an anaerobic biodegradability test and its use for assessment of 13 chemicals. Chemosphere, 39 (12), 2007-2018.

(12) International Organization for Standardization (1995) ISO 10 634 Water Quality – Guidance for the preparation and treatment of poorly water-soluble organic compounds for the subsequent evaluation of their biodegradability in an aqueous medium.

(13) International Organization for Standardization (1997) ISO 11 923 Water Quality - Determination of suspended solids by filtration through glass-fibre filters.

Appendix 1

EXAMPLE OF AN APPARATUS TO MEASURE BIOGAS PRODUCTION BY GAS PRESSURE



Key:
1 - Pressure-meter
2 - 3-way gas-tight valve
3 - Syringe needle
4 - Gastight seal (crimp cap and septum)
5 – Head space
6 - Digested sludge inoculum

Test vessels in an environment of 35 °C ± 2 °C

Appendix 2

CONVERSION OF THE PRESSURE-METER

The pressure-meter readings may be related to gas volumes by means of a standard curve and from this the volume of gas produced per g dry sludge per 48 hours may be calculated. This activity index is used as one of the criteria by which to assess the validity of test results. The calibration curve is produced by injecting known volumes of gas at 35 ^{o}C ± 2^{o}C in serum bottles containing a volume of water equal to that of the reaction mixture, V_R;

- Dispense V_R ml aliquots of water, kept at 35 ^{o}C ± 2^{o}C into five serum bottles. Seal the bottles and place in a water bath at 35 ^{o}C ± 2^{o}C for 1 hour to equilibrate;
- Switch on the pressure-meter, allow to stabilise, and adjust to zero;
- Insert the syringe needle through the seal of one of the bottles, open the valve until the pressure-meter reads zero and close the valve;
- Repeat the procedure with the remaining bottles;
- Inject 1 ml of air at 35 ^{o}C ± 2^{o}C into each bottle. Insert the needle (on the meter) through the seal of one of the bottles and allow the pressure reading to stabilise. Record the pressure, open the valve until the pressure reads zero and then close the valve;
- Repeat the procedure with the remaining bottles;
- Repeat the total procedure using 2 ml, 3 ml, 4 ml, 5 ml, 6 ml, 8 ml, 10 ml, 12 ml, 16 ml, 20 ml, and 50 ml of air;
- Plot a conversion curve of pressure (Pa) against gas volume injected (ml). The response of the instrument is linear over the range 0 Pa to 70 000 Pa, and 0 ml to 50 ml of gas production.

Appendix 3

IDENTIFIED FACTORS WHICH CAN LEAD TO FALSE RESULTS

(a) Quality of the bottle-caps

Different types of septa for the serum bottles are available commercially; many of them, including butyl rubber, lose tightness when pierced with a needle under the conditions of this test. Sometimes the pressure falls very slowly once the septum has been pierced with the syringe needle. The use of gas-tight septa is recommended to overcome leaks (paragraph 12(b)).

(b) Moisture in the syringe needle

Moisture sometimes accumulates in the syringe needle and tubing, and is indicated by a small negative pressure reading. To rectify this remove the needle and shake the tubing, dry with a tissue and fit a new needle (paragraphs 12(c) and 35).

(c) Oxygen contamination

Anaerobic methods are subject to error from contamination by oxygen, which can cause lower gas production. In this method this possibility should be minimised by the use of strictly anaerobic techniques, including use of a glove box.

(d) Gross substrates in sludge

The anaerobic gas production and the sensitivity of the sludge are influenced by substrates which are transferred with the inoculum into the test bottles. Digested sludge from domestic anaerobic digesters still often contains recognisable matter like hair and plant residues of cellulose, which tend to make it difficult to take representative samples. By sieving the sludge gross insoluble matter can be removed, which makes representative sampling more likely (paragraph 16).

(e) Volatile test chemicals

Volatile test chemicals will be released into the headspace of the test bottles. This may result in the loss of some of the test material from the system during venting after pressure measurements, yielding falsely high EC_{50} values. By suitable choice of ratio of headspace volume to liquid volume and by not venting after taking pressure measurements, the error can be reduced (10).

(f) Non-linearity of gas production

If the plot of mean cumulative gas production against incubation time is not approximately linear over the 48h period, the accuracy of the test may be lowered. To overcome this, it may be advisable to use digesting sludge from a different source and/or to add an increased concentration of the test substrate-nutrient broth, yeast extract and glucose (paragraph 29).

Appendix 4

APPLICATION TO ENVIRONMENTAL SAMPLES OF LOW BIOMASS CONCENTRATION – ANAEROBIC MUDS, SEDIMENTS, ETC.

Introduction

A.1 In general, the specific microbial activity (volume of gas produced per g dry solids) of naturally occurring anaerobic muds, sediments, soils, etc, is much lower than that of anaerobic sludge derived from sewage. Because of this, when the inhibitory effects of chemicals on these less active samples are to be measured some of the experimental conditions have to be modified. For these less active samples there are two general course of action possible:

(a) Carry out a modified preliminary test (paragraph 25) with the undiluted sample of mud, soil, etc at 35 ^{o}C ± 2^{o}C or at the temperature at the sample site of collection, for more accurate simulation (as in Part 1 of ISO 13 641);

(b) Or make the test with a dilute (1 in 100) digester sludge to simulate the low activity expected from the environment sample, but maintain the temperature at 35 ^{o}C ± 2^{o}C (as in Part 2 of ISO 13 641).

A.2 Option (a) may be achieved by following the method described here (equivalent to Part 1 of ISO 13 641), but it is essential to make a preliminary test (paragraph 25) to ascertain optimal conditions, unless these are already known from previous testing. The mud or sediment sample should be thoroughly mixed, e.g. in a blender, and , if necessary, diluted with a small proportion of de-aerated dilution water (paragraph 14) so that it is sufficiently mobile to be transferred by a coarse-tipped pipette or a measuring cylinder. If it is considered that nutrients may be lacking, the mud sample may be centrifuged (under anaerobic conditions) and re-suspended in the mineral medium containing yeast extract (A.11)

A.3 Option (b). This reasonably mimics the low activity of environmental samples but lacks the high concentration of suspended solids present in these samples. The role of these solids in inhibition is not known, but it is possible that reaction between the test chemicals and constituents of the mud, as well as adsorption of the test chemicals onto the solids, could result in a lowering of toxicity of the test chemical.

A.4 Temperature is another important factor: for strict simulation, tests should be made at the temperature of the sample site, since different groups of methane-producing consortia of bacteria are known to operate within different temperature ranges, namely thermophiles (~30-35oC), mesophiles (20-25oC) and psychrophiles (<20 oC), which may display different inhibitory patterns.

A.5 Duration. In the general test, Part 1, using undiluted sludge, the production of gas in the 2-4 days was always sufficient, while in Part 2 with one-hundred diluted sludge insufficient gas, if any, was produced in this period in the ring test. Madsen et al (1996), in describing this latter test, say at least 7 days should be allowed.

Testing with low biomass concentration (Option b)

The following changes and amendments should be made, adding to or replacing some existing paragraphs and sub-paragraphs of the main text.

A.6 Add to Paragraph 6: Principle of the test;

"This technique may be used with 1 in 100 diluted anaerobic sludge, partially to simulate the low activity of muds and sediments. The incubation temperature may be either 35 ^{o}C or that of the site from which the sample was collected. Since the bacterial activity is much less than in undiluted sludge, the incubation period should be extended to at least 7 days."

A.7 Add to paragraph 12 (a):

"the incubator should be capable of operating down to temperatures of 15 ^{o}C."

A.8 Add an extra reagent after Paragraph 13:

"Phosphoric acid (H_3PO_4), 85% by mass in water."

A.9 Add at end of Paragraph 16:

"Use a final concentration of 0.20± 0.05 g/l of total dry solids in the test."

A.10 Paragraph 17. Test substrate

This substrate is not to be used, but is replaced by yeast extract (see paragraphs 17; A.11, A.12, A.13).

A.11 A mineral medium, including trace elements, for diluting anaerobic sludge, is required and for convenience the organic substrate, yeast extract, is added to this medium.

Add after Paragraph 17

"(a) Test mineral medium, with yeast extract.

This is prepared from a 10-fold concentrated test medium (paragraph 17 (b); A.12) with a trace element solution (paragraph 17 (c); A.13). Use freshly supplied sodium sulphide nonahydrate (paragraph 17 (b); A.12) or wash and dry it before use, to ensure that it has sufficient reducing capacity. If the test is performed without using a glove box (paragraph 12 (j)), the concentration of sodium sulphide in the stock solution should be increased to 2 g/l (from 1 g/l). Sodium sulphide may also be added from an appropriate stock solution through the septum of the closed test bottles, as this procedure will decrease the risk of oxidation, to obtain a final concentration of 0.2 g/l. Alternatively titanium (III) citrate (paragraph 17 (b)) may be used. Add it through the septum of closed test bottles to obtain a concentration of 0.8 mmol/l to 1.0 mmol/l. Titanium (III) citrate is a highly effective and a low-toxicity reducing agent, which is prepared as follows: Dissolve 2.94 g of trisodium citrate dihydrate in 50 ml of oxygen-free dilution water (paragraph 14) (which results in a 200 mmol/l solution) and add 5 ml of a titanium (III) chloride solution (15 g/100 ml dilution water). Neutralise to pH

7± 0.5 with sodium carbonate and dispense to an appropriate serum bottle under a stream of nitrogen gas. The concentration of titanium (III) citrate in this stock solution is 164 mmol/l. Use the test medium immediately or store at 4 ^{o}C for no longer than 1 day.

A.12 (b) Tenfold concentrated test medium, prepared with the following:

anhydrous potassium dihydrogenphosphate (KH_2PO_4)	2.7 g
Disodium hydrogen phosphate (Na_2HPO_4)	4.4 g
(or 11.2 g dodecahydrate)	
ammonium chloride (NH_4Cl)	5.3 g
calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$)	0.75 g
magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$)	1.0 g
iron (II) chloride tetrahydrate ($FeCl_2 \cdot 4H_2O$)	0.2 g
resazurin (redox indicator)	0.01 g
sodium sulphide nonahydrate ($Na_2S \cdot 9H_2O$)	1.0 g
(or titanium (III) citrate) final concentration	0.8 mmol/l to 1.0 mmol/l
trace element solution (see paragraph 17 (c); A.13)	10.0 ml
yeast extract	100 g
Dissolve in dilution water (paragraph 14) and make up to:	1000 ml

A.13 (c) Trace element solution, prepared with the following:

manganese (II) chloride tetrahydrate ($MnCl_2.4H_2O$)	0.5 g
ortho-boric acid (H_3BO_3)	0.05 g
zinc chloride ($ZnCl_2$)	0.05 g
copper (II) chloride ($CuCl_2$)	0.03 g
sodium molybdate dihydrate ($Na_2MoO_4.2H_2O$)	0.01 g
cobalt (II) chloride hexahydrate ($CoCl_2.6H_2O$)	1.0 g
nickel (II) chloride hexahydrate ($NiCl_2.6H_2O$)	0.1 g
disodium selenite (Na_2SeO_3)	0.05 g
Dissolve in dilution water (paragraph 14) and make up to:	1000 ml”

A.14 Paragraph 25: Preliminary test

It is essential that a preliminary test is made as described in paragraph 24, except that the concentration of sludge solids should be one hundredth of those given, that is 0.1g/l, 0.2g/l and 0.4g/l. The duration of incubation should be at least 7 days.

NOTE: In the ring test (5) the headspace volume was much too high at 75% total volume; it should be in the recommended range of 10%-40%. The relevant criterion is that the volume of gas produced at around 80% inhibition should be measurable with acceptable precision (e.g. ±5% to ±10%).

A.15 Paragraph 26 to 30: Addition of test chemical, inoculum and substrate.

The additions are made in the same way as described in these paragraphs, but the substrate solution (paragraph 17) is replaced by the test medium plus yeast extract substrate (A.11).

Also, the final concentration of dry sludge solids is reduced from 2 g/l - 4 g/l to 0.2 ± 0.05 g/l (A.9). Two examples of the addition of components to the test mixture are given in Table A.1, which replaces the table in paragraph 29.

A.16 Paragraph 33: Incubation of bottles

Because of the expected lower rate of gas production, incubation is carried on for at least 7 days.

A.17 Paragraph 34: Pressure measurements

The same procedure for measuring the pressure in the headspace of the bottles is used as described in paragraph 34 if the amounts in the gaseous phase are required. If total amounts of CO_2 plus CH_4 are to be measured, the pH of the liquid phase is reduced to about pH 2 by the injection of H_3PO_4 into each relevant bottle and measuring the pressure after 30 minutes shaking at the temperature of the test. However, more information on the quality of the inoculum may be obtained by measuring the pressure in each bottle before and after acidification. For example when the rate of CO_2 production is much higher than that of methane, the sensitivity of the fermentative bacteria may be altered and/or methanogenic bacteria are preferentially affected by the test chemical.

A.18 Paragraph 36: pH measurement

If H_3PO_4 is to be used some extra bottles, to which no H_3PO_4 is added, would have to be set up especially for the pH measurement.

Reference: Madsen, T, Rasmussen, HB; and Nilsson, L (1996), Methods for screening anaerobic biodegradability and toxicity of organic chemicals. Project No.336, Water Quality Institute, Danish Environment Protection Agency, Copenhagen.

Table A.1. Examples of the test set-up for test batches

Reaction Mixture constituents	**Example 1**	**Example 2**	**Normal order of addition**
Concentration of prepared inoculum (g/l)	0.42	2.1	-
Volume of inoculum added (ml)	45	9	4
Concentration of inoculum in test bottles (g/l)	0.20	0.20	-
Volume of test medium added (ml)	9	9	2
Volume of dilution water added (ml)	36	72	3
Concentration of yeast extract in test bottles (g/l)	9.7	9.7	-
Volume of test chemical stock solution (ml)	3	3	1
Total liquid volume (ml)	93	93	-

Appendix 5

DEFINITIONS

For the purpose of this test method the following definitions are used:

Chemical means a substance or a mixture.

Test chemical means any substance or mixture tested using this test method.

C.35 Sediment-Water *Lumbriculus* Toxicity Test Using Spiked Sediment

INTRODUCTION

1. This test method is equivalent to OECD test guideline (TG) 225 (2007). Sediment-ingesting endobenthic animals are subject to potentially high exposure to sediment bound chemicals 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. By bioturbation of the sediment and by serving as prey these animals can have a strong influence on the bioavailability of such chemicals 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 chemical 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 method is designed to assess the effects of prolonged exposure of the endobenthic oligochaete *Lumbriculus variegatus* (Müller) to sediment-associated 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 test method is spiking of sediment with the test chemical. Using spiked sediment is intended to simulate a sediment contaminated with the test chemical.

3. Chemicals 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, depends on the physical-chemical properties of the chemical concerned and its ultimate fate in the animal. For strongly adsorbing chemicals (e.g. with log K_{ow} > 5) or for chemicals covalently binding to sediment, ingestion of contaminated food may be a significant exposure route. In order not to underestimate the toxicity of such chemicals, the food necessary for reproduction and growth of the test organisms is added to the sediment before application of the test chemical (11). The test 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 chemicals.

4. The test method is aimed to determine effects of a test chemical 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 the No Observed Effect Concentration (NOEC) and the Lowest Observed Effect Concentration (LOEC).

5. Chapter C.27 of this Annex, "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 test method ((13), 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 chemical such as safety precautions, proper storage conditions and analytical methods should be obtained before beginning the study. Guidance for testing chemicals with physical-chemical properties that make them difficult to perform the test is provided in (14).

7. Before carrying out a test, the following information about the test chemical should be known:

- common name, chemical name (preferably IUPAC name), structural formula, CAS registry number, purity;
- vapour pressure;
- solubility in water.

8. The following additional information is considered useful before starting the test:

- octanol-water partition coefficient, K_{ow};
- organic carbon-water partitioning coefficient, expressed as K_{oc};
- hydrolysis;
- phototransformation in water;
- biodegradability;
- surface tension.

9. Information on certain characteristics of the sediment to be used should be acquired before the start of the test (7). For details see paragraphs 22 to 25.

PRINCIPLE OF THE TEST

10. Worms of similar physiological state (synchronised as described in Appendix 5) 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 chemical serve as controls. The test chemical 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 29). 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 paragraphs 22 to 23, and Appendix 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. (15)) 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 (see paragraphs 34 to 38). The purpose of the study, EC_x or NOEC derivation, will determine the test design.

REFERENCE TESTING

12. Performance of the control organisms is expected to demonstrate sufficiently the ability of a laboratory to perform the test, and if historical data are available, the repeatability of the test. In addition, reference toxicity tests may be conducted in regular intervals using a reference toxicant to assess the sensitivity of the test organisms. 96 h reference toxicity tests in water only may satisfactorily demonstrate the sensitivity and condition of the test animals (4)(7). Information on the toxicity of pentachlorophenol (PCP) in complete tests (28 d exposure to spiked sediment) is included in Appendix 6, and in the report on the ring test of the Test Method (13). The acute, water-only toxicity of PCP is described e.g. in (16). This information can be used for comparison of test organism sensitivity in reference tests with PCP as reference toxicant. Potassium chloride (KCl) or copper sulphate ($CuSO_4$) have been recommended as reference toxicants with *L. variegatus* (4)(7). To date, establishment of quality criteria based on toxicity data for KCl is difficult due to lack of literature data for *L. variegatus*. Information on the toxicity of copper towards *L. variegatus* can be found in (17) to (21).

VALIDITY OF THE TEST

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

- A ring-test (13) 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 throughout the test.
- The oxygen concentration in the overlying water should not be below 30% of air

saturation value (ASV) at test temperature during the test.

DESCRIPTION OF THE TEST METHOD

Test system

14. Static systems without renewal of the overlying water are recommended. If the sediment-to-water ratio (see paragraph 15) is appropriate, gentle aeration will normally suffice to keep the water quality at acceptable levels for the test organisms (e.g. maximise dissolved oxygen levels, minimise build-up of excretory products). Semi-static or flow-through systems with intermittent or continuous renewal of overlying water should only be used in exceptional cases, since regular renewal of overlying water is expected to affect chemical equilibrium (e.g. losses of test chemical from the test system).

Test vessels and apparatus

15. The exposure should be conducted in glass beakers of e.g. 250 ml measuring 6 cm in diameter. Other suitable glass 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 39).

16. Test vessels and other apparatus that will come into contact with the test chemical should be made entirely of glass or other chemically inert material. Care should be taken to avoid the use of materials, for all parts of the equipment that can dissolve, absorb test chemicals or leach other chemicals and have an adverse effect on the test animals. Polytetrafluoroethylene (PTFE), stainless steel and/or glass should be used for any equipment having contact with the test media. For organic chemicals known to adsorb to glass, silanised glass may be required. In these situations the equipment will have to be discarded after use.

Test species

17. 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), (13), (15), (16), (22), (23), (24), (25), (26), (27), (28), (29), (30), (31), (32), (33), (34), (35)]. The origin of the test animals, the confirmation of species identity (e.g. (36)) 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

18. 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 Appendix 5. For details on culturing this species see references (3), (7), (27).

19. 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.

Water

20. Reconstituted water according to Chapter C.1 of this Annex (37) is recommended for use as overlying water in the tests; it can also be used for the laboratory cultures of the worms (see Appendix 2 for preparation). If required, natural water may be used. 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* has been demonstrated to survive, grow, and reproduce in this type of water (30), and maximum standardisation of test and culture conditions is provided. If a reconstituted water is used, its composition should be reported, and the water should be characterised prior to use at least by pH, oxygen content, and hardness (expressed as mg $CaCO_3$/l). Analysis of the water for micropollutants prior to use might provide useful information (see, e.g., Appendix 3).

21. The pH of the overlying water should be in the range of 6.0 to 9.0 (see paragraph 13). If increased ammonia development is expected, it is considered useful to keep the pH between 6.0 and 8.0. For testing of e.g. weak organic acids, it is advisable to adjust the pH by buffering the water to be used in the test, as described e.g. by (16). The total hardness of the water to be used in the test should be between 90 and 300 mg $CaCO_3$ per liter for natural water. Appendix 3 summarises additional criteria for acceptable dilution water according to OECD Guideline No. 210 (38).

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. Use of a formulated sediment minimises variability of test conditions as well as introduction of indigenous fauna. The following formulated sediment is based on the artificial sediment according to (6), (39) and (40). It is recommended for use in this type of test ((6), (10), (30), (41), (42), (43)):

    (a) 4-5 % (dry weight) sphagnum peat; it is important to use peat in powder form, degree of decomposition: “medium”, 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., in accordance with pharmacy standards, for human consumption), 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 Appendix 4.

23. The source of peat, kaolin clay, food material, and sand should be known. In addition to item g), Chapter C.27 of this Annex (6) lists alternative plant materials to be used as a source of nutrition: dehydrated leaves of mulberry (*Morus alba*), white clover (*Trifolium repens*), spinach (*Spinacia oleracea*), or cereal grass.

24. The chosen food source should be added prior to or during spiking the sediment with the test chemical. The chosen food source should allow for at least acceptable reproduction in the controls. Analysis of the artificial sediment or its constituents for micro-pollutants prior to use might provide useful information. An example for the preparation of the formulated sediment is described in Appendix 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 25 and Appendix 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.

25. 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 and cation exchange capacity is optional. It is also recommended that, before it is spiked with the test chemical, 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.

26. 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 (24), (25), (44), and (45), which specify sediment ingestion or particle selection in the test organisms.

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

Application of the test chemical

28. The test chemical is to be spiked to the sediment. As most test chemicals are expected to have low water solubility, they should be dissolved in a suitable organic solvent (e.g. acetone, n-hexane, cyclohexane) at a volume 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 chemical in the chosen solvent should be the main criteria for the selection of a suitable solubilising agent. For each concentration level the same volume of the corresponding solution should be used. The sediment should be spiked in bulk for each concentration level in order to minimise between-replicate variability of the test chemical concentration. Each of the test solutions is then mixed with quartz sand as described in paragraph 22 (e.g. 10 g of quartz sand per test vessel). In order to soak the quartz sand completely, a volume of 0.20 - 0.25 ml per g of sand has been found sufficient. Thereafter, the solvent must be evaporated to dryness. In order to minimise losses of the test chemical through co-evaporation (e.g. depending on the chemical's vapour pressure), the coated sand should be used immediately after drying. 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-chemical-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). Alternatively, e.g. for field sediment, the test chemical may be added by spiking a dried and finely ground portion of the sediment as described above for the quartz sand, or by stirring the test chemical into the wet sediment, with subsequent evaporating of any solubilising agent used. 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 chemical 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 other than described above should be reported. The method of spiking may be adapted to the test chemical's physical-chemical properties, e.g. to avoid losses due to volatilisation during spiking or equilibration. Additional guidance on spiking procedures is given in Environment Canada (1995) (46).

29. Once the spiked sediment has been prepared, distributed to the replicate test vessels, and topped with the test water, it is desirable to allow partitioning of the test chemical 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. (27)(47)). In this test, equilibrium is not awaited but an equilibration period of 48 hours to 7 days is recommended. Thus, time for degradation of the test chemical will be minimised. 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.

30. At the end of this equilibration period, samples should be taken at least of the overlying water and the bulk sediment, at least at the highest concentration and a lower one, for analysis of the test chemical concentration. These analytical determinations of the test chemical should allow for calculation of mass balance and expression of results based on measured initial concentrations. In general, sampling disturbs or destroys the sediment water system. Therefore it is usually not possible to use the same replicates for sampling of sediment and worms. Additional "analytical" vessels of appropriate dimensions have to be set up, which are treated in the same way (including the presence of test organisms) but not used for biological observations. The vessel dimensions should be selected to provide the sample amounts required by the analytical method. Details of sampling are described in paragraph 53.

PERFORMANCE OF THE TEST

Preliminary test

31. If no information is available on the toxicity of the test chemical 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 chemical are used. The worms are exposed to each concentration of the test chemical 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

32. 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 31), and as described in paragraphs 35, 36, 37 and 38.

33. A control (for replication see paragraphs 36, 37 and 38) containing all constituents, except for the test chemical, is run in addition to the test series. If any solubilising agent is used for application of the test chemical, it should have no significant effect on the test organisms as revealed by an additional solvent-only control.

Test design

34. 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 35, 36, 37 and 38.

35. The effect concentration (e.g. EC_{50} EC_{25}, EC_{10}) and the concentration range, over which the effect of the test chemical 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 avoided. If - in exceptional cases - such an extrapolation is done, a full explanation must be given in the report.

36. If the EC_x is to be estimated, at least five concentrations and a minimum of 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.

37. If the LOEC/NOEC values are to be estimated, at least 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 test method is given in Appendix 6.

38. 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), or if testing at a single concentration will be adequate to confirm a NOEC value of interest. In the latter case, a detailed rationale for selection of limit concentration should be included in the test report. 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 chemical, 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 test method is given in Appendix 6.

Exposure conditions

Test organisms

39. 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% (48), 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.

40. The worms used in a test should all come from the same source, and should be animals of similar physiological state (see Appendix 5). Worms of similar size should be selected (see paragraph 39). 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.

41. The worms to be used in a test are removed from the culture (see Appendix 5 for details). Large (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 Appendix 5. After regenerating for a period of 10 to 14 d, intact complete worms of similar size, which are actively swimming or crawling after 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

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

Light and temperature

43. The photoperiod in the culture and the test is usually 16 hours (3), (7). Light intensity should be kept low (e.g. 100-500 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 bias of reproduction due to vessel location.

Aeration

44. The overlying water of the test vessels should be gently aerated (e.g. 2 - 4 bubbles per second) via a pasteur pipette positioned approx. 2 cm above the sediment surface so as to minimise perturbation of the sediment. Care should be taken that the dissolved oxygen concentration does not fall below 30% of air saturation value (ASV). Air supply should be controlled and - if necessary - adjusted at least once daily on workdays.

Water quality measurements

45. 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. at 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 replicate 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.

If measurement of water quality parameters requires removal of significant water samples from the vessels, it may be advisable to set up separate vessels for water quality measurements so as not to alter the water-to-sediment volume ratio.

Biological observations

46. During the exposure, 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.

47. At the end of the test, 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, 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 wide-mouth 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.

48. 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).

49. 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 additional observations are not mandatory, but can be used for additional interpretation of the biological results (for example, a high number of worms assigned to group c may indicate a delay of reproduction or regeneration in a given treatment). Additionally, if any differences in appearance (e.g. lesions of the integument, oedematous body sections) are observed between treated and control worms, these should be recorded.

50. Immediately after counting/assessment, the living 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).

51. In addition to the total dry weight, the ash-free dry weight may be determined as described in (49) in order to account for inorganic components originating from ingested sediment present in the alimentary tract of the worms.

52. 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.

Verification of test chemical concentrations

Sampling

53. Samples for chemical analysis of the test chemical 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 bulk sediment and the overlying water should be sampled for analysis. At least two samples should be taken per matrix and treatment on each sampling date. One of the duplicate samples may be stored as a reserve (to be analysed e.g. in the event that initial analysis falls outside the ± 20 % range from the nominal concentration). In case of specific chemical properties, e.g. if rapid degradation of the test chemical is expected, the analytical schedule may be refined (e.g. more frequent sampling, analysis of more concentration levels) on the basis of expert judgment. Samples may then be taken on intermediate sampling dates (e.g. on day seven after start of exposure).

54. 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.

55. 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.

56. If analysis of the test chemical 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 without filtration to isolate the porewater, e.g. at 10000 ± 600 x *g* for 30 - 60 min at a temperature not exceeding the temperature used in the test. After centrifugation, the supernatant is decanted or pipetted taking care that no sediment particles are introduced, and the volume is recorded. The weight of the remaining sediment pellet is recorded. It may facilitate the estimation of the mass balance or recovery of the test chemical 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.

57. Failing immediate analysis, all samples should be stored by an appropriate method, e.g. under the storage conditions recommended for minimum degradation of the particular test chemical (e.g., environmental samples are commonly stored at -18°C in the dark). Obtain information on the proper storage conditions for the particular test chemical - for example, duration and temperature of storage, extraction procedures, etc. - before beginning the study.

Analytical method

58. Since the whole procedure is governed essentially by the accuracy, precision and sensitivity of the analytical method used for the test chemical, check experimentally that the precision and reproducibility of the chemical analysis, as well as the recovery of the test chemical from water and sediment samples are satisfactory for the particular method at least at the lowest and highest test concentrations. Also, check that the test chemical is not detectable in the control chambers in concentrations higher than the limit of quantification. If necessary, correct the nominal concentrations for the recoveries of quality control spikes (e.g. where recovery is outside 80 - 120% of spiked amount). 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 chemical on the sampling device).

59. The recovery of test chemical, the limit of quantification, and the limit of detection in sediment and water should be recorded and reported.

DATA AND REPORTING

Treatment of results

60. The main mandatory response variables of the test to be evaluated statistically are the biomass and the total number of worms per replicate. Optionally, reproduction (as increase of worm numbers) and growth (as increase of dry biomass) could be also evaluated. 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 sub-sample of the batch of synchronised worms to be used for the test.

61. Although mortality is not an endpoint of this test, 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, and the missing

worms should be considered dead. Mortalities should at least be recorded and considered when interpreting the test results.

62. Effect concentrations should be expressed in mg/kg sediment dry weight. If the recovery of test chemical measured in the sediment, or in sediment and overlying water at start of exposure, is between 80 and 120% 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 chemical in the test system (see paragraph 30). In these cases, additional information can be obtained from analysis of stock and/or application solutions in order to confirm that the test sediments were prepared correctly.

EC_x

63. EC_x-values for the parameters described in paragraph 60 are calculated using appropriate statistical methods (e.g. probit analysis, logistic or Weibull function, trimmed Spearman-Karber method, or simple interpolation). Guidance on statistical evaluation is given in (15) and (50). 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 ($\bar{X}$) 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. Guidance on selection of appropriate statistical methods is given in (15) and (50).

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 Appendix 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.

Interpretation of results

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

Test report

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

Test chemical:

- chemical identification data (common name, chemical name, structural formula, CAS number, etc.) including purity and analytical method for quantification of test chemical; source of the test chemical, 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 per vessel, sediment mass and volume per vessel, (for flow-through or semi-static procedures: water volume replacement rate), 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);
- depth of sediment and overlying water;
- method of test chemical 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 chemical were obtained;
- sediment characteristics as described in paragraphs 24 - 25, 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) before the start of the test;
- detailed information on feeding including type of food, preparation, amount and feeding regimen;
- light intensity and photoperiod(s);
- 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 chemical, and recoveries of the test chemical.

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 if determined, 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, and if measured, dry weight of a sub-sample 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 chemical in the test vessels;
- any deviations from the validity criteria.

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 test method.

LITERATURE

(1) EC (2003). Technical Guidance Document in support of Commission Directive 93/67/EEC on Risk Assessment for new notified substances, Commission Regulation (EC) No 1488/94 on Risk Assessment for existing substances and Directive 98/8/EC of the European Parliament and of the Council concerning the placing of biocidal products on the market; Part I - IV. Office for Official Publications of the EC (European Commission), Luxembourg.

(2) OECD (1992a). Report of the OECD workshop on effects assessment of chemicals in sediment. OECD Monographs No. 60. Organisation for Economic Co-operation and Development (OECD), Paris.

(3) ASTM International (2000). Standard guide for the determination of the bioaccumulation of sediment-associated contaminants by benthic invertebrates, E 1688-00a. In ASTM International 2004 Annual Book of Standards. Volume 11.05. Biological Effects and Environmental Fate; Biotechnology; Pesticides. ASTM International, West Conshohocken, PA.

(4) ASTM International (2002). Standard Test Method for Measuring the Toxicity of Sediment-Associated Contaminants with Freshwater Invertebrates, E1706-00. In ASTM International 2004 Annual Book of Standards. Volume 11.05. Biological Effects and Environmental Fate; Biotechnology; Pesticides. ASTM International, West Conshohocken, PA.

(5) Phipps, G.L., Ankley, G.T., Benoit, D.A. and Mattson, V.R. (1993). Use of the aquatic Oligochaete *Lumbriculus variegatus* for assessing the toxicity and bioaccumulation of sediment-associated contaminants. Environ.Toxicol. Chem. 12, 269-279.

(6) Chapter C.27 of this Annex, "Sediment-water chironomid toxicity test using spiked sediment".

(7) U.S. EPA (2000). Methods for measuring the toxicity and bioaccumulation of sediment-associated contaminants with freshwater invertebrates. Second Edition. EPA 600/R-99/064, U.S. Environmental Protection Agency, Duluth, MN, March 2000.

(8) Environment Canada (1997). Test for Growth and Survival in Sediment using Larvae of Freshwater Midges (*Chironomus tentans* or *Chironomus riparius*). Biological Test Method. Report SPE 1/RM/32. December 1997.

(9) Hill, I.R., Matthiessen, P., Heimbach, F. (eds), 1993, Guidance document on Sediment Toxicity Tests and Bioassays for freshwater and

Marine Environments, From the SETAC-Europe Workshop On Sediment Toxicity Assessment, 8-10 November 1993, Renesse (NL).

(10) BBA (1995). Long-term toxicity test with *Chironomus riparius*: Development and validation of a new test system. Edited by M. Streloke and H.Köpp. Berlin 1995.

(11) Riedhammer C. & B. Schwarz-Schulz (2001). The Newly Proposed EU Risk Assessment Concept for the Sediment Compartment. J. Soils Sediments 1(2), 105-110.

(12) ASTM International (2004). Standard guide for collection, storage, characterisation, and manipulation of sediment for toxicological testing and for selection of samplers used to collect benthic invertebrates. American Society for Testing and Materials, E 1391-03.

(13) Egeler, Ph., Meller, M., Schallnaß, H.J. & Gilberg, D. (2005). Validation of a sediment toxicity test with the endobenthic aquatic oligochaete *Lumbriculus variegatus* by an international ring test. In co-operation with R. Nagel and B. Karaoglan. Report to the Federal Environmental Agency (Umweltbundesamt Berlin), R&D No.: 202 67 429.

(14) OECD (2000). Guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixtures. OECD Environment, Health and Safety Publications, Series on Testing and Assessment No. 23.

(15) Environment Canada (2003). Guidance Document on Statistical Methods for Environmental Toxicity Tests; fifth draft, March 2003; Report EPS 1/RM/___

(16) Nikkilä A., Halme A., Kukkonen J.V.K. (2003). Toxicokinetics, toxicity and lethal body residues of two chlorophenols in the oligochaete worm, *Lumbriculus variegatus*, in different sediments. Chemosphere 51: 35-46.

(17) Baily H.C., & Liu D.H.W. (1980). *Lumbriculus variegatus*, a Benthic Oligochaete, as a Bioassay Organism. p. 205-215. In J.C. Eaton, P.R. Parrish, and A.C. Hendricks (eds). Aquatic Toxicology, ASTM STP 707. American Society for Testing and Materials.

(18) Chapman K. K., Benton M. J., Brinkhurst R. O. & Scheuerman P. R. (1999). Use of the aquatic oligochaetes *Lumbriculus variegatus* and *Tubifex tubifex* for assessing the toxicity of copper and cadmium in a spiked-artificial-sediment toxicity test. Environmental Toxicology. 14(2): 271-278.

(19) Meyer J.S., Boese C.J. & Collyard S.A. (2002). Whole-body accumulation of copper predicts acute toxicity to an aquatic oligochaete (*Lumbriculus variegatus*) as pH and calcium are varied. Comp. Biochem. Physiol. Part C 133:99-109.

(20) Schubauer-Berigan M.K., Dierkes J.R., Monson P.D. & Ankley G.T. (1993). pH-dependent toxicity of cadmium, copper, nickel, lead and zinc to *Ceriodaphnia dubia*, *Pimephales promelas*, *Hyalella azteca* and *Lumbriculus variegatus*. Environ. Toxciol. Chem. 12(7):1261-1266.

(21) West, C.W., V.R. Mattson, E.N. Leonard, G.L. Phipps & G.T. Ankley (1993). Comparison of the relative sensitivity of three benthic invertebrates to copper-contaminated sediments from the Keweenaw Waterway. Hydrobiol. 262:57-63.

(22) Ingersoll, C.G., Ankley, G.T., Benoit D.A., Brunson, E.L., Burton, G.A., Dwyer, F.J., Hoke, R.A., Landrum, P. F., Norberg-King, T. J. and Winger, P.V. (1995). Toxicity and bioaccumulation of sediment-associated contaminants using freshwater invertebrates: A review of methods and applications. Environ. Toxicol. Chem. 14, 1885-1894.

(23) Kukkonen, J. and Landrum, P.F. (1994). Toxicokinetics and toxicity of sediment-associated Pyrene to *Lumbriculus variegatus* (Oligochaeta). Environ. Toxicol. Chem. 13, 1457-1468.

(24) Leppänen, M.T. & Kukkonen, J.V.K. (1998a). Relationship between reproduction, sediment type and feeding activity of *Lumbriculus variegatus* (Müller): Implications for sediment toxicity testing. Environ. Toxicol. Chem. 17: 2196-2202.

(25) Leppänen, M.T. & Kukkonen, J.V.K. (1998b). Factors affecting feeding rate, reproduction and growth of an oligochaete *Lumbriculus variegatus* (Müller). Hydrobiologia 377: 183-194.

(26) Landrum, P.F., Gedeon, M.L., Burton, G.A., Greenberg. M.S., & Rowland, C.D. (2002). Biological Responses of *Lumbriculus variegatus* Exposed to Fluoranthene-Spiked Sediment. Arch. Environ. Contam. Toxicol. 42: 292-302.

(27) Brunson, E.L., Canfield, T.J., Ingersoll, C.J. & Kemble, N.E. (1998). Assessing the bioaccumulation of contaminants from sediments of the Upper Mississippi river using field-collected oligochaetes and laboratory-exposed *Lumbriculus variegatus*. Arch. Environ. Contam. Toxicol. 35, 191-201.

(28) Ingersoll, C.G., Brunson, E.L., Wang N., Dwyer, F.J., Ankley, G.T., Mount D.R., Huckins J., Petty. J. and Landrum, P. F. (2003). Uptake and depuration of non-ionic organic contaminants from sediment by the oligochaete, *Lumbriculus variegatus*. Environmental Toxicology and Chemistry 22, 872-885.

(29) Rodriguez, P. & Reynoldson, T.B. (1999). Laboratory methods and criteria for sediment bioassessment. In: A. Mudroch, J.M. Azcue & P. Mudroch (eds.): Manual of Bioassessment of aquatic sediment quality. Lewis Publishers, Boca Raton, CRC Press LLC.

(30) Liebig, M., Egeler, Ph. Oehlmann, J., & Knacker, Th. (2005). Bioaccumulation of ^{14}C-17α-ethinylestradiol by the oligochaete *Lumbriculus variegatus* in artificial sediment. Chemosphere 59, 271-280.

(31) Brust, K., O. Licht, V. Hultsch, D. Jungmann & R. Nagel (2001). Effects of Terbutryn on Aufwuchs and *Lumbriculus variegatus* in Artificial Indoor Streams. Environ. Toxicol. Chemistry, Vol. 20, pp. 2000–2007.

(32) Oetken, M., K.-U. Ludwichowski & R. Nagel (2000). Sediment tests with *Lumbriculus variegatus* and *Chironomus riparius* and 3,4-dichloroaniline (3,4-DCA) within the scope of EG-AltstoffV. By order of the Federal Environmental Agency (Umweltbundesamt Berlin), FKZ 360 12 001, March 2000.

(33) Leppänen M.T. & Kukkonen J.V.K. (1998). Relative importance of ingested sediment and porewater as bioaccumulation routes for pyrene to oligochaete (*Lumbriculus variegatus*, Müller). Environ. Sci. Toxicol. 32, 1503-1508.

(34) Dermott R. & Munawar M. (1992). A simple and sensitive assay for evaluation of sediment toxicity using *Lumbriculus variegatus* (Müller). Hydrobiologia 235/236: 407-414.

(35) Drewes C.D. & Fourtner C.R. (1990). Morphallaxis in an aquatic oligochaete, *Lumbriculus variegatus*: Reorganisation of escape reflexes in regenerating body fragments. Develop. Biol. 138: 94-103.

(36) Brinkhurst, R.O. (1971). A guide for the identification of British aquatic oligochaeta. Freshw. Biol. Assoc., Sci. Publ. No. 22.

(37) Chapter C.1 of this Annex, Fish, Acute Toxicity Test.

(38) OECD (1992c). Guidelines for Testing of Chemicals No. 210. Fish, Early-life Stage Toxicity Test. OECD, Paris.

(39) Egeler, Ph., Römbke, J., Meller, M., Knacker, Th., Franke, C., Studinger, G. & Nagel, R. (1997). Bioaccumulation of lindane and hexachlorobenzene by tubificid sludgeworms (Oligochaeta) under standardised laboratory conditions. Chemosphere 35, 835-852.

(40) Meller, M., P. Egeler, J. Roembke, H. Schallnass, R. Nagel and B. Streit. (1998). Short-term Toxicity of Lindane, Hexachlorobenzene and Copper Sulphate on Tubificid Sludgeworms (Oligochaeta) in Artificial Media. Ecotox. and Environ. Safety, 39, 10-20.

(41) Egeler, Ph., Römbke, J., Knacker, Th., Franke, C. & Studinger, G. (1999). Workshop on "Bioaccumulation: Sediment test using benthic oligochaetes", 26.-27.04.1999, Hochheim/Main, Germany. Report on the R+D-project No. 298 67 419, Umweltbundesamt, Berlin.

(42) Suedel, B.C. and Rodgers, J.H. (1993). Development of formulated reference sediments for freshwater and estuarine sediment testing. Environ. Toxicol. Chem. 13, 1163-1175.

(43) Naylor, C. and C. Rodrigues. (1995). Development of a test method for *Chironomus riparius* using a formulated sediment. Chemosphere 31: 3291-3303.

(44) Kaster, J.L., Klump, J.V., Meyer, J., Krezoski, J. & Smith, M.E. (1984). Comparison of defecation rates of *Limnodrilus hoffmeisteri* using two different methods. Hydrobiologia 11, 181-184.

(45) Martinez-Madrid, M., Rodriguez, P., Perez-Iglesias, J.I. & Navarro, E. (1999). Sediment toxicity bioassays for assessment of contaminated sites in the Nervion river (Northern Spain). 2. *Tubifex tubifex* (Müller) reproduction sediment bioassay. Ecotoxicology 8, 111-124.

(46) Environment Canada (1995). Guidance document on measurement of toxicity test precision using control sediments spiked with a reference toxicant. Environmental Protection Series Report EPS 1/RM/30.

(47) Landrum, P.F. (1989). Bioavailability and toxicokinetics of polycyclic aromatic hydrocarbons sorbed to sediments for the amphipod *Pontoporeia hoyi*. Environ. Sci. Technol. 23, 588-595.

(48) Brooke, L.T., Ankley, G.T., Call, D.J. & Cook, P.M. (1996). Gut content and clearance for three species of freshwater invertebrates. Environ. Toxicol. Chem. 15, 223-228.

(49) Mount, D.R., Dawson, T.D. & Burkhard, L.P. (1999). Implications of gut purging for tissue residues determined in bioaccumulation testing of sediment with *Lumbriculus variegatus*. Environ. Toxicol. Chem. 18, 1244-1249.

(50) OECD 2006. Current approaches in the statistical analysis of ecotoxicity data: A guidance to application. OECD Series on Testing and Assessment No. 54, OECD, Paris, France.

(51) Liebig M., Meller M. & Egeler P. (2004). Sedimenttoxizitätstests mit aquatischen Oligochaeten - Einfluss verschiedener Futterquellen im künstlichen Sediment auf Reproduktion und Biomasse von *Lumbriculus variegatus*. Proceedings 5/2004: Statusseminar Sedimentkontakttests. March 24-25, 2004. BfG (Bundesanstalt für Gewässerkunde), Koblenz, Germany. pp. 107-119.

Additional literature on statistical procedures:

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

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

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

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

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

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

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

Miller, R.G., Jr. (1986). Beyond ANOVA, basics of applied statistics. John Wiley & Sons. New York.

Shapiro S.S. & Wilk M.B (1965). An analysis of variance test for normality (complete samples). Biometrika 52: 591-611.

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

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

Appendix 1

DEFINITIONS

For the purpose of this test method the following definitions are used:

A **chemical** means a substance or a mixture.

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 chemical. Usually, the overlying water is discarded after conditioning.

The $\mathbf{EC_x}$ is the concentration of the test chemical in the sediment that results in X% (e.g. 50%) effect on a biological parameter within a stated exposure period.

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

The **exposure phase** is the time during which the test organisms are exposed to the test chemical.

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

The **Lowest Observed Effect Concentration (LOEC)** is the lowest tested concentration of a test chemical at which the chemical 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.

The **octanol-water partitioning coefficient** (K_{ow}; also sometimes expressed as P_{ow}) is the ratio of the solubility of a chemical in n-octanol and water at equilibrium and represents the lipophilicity of a chemical (Chapter A.24 of this Annex). The K_{ow} or its logarithm of K_{ow} ($\log K_{ow}$) is used as an indication of the potential of a chemical for bioaccumulation by aquatic organisms.

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

Overlying water is the water covering the sediment in the test vessel.

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

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

Test chemical means any substance or mixture tested using this test method.

Appendix 2

COMPOSITION OF THE RECOMMENDED RECONSTITUTED WATER

(adopted from Chapter C.1 of this Annex (1))

(a) Calcium chloride solution

Dissolve 11.76 g $CaCl_2 \cdot 2H_2O$ in deionised water; make up to 1 l with deionised water

(b) Magnesium sulphate solution

Dissolve 4.93 g $MgSO_4 \cdot 7H_2O$ 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 these 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.

REFERENCE

(1) Chapter C.1 of this Annex, Fish Acute Toxicity Test.

Appendix 3

PHYSICAL-CHEMICAL CHARACTERISTICS OF AN ACCEPTABLE DILUTION WATER

Component	Concentrations
Particulate 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 OECD (1992) (1))

Reference

(1) OECD (1992). Guidelines for Testing of Chemicals No. 210. Fish, Early-life Stage Toxicity Test. OECD, Paris.

Appendix 4

RECOMMENDED ARTIFICIAL SEDIMENT - GUIDANCE ON PREPARATION AND STORAGE

Sediment constituents

Constituent	*Characteristics*	*% of sediment dry weight*
Peat	*Sphagnum moss peat, degree of decomposition: "medium", 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*
Food source	*e.g. 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*

NOTE: If elevated ammonia concentrations are expected, e.g. if the test chemical is known to inhibit 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; (1) (2)).

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 chemical, 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 chemical. 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 chemical, the food source should be mixed with the sediment not later than on the day the test chemical is spiked to the sediment.

Storage

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

REFERENCES

(1) Egeler, Ph., Meller, M., Schallnaß, H.J. & Gilberg, D. (2005). Validation of a sediment toxicity test with the endobenthic aquatic oligochaete *Lumbriculus variegatus* by an international ring test. In co-operation with R. Nagel and B. Karaoglan. Report to the Federal Environmental Agency (Umweltbundesamt Berlin), R&D No.: 202 67 429.

(2) Liebig M., Meller M. & Egeler P. (2004). Sedimenttoxizitätstests mit aquatischen Oligochaeten - Einfluss verschiedener Futterquellen im künstlichen Sediment auf Reproduktion und Biomasse von *Lumbriculus variegatus*. Proceedings 5/2004: Statusseminar Sedimentkontakttests. March 24-25, 2004. BfG (Bundesanstalt für Gewässerkunde), Koblenz, Germany. pp. 107-119.

Appendix 5

CULTURE METHODS FOR LUMBRICULUS VARIEGATUS

Lumbriculus variegatus (MÜLLER), 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) (1), Brunson et al. (1998) (2), ASTM (2000) (3), U.S. EPA (2000) (4). 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. (1), (3), (4), (5)).

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 de-ionized water.

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 (4), or in the following.

An issue to be regarded critically when using *L. variegatus* in sediment tests is its reproduction mode (architomy or morphallaxis, e.g. (6)). 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., (7), (8)). 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 chemical than other individuals, which do not fragment during the test (9), (10), (11). 10 to 14 days before the start of exposure, the worms should be artificially fragmented (synchronisation). Large (adult) worms, which preferably do not show signs of recent morphallaxis should be selected for synchronisation. 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, 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 (4))

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

REFERENCES

(1) Phipps, G.L., Ankley, G.T., Benoit, D.A. and Mattson, V.R. (1993). Use of the aquatic Oligochaete *Lumbriculus variegatus* for assessing the toxicity and bioaccumulation of sediment-associated contaminants. Environ.Toxicol. Chem. 12, 269-279.

(2) Brunson, E.L., Canfield, T.J., Ingersoll, C.J. & Kemble, N.E. (1998). Assessing the bioaccumulation of contaminants from sediments of the Upper Mississippi river using field-collected oligochaetes and laboratory-exposed Lumbriculus variegatus. Arch. Environ. Contam. Toxicol. 35, 191-201.

(3) ASTM International (2000). Standard guide for the determination of the bioaccumulation of sediment-associated contaminants by benthic invertebrates, E 1688-00a. In ASTM International 2004 Annual Book of Standards. Volume 11.05. Biological Effects and Environmental Fate; Biotechnology; Pesticides. ASTM International, West Conshohocken, PA.

(4) U.S. EPA (2000). Methods for measuring the toxicity and bioaccumulation of sediment-associated contaminants with freshwater invertebrates. Second Edition. EPA 600/R-99/064, U.S. Environmental Protection Agency, Duluth, MN, March 2000.

(5) Kukkonen, J. and Landrum, P.F. (1994). Toxicokinetics and toxicity of sediment-associated Pyrene to Lumbriculus variegatus (Oligochaeta). Environ. Toxicol. Chem. 13, 1457-1468.

(6) Drewes C.D. & Fourtner C.R. (1990). Morphallaxis in an aquatic oligochaete, Lumbriculus variegatus: Reorganisation of escape reflexes in regenerating body fragments. Develop. Biol. 138: 94-103.

(7) Leppänen, M.T. & Kukkonen, J.V.K. (1998a). Relationship between reproduction, sediment type and feeding activity of Lumbriculus variegatus (Müller): Implications for sediment toxicity testing. Environ. Toxicol. Chem. 17: 2196-2202.

(8) Leppänen, M.T. & Kukkonen, J.V.K. (1998b). Factors affecting feeding rate, reproduction and growth of an oligochaete Lumbriculus variegatus (Müller). Hydrobiologia 377: 183-194.

(9) Brust, K., O. Licht, V. Hultsch, D. Jungmann & R. Nagel (2001). Effects of Terbutryn on Aufwuchs and Lumbriculus variegatus in Artificial Indoor Streams. Environ. Toxicol. Chemistry, Vol. 20, pp. 2000–2007.

(10) Oetken, M., K.-U. Ludwichowski & R. Nagel (2000). Sediment tests with Lumbriculus variegatus and Chironomus riparius and 3,4-dichloroaniline (3,4-DCA) within the scope of EG-AltstoffV. By order

of the Federal Environmental Agency (Umweltbundesamt Berlin), FKZ 360 12 001, March 2000.

(11) Leppänen M.T. & Kukkonen J.V.K. (1998). Relative importance of ingested sediment and porewater as bioaccumulation routes for pyrene to oligochaete (Lumbriculus variegatus, Müller). Environ. Sci. Toxicol. 32, 1503-1508.

Appendix 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 EC50, NOEC and LOEC; SD = standard deviation; CV = coefficient of variation.

biological parameter		**Inter-laboratory mean (mg/kg)**	**min**	**max**	**Inter-laboratory factor**	**SD**	**CV (%)**	**geometr. mean (mg/kg)**
total number	**EC_{50}**	**23.0**	4.0	37.9	9.4	10.7	46.3	19.9

of worms	**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

REFERENCE

Egeler, Ph., Meller, M., Schallnaß, H.J. & Gilberg, D. (2005). Validation of a sediment toxicity test with the endobenthic aquatic oligochaete *Lumbriculus variegatus* by an international ring test. In co-operation with R. Nagel and B. Karaoglan. Report to the Federal Environmental Agency (Umweltbundesamt Berlin), R&D No.: 202 67 429.

C.36 Predatory mite (*Hypoaspis (Geolaelaps) aculeifer*) reproduction test in soil

INTRODUCTION

1. This test method is equivalent to OECD test guideline (TG) 226 (2008). This test method is designed to be used for assessing the effects of chemicals in soil on the reproductive output of the soil mite species *Hypoaspis (Geolaelaps) aculeifer* Canestrini (Acari: Laelapidae), hence allowing for the estimation of the inhibition of the specific population growth rate (1,2). Reproductive output here means the number of juveniles at the end of the testing period. *H. aculeifer* represents an additional trophic level to the species for which test methods are already available. A reproduction test without discrimination and quantification of the different stages of the reproductive cycle is considered adequate for the purpose of this test method. For chemicalsubstances with another exposure scenario than via the soil other approaches might be appropriate (3).

2. *Hypoaspis (Geolaelaps) aculeifer* is considered to be a relevant representative of soil fauna and predatory mites in particular. It is worldwide distributed (5) and can easily be collected and reared in the laboratory. A summary on the biology of *H. aculeifer* is provided in Appendix 7. Background information on the ecology of the mite species and the use in ecotoxicological testing is available (4), (5), (6), (7), (8), (9), (10), (11), (12).

PRINCIPLE OF THE TEST

3. Adult females are exposed to a range of concentrations of the test chemical mixed into the soil. The test is started with 10 adult females per replicate vessel. Males are not introduced in the test, because experience has shown that females mate immediately or shortly after hatching from the deutonymph stage, if males are present. In addition, inclusion of males would prolong the test in a way that the demanding discrimination of age stages would become necessary. Thus, mating itself is not part of the test. The females are introduced into the test 28-35 days after the start of the egg laying period in the synchronisation (see Appendix 4), as the females can then be considered as already mated and having passed the pre-oviposition stage. At 20°C the test ends at day 14 after introducing the females (day 0), which allows the first control offspring to reach the deutonymph stage (see Appendix 4). For the main measured variable, the number of juveniles per test vessels and additionally the number of surviving females are determined. The reproductive output of the mites exposed to the test chemical is compared to that of the controls in order to determine the ECx (e.g. EC10, EC50) or the no observed effect concentration (NOEC) (see Appendix 1 for definitions), depending on the experimental design (see paragraph 29). An overview of the test schedule is given in Appendix 8.

INFORMATION ON THE TEST CHEMICAL

4. The water solubility, the log K_{ow}, the soil water partition coefficient and the vapour

pressure of the test chemical should preferably be known. Additional information on the fate of the test chemical in soil, such as the rates of biotic and abiotic degradation, is desirable.

5. This test method can be used for water soluble or insoluble chemicals. However, the mode of application of the test chemical will differ accordingly. The test method is not applicable to volatile chemicals, i.e. chemicals for which the Henry's constant or the air/water partition coefficient is greater than one, or chemicals for which the vapour pressure exceeds 0.0133 Pa at 25°C.

VALIDITY OF THE TEST

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

- Mean adult female mortality should not exceed 20% at the end of the test;
- The mean number of juveniles per replicate (with 10 adult females introduced) should be at least 50 at the end of the test;
- The coefficient of variation calculated for the number of juvenile mites per replicate should not be higher than 30% at the end of the definitive test.

REFERENCE CHEMICAL

7. The ECx and/or NOEC of a reference chemical must be determined to provide assurance that the laboratory test conditions are adequate and to verify that the response of the test organisms did not change over time. Dimethoate (CAS 60-51-5) is a suitable reference chemical that has shown to affect population size (4). Boric acid (CAS 10043-35-3) may be used as an alternative reference chemical. Less experience has been gained with this chemical. Two design options are possible:

- The reference chemical can be tested in parallel to the determination of the toxicity of each test chemical at one concentration, which has to be demonstrated beforehand in a dose response study to result in an effect of > 50% reduction of offspring. In this case, the number of replicates should be the same as that in the controls (see paragraph 29).
- Alternatively, the reference chemical is tested 1 – 2 times a year in a dose-response test. Depending on the design chosen, the number of concentrations and replicates and the spacing factor differ (see paragraph 29), but a response of 10 - 90 % effect should be achieved (spacing factor of 1.8). The EC_{50} for dimethoate based on the number of juveniles should fall in the range between 3.0 and 7.0 mg a.s./kg soil (dw). Based on the results obtained with boric acid so far, the EC50 based on the number of juveniles should fall in the range between 100 and 500 mg/kg dw soil.

DESCRIPTION OF THE TEST

Test vessels and equipment

8. Test vessels of 3 - 5 cm diameter (height of soil ≥1.5 cm), made of glass or other chemically inert material and having a close fitting cover, should be used. Screw lids

are preferred and in that case, the vessels could be aerated twice a week. Alternatively, covers that permit direct gaseous exchange between the substrate and the atmosphere (e.g. gauze) can be used. Since moisture content must be kept high enough during the test, it is essential to control the weight of each experimental vessel during the test and replenish water if necessary. This may be especially important if no screw lids are available. If a non-transparent test vessel is used, the cover should be made of material that allows for access to light (e.g. by means of a perforated transparent cover) whilst preventing the mites from escaping. The size and type of the test vessel depends on the extraction method (see Appendix 5 for details). If heat extraction is applied directly to the test vessel, then a bottom mesh of appropriate mesh size could be added (sealed until extraction), and soil depth should be sufficient to allow for a temperature and moisture gradient.

9. Standard laboratory equipment is required, specifically the following:

- preferably glass vessels with screw lids;
- drying cabinet;
- stereomicroscope;
- brushes for transferring mites
- pH-meter and luxmeter;
- suitable accurate balances;
- adequate equipment for temperature control;
- adequate equipment for air humidity control (not essential if exposure vessels are covered by lids);
- temperature-controlled incubator or small room;
- equipment for extraction (see Appendix 5) (13)
- overhead light panel with light control
- collection jars for extracted mites.

Preparation of the artificial soil

10. For this test, an artificial soil is used. The artificial soil consists of the following components (all values based on dry mass):

- 5% sphagnum peat, air-dried and finely ground (a particle size of 2 ±1 mm is acceptable);
- 20% kaolin clay (kaolinite content preferably above 30%);
- approximately 74% air-dried industrial sand (depending on the amount of $CaCO_3$ needed), predominantly fine sand with more than 50% of the particles between 50 and 200 microns. The exact amount of sand depends on the amount of $CaCO_3$ (see below), together they should add up to 75 %.
- < 1.0% calcium carbonate ($CaCO_3$, pulverised, analytical grade) to obtain a pH of 6.0 ± 0.5; the amount of calcium carbonate to be added may depend principally on the quality/nature of the peat (see Note 1).

Note 1: The amount of $CaCO_3$ required will depend on the components of the soil substrate and should be determined by measuring the pH of soil sub-samples immediately before the test (14).

Note 2: The peat content of the artificial soil deviates from other test methods on soil organisms, where in most cases 10% peat is used (e.g. (15)). However, according to

EPPO (16) a typical agricultural soil has not more than 5% organic matter, and the reduction in peat content thus reflects the decreased possibilities of a natural soil for sorption of the test chemical to organic carbon.

Note 3: If required, e.g. for specific testing purposes, natural soils from unpolluted sites may also serve as test and/or culture substrate. However, if natural soil is used, it should be characterised at least by origin (collection site), pH, texture (particle size distribution) and organic matter content. If available, the type and name of the soil according to soil classification should be included, and the soil should be free from any contamination. In case the test chemical is a metal or organo-metal, the cation exchange capacity (CEC) of the natural soil should also be determined. Special attention should be paid to meet the validity criteria as background information on natural soils typically is rare.

11. The dry constituents of the soil are mixed thoroughly (e.g. in a large-scale laboratory mixer). For the determination of pH a mixture of soil and 1 M potassium chloride (KCl) or 0.01 M calcium chloride ($CaCl_2$) solution in a 1:5 ratio is used (see (14) and Appendix 3). If the soil is more acidic than the required range (see paragraph 10), it can be adjusted by addition of an appropriate amount of $CaCO_3$. If the soil is too alkaline it can be adjusted by the addition of more of the mixture comprising the first three components described in paragraph 10, but excluding the $CaCO_3$.

12. The maximum water holding capacity (WHC) of the artificial soil is determined in accordance with procedures described in Appendix 2. Two to seven days before starting the test, the dry artificial soil is pre-moistened by adding enough distilled or de-ionised water to obtain approximately half of the final water content, that being 40 to 60% of the maximum WHC. The moisture content is adjusted to 40-60 % of the maximum WHC by the addition of the test chemical solution and/or by adding distilled or de-ionised water (see paragraphs 16-18). An additional rough check of the soil moisture content should be obtained by gently squeezing the soil in the hand, if the moisture content is correct small drops of water should appear between the fingers.

13. Soil moisture content is determined at the beginning and at the end of the test by drying to constant weight at 105°C in accordance with ISO 11465 (17) and soil pH in accordance with Appendix 3 or ISO 10390 (14) . These measurements should be carried out in additional samples without mites, both from the control soil and from each test concentration soil. The soil pH should not be adjusted when acidic or basic chemicals are tested. The moisture content should be monitored throughout the test by weighing the vessels periodically (see paragraphs 20 and 24).

Selection and preparation of test animals

14. The species used in the test is *Hypoaspis (Geolaelaps) aculeifer* (Canestrini, 1883). Adult female mites, obtained from a synchronised cohort are required to start the test. Mites should be introduced ca. 7-14 days after becoming adult, 28 – 35 days after the start of the egg laying in the synchronisation (see paragraph 3 and Appendix 4). The source of the mites or the supplier and maintenance of the laboratory culture should be recorded. If a laboratory culture is kept, it is recommended that the identity of the species is confirmed at least once a year. An identification sheet is included as Appendix 6.

Preparation of test concentrations

15. The test chemical is mixed into the soil. Organic solvents used to aid treatment of the soil with the test chemical should be selected on the basis of their low toxicity to mites and appropriate solvent control must be included in the test design (see paragraph 29).

Test chemical soluble in water

16. A solution of the test chemical is prepared in deionised water in a quantity sufficient for all replicates of one test concentration. It is recommended to use an appropriate quantity of water to reach the required moisture content, i.e. 40 to 60% of the maximum WHC (see paragraph 12). Each solution of test chemical is mixed thoroughly with one batch of pre-moistened soil before being introduced into the test vessel.

Test chemical insoluble in water

17. For chemicals insoluble in water but soluble in organic solvents, the test chemical can be dissolved in the smallest possible volume of a suitable vehicle (e.g. acetone). Only volatile solvents should be used. When such vehicles are used, all test concentrations and the control should contain the same minimum amount of the vehicle. The vehicle is sprayed on or mixed with a small amount, for example 10 g, of fine quartz sand. The total sand content of the substrate should be corrected for this amount. The vehicle is eliminated by evaporation under a fume hood for at least one hour. This mixture of quartz sand and test chemical is added to the pre-moistened soil and thoroughly mixed by adding an appropriate amount of de-ionised water to obtain the moisture required. The final mixture is introduced into the test vessels. Note that some solvents may be toxic to mites. It is therefore recommended to use an additional water control without vehicle if the toxicity of the solvent to mites is not known. If it is adequately demonstrated that the solvent (in the concentrations to be applied) has no effects, the water control may be excluded.

Test chemical poorly soluble in water and organic solvents

18. For chemicals that are poorly soluble in water and organic solvents, the equivalent of 2.5 g of finely ground quartz sand per test vessel (for example 10 g of fine quartz sand for four replicates) is mixed with the quantity of test chemical to obtain the desired test concentration. The total sand content of the substrate should be corrected for this amount. This mixture of quartz sand and test chemical is added to the pre-moistened soil and thoroughly mixed after adding an appropriate amount of deionised water to obtain the required moisture content. The final mixture is divided between the test vessels. The procedure is repeated for each test concentration and an appropriate control is also prepared.

PROCEDURE

Test groups and controls

19. Ten adult females in 20 g dry mass of artificial soil are recommended for each control

and treatment vessel. Test organisms should be added within two hours after preparation of the final test substrate (i.e. after application of the test item). In specific cases (e.g. when ageing is considered to be a determining factor), the time between preparation of the final test substrate and the addition of the mites can be prolonged (for details of such ageing, see (18)). However, in such cases a scientific justification must be provided.

20. After the addition of the mites to the soil, the mites are provided with food and the initial weight of each test vessel should be measured to be used as reference for monitoring soil moisture content throughout the test as described in paragraph 24. The test vessels are then covered as described in paragraph 8 and placed in the test chamber.

21. Appropriate controls are prepared for each of the methods of test chemical application described in paragraphs 15 to 18. The relevant procedures described are followed for preparing the controls except that the test chemical is not added. Thus, where appropriate, organic solvents, quartz sand or other vehicles are applied to the controls in concentrations/amounts like in the treatments. Where a solvent or other vehicle is used to add the test chemical, an additional control without the vehicle or test chemical should also be prepared and tested in case the toxicity of the solvent is not known (see paragraph 17).

Test conditions

22. The test temperature should be 20 ± 2°C. Temperature should be recorded at least daily and adjusted, if necessary. The test is carried out under controlled light-dark cycles (preferably 16 hours light and 8 hours dark) with illumination of 400 to 800 lux in the vicinity of the test vessels. For reasons of comparability, these conditions are the same as in other soil ecotoxicological tests (e.g. (15)).

23. Gaseous exchange should be guaranteed by aerating the test vessels at least twice a week in case screw lids are used. If gauze covers are used, special attention should be paid to the maintenance of the soil moisture content (see paragraphs 8 and 24).

24. The water content of the soil substrate in the test vessels is maintained throughout the test by weighing and if needed re-watering the test vessels periodically (e.g. once per week). Losses are replenished as necessary with de-ionised water. The moisture content during the test should not differ by more than 10% from the start value.

Feeding

25. Cheese mites (*Tyrophagus putrescentiae* (Schrank, 1781)) have been shown to be a suitable food source. Small collembolans (e.g. juvenile *Folsomia candida* Willem, 1902 or Onychiurus *fimatus* (19), (20), enchytraeids (e.g. *Enchytraeus crypticus* Westheide & Graefe, 1992) or nematodes (e.g. *Turbatrix silusiae* de Man, 1913)) may be also suitable (21). It is recommended to check the food before using it in a test. The type and amount of food should secure an adequate number of juveniles in order to fulfil the validity criteria (paragraph 6). For the prey selection, the mode of action of the test item should be considered (e.g. an acaricide may be toxic to the food mites too,

see paragraph 26).

26. Food should be provided *ad libitum* (i.e. each time a small amount (tip of a spatula)). For this purpose, also low suction exhaustor as proposed in the collembolan test or a fine paint brush can also be used. Supplying food at the beginning of the test and two to three times a week will usually be sufficient. When the test item appears to be toxic to the prey, an increased feeding rate and/or an alternative food source should be considered.

Selection of test concentrations

27. Prior knowledge of the toxicity of the test chemical should help in selecting appropriate test concentrations, e.g. from range-finding studies. When necessary, a range-finding test is conducted with five concentrations of the test chemical in the range of 0.1 – 1000 mg/kg dry soil, with at least one replicate for treatments and control. The duration of the range finding test is 14 days, after which mortality of the adult mites and the number of juveniles is determined. The concentration range in the final test should preferably be chosen so that it includes concentrations at which juvenile numbers are affected while survival of the maternal generation is not. This, however, may not be possible for chemicals that cause lethal and sub-lethal effects at almost similar concentrations. The effect concentration (e.g. EC_{50} EC_{25}, EC_{10}) and the concentration range, over which the effect of the test chemical 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.

Experimental design

Dose response tests

28. Three test designs are proposed, based on the recommendations arising from another ring test (Enchytraeid reproduction test (22)). The general suitability of all these designs was confirmed by the outcome of *H. aculeifer* validation.

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

- For determination of the EC_x (e.g. EC_{10}, EC_{50}), twelve concentrations should be tested. At least two replicates for each test concentration and six control replicates are recommended. The spacing factor may vary, i.e. less than or equal to 1.8 in the expected effect range and above 1.8 at the higher and lower concentrations.
- For determination of the NOEC, at least five concentrations in a geometric series should be tested. Four replicates for each test concentration plus eight controls are recommended. The concentrations should be spaced by a factor not exceeding 2.0.
- A combined approach allows for determination of both the NOEC and ECx. Eight treatment concentrations in a geometric series should be used. Four replicates for each treatment plus eight controls are recommended. The concentrations should be spaced by a factor not exceeding 1.8.

Limit test

30. If no effects are observed at the highest concentration in the range-finding test (i.e. 1000 mg/kg dw soil), the definitive reproduction test can be performed as a limit test, using a test concentration of 1000 mg/kg dw soil. A limit test will provide the opportunity to demonstrate that the NOEC or the EC_{10} for reproduction is greater than the limit concentration, whilst minimising the number of mites used in the test. Eight replicates should be used for both the treated soil and the control.

Test duration and measurements

31. Any observed differences between the behaviour and the morphology of the mites in the control and the treated vessels should be recorded.

32. On day 14 the surviving mites are extracted from the soil via heat/light extraction or by another appropriate method (see Appendix 5). The numbers of juveniles (i.e. larvae, protonymphs and deutonymphs) and adults are counted separately. Any adult mites not found at this time are to be recorded as dead, assuming that such mites have died and decomposed prior to the assessment. Extraction efficiency must be validated once or twice a year in controls with known numbers of adults and juveniles. Efficiency should be above 90% on average combined for all developmental stages (see Appendix 5). Adult and juvenile counts are not adjusted for efficiency.

DATA AND REPORTING

Treatment of results

33. Information on the statistical methods that may be used for analysing the test results is given in paragraphs 36 to 41. In addition, OECD Document 54 on the "Current Approaches in the Statistical Analysis of Ecotoxicity Data: a Guidance to Application" (31) should be consulted.

34. Test main endpoint is the reproductive output, here the number of juveniles produced per replicate test vessel (with 10 adult females introduced). The statistical analysis requires the arithmetic mean (X) and the variance (s^2) for the reproductive output to be calculated per treatment and per control. X and s^2 are used for ANOVA procedures such as the Student t test, Dunnett test, or Williams' test as well as for the computation of 95% confidence intervals.

    Note: This main endpoint is equivalent with fecundity measured as the number of living juveniles produced during the test divided by the number of parental females introduced at the start of the test.

35. The number of surviving females in the untreated controls is a major validity criterion and has to be documented. As in the range-finding test, all other harmful signs should be recorded in the final report as well.

ECx

36. ECx-values including their associated lower and upper 95% confidence limits for the

parameter described in paragraph 34 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 EC50 or any other EC_x, the per treatment means (X) should be subjected to regression analysis.

NOEC/LOEC

37. 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 (according to OECD Document 54 on the Current Approaches in the Statistical Analysis of Ecotoxicity Data: A Guidance to Application). 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.

38. 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. Multiple comparisons (e.g. Dunnett's t-test) or step-down trend tests (e.g. Williams' test in case of a monotonous dose-response relationship) can be used to calculate whether there are significant differences ($p \leq 0.05$) between the controls and the various test item concentrations (selection of the recommended test according to OECD Document 54 on the Current Approaches in the Statistical Analysis of Ecotoxicity Data: a Guidance to Application). 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

39. 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 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.

40. 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.

Test report

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

Test chemical

- the identity of the test chemical, name, batch, lot and CAS-number, purity;
- physico-chemical properties of the test chemical (e.g. log K_{ow}, water solubility, vapour pressure, Henry's constant (H) and preferably information on the fate of the test chemical in soil).

Test organisms

- identification and supplier of the test organisms, description of the culturing conditions;
- age range of test organisms.

Test conditions

- description of the experimental design and procedure;
- preparation details for the test soil; detailed specification if natural soil is used (origin, history, particle size distribution, pH, organic matter content and if available the soil classification)
- the maximum water holding capacity of the soil;
- a description of the technique used to apply the test chemical to the soil;
- details of auxiliary chemicals used for administering the test chemical;
- size of test vessels and dry mass of test soil per vessel;
- test conditions: light intensity, duration of light-dark cycles, temperature;
- a description of the feeding regime, the type and amount of food used in the test, feeding dates;
- pH and water content of the soil at the start and during the test (control and each treatment)
- detailed description of the extraction method and extraction efficiency.

Test results

- the number of juveniles determined in each test vessel at the end of the test;
- number of adult females and adult mortality (%) in each test vessel at the end of the test
- a description of obvious symptoms or distinct changes in behaviour;
- the results obtained with the reference test chemical;
- summary statistics (ECx and/or NOEC) including 95%-confidence limits and a description of the method of calculation;
- a plot of the concentration-response-relationship;
- deviations from procedures described in this test method and any unusual occurrences during the test.

LITERATURE

(1) Casanueva, M.E. (1993). Phylogenetic studies of the free-living and arthropod associated Laelapidae (Acari: Mesostigmata). Gayana Zool. 57, 21-46.

(2) Tenorio, J. M. (1982). Hypoaspidinae (Acari: Gamasida: Laelapidae) of the Hawaiian Islands. Pacific Insects 24, 259-274.

(3) Bakker, F.M., Feije, R., Grove, A. J., Hoogendorn, G., Jacobs, G., Loose, E.D. and van Stratum, P. (2003). A laboratory test protocol to evaluate effects of plant protection products on mortality and reproduction of the predatory mite *Hypoaspis aculeifer* Canestrini (Acari: Laelapidae) in standard soil. JSS – Journal of Soils and Sediments 3, 73-77.

(4) Karg, W. (1993). Die freilebenden Gamasina (Gamasides), Raubmilben. 2nd edition In: Dahl, F. (Hrsg.): Die Tierwelt Deutschlands 59. Teil, G. Fischer, Jena, 523 pp.

(5) Ruf, A. (1991). Do females eat males?: Laboratory studies on the popualation development of *Hypoaspis aculeifer* (Acari: Parasitiformes). In: F. Dusbabek & V. Bukva (eds.): Modern Acarology. Academia Prague & SPD Academic Publishing bv, The Hague, Vol. 2, 487-492

(6) Ruf, A. (1995). Sex ratio and clutch size control in the soil inhabiting predatory mite *Hypoaspis aculeifer* (Canestrini 1883) (Mesostigmata, Dermanyssidae). Proc. 2nd Symp. EURAAC: p 241-249.

(7) Ruf, A. (1996). Life-history patterns in soil-inhabiting mesostigmatid mites. Proc. IXth Internat. Congr. Acarol. 1994, Columbus, Ohio: p 621-628.

(8) Krogh, P.H. and Axelsen, J.A. (1998). Test on the predatory mite *Hypoaspis aculeifer* preying on the collembolan *Folsomia fimetaria*. In: Lokke, H. and van Gestel, C.A.M.: Handbook of soil invertebrate toxicity tests. John Wiley Sons, Chichester, p 239-251.

(9) Løkke, H., Janssen, C.R., Lanno, R.P., Römbke, J., Rundgren, S. and Van Straalen, N.M. (2002). Soil Toxicity Tests – Invertebrates. In: Test Methods to Determine Hazards of Sparingly Soluble Metal Compounds in Soils. Fairbrother, A., Glazebrook, P.W., Van Straalen, N.M. and Tarazona, J.V. (eds.). SETAC Press, Pensacola, USA. 128 pp.

(10) Schlosser, H.-J. and Riepert, F. (1991/92). Entwicklung eines Prüfverfahrens für Chemikalien an Bodenraubmilben (Gamasina). Teil 1: Biologie der Bodenraubmilbe Hypoaspis aculeifer Canestrini, 1883 (Gamasina) unter Laborbedingungen. Zool. Beiträge, 34, 395-433.

(11) Schlosser, H.-J. and Riepert, F. (1992). Entwicklung eines Prüfverfahrens für Chemikalien an Boden-raubmilben (Gamasina). Teil 2: Erste Ergebnisse mit Lindan und Kaliumdichromat in subletaler Dosierung. Zool. Beitr. N.F. 34, 413-433.

(12) Heckmann, L.-H., Maraldo, K. and Krogh, P. H. (2005). Life stage specific impact of dimethoate on the predatory mite Hypoaspis aculeifer Canestrini (Gamasida: Laelapidae). Environmental Science & Technology 39, 7154-7157.

(13) Petersen, H. (1978). Some properties of two high-gradient extractors for soil microarthropods, and an attempt to evaluate their extraction efficiency. Natura Jutlandica 20, 95-122.

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

(15) Chapter C.8 of this Annex - . Toxicity for Earthworms. .

(16) EPPO (2003): EPPO Standards. Environmental risk assessment scheme for plant protection products. Chapter 8. Soil Organisms and Functions. Bull. OEPP/EPPO Bull. 33, 195-209.

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

(18) Fairbrother, A., Glazebrock, P.W., Van Straalen, N.M. and Tarazona, J.V. 2002. Test methods to determine hazards of sparingly soluble metal compounds in soils. SETAC Press, Pensacola, FL, USA.

(19) Chi, H. 1981. Die Vermehrungsrate von Hypoaspis aculeifer Canestrini (Acarina, Laelapidae) bei Ernährung mit Onychiurus fimatus Gisin (Collenbola). Ges.allg..angew. Ent. 3:122-125.

(20) Schlosser, H.J., und Riepert, F. 1992. Entwicklung eines Prüfverfahrens für Chemikalien an Bodenraubmilden (Gamasina). Zool.Beitr. N.F. 34(3):395-433.

(21) Heckmann, L.-H., Ruf, A., Nienstedt, K. M. and Krogh, P. H. 2007. Reproductive performance of the generalist predator Hypoaspis aculeifer (Acari: Gamasida) when foraging on different invertebrate prey. Applied Soil Ecology 36, 130-135.

(22) Chapter C.32 of this Annex- Enchytraeid reproduction test.

(23) ISO (International Organization for Standardization) (1994). Soil Quality - Effects of pollutants on earthworms (Eisenia fetida). Part 2: Determination of effects on reproduction, No. 11268-2. ISO, Geneve.

(24) Southwood, T.R.E. (1991). Ecological methods. With particular reference to the study of insect populations. (2nd ed.). Chapman & Hall, London, 524 pp.

(25) Dunger, W. and Fiedler, H.J. (1997). Methoden der Bodenbiologie (2nd ed.). G. Fischer, Jena, 539 pp.

(26) Lesna, I. and Sabelis, M.W. (1999). Diet-dependent female choice for males with "good genes" in a soil predatory mite. Nature 401, 581-583.

(27) Ruf, A. (1989). Die Bedeutung von Arrhenotokie und Kannibalismus für die Populationsentwicklung von Hypoaspis aculeifer (Canestrini 1883) (Acari, Gamasina). Mitt. Deut. Ges. Allg. Angew. Ent. 7, 103-107.

(28) Ruf, A. (1993). Die morphologische Variabilität und Fortpflanzungsbiologie der Raubmilbe Hypoaspis aculeifer (Canestrini 1883) (Mesostigmata, Dermanyssidae). Dissertation, Universität Bremen.

(29) Ignatowicz, S. (1974). Observations on the biology and development of Hypoaspis aculeifer Canestrini, 1885 (Acarina, Gamasides). Zoologica Poloniae 24, 11-59.

(30) Kevan, D.K. McE. and Sharma, G.D. (1964). Observations on the biology of Hypoaspis aculeifer (Canestrini, 1884), apparently new to North America (Acarina : Mesostigmata : Laelaptidae). Acarologia 6, 647-658.

(31) OECD (2006c). Current Approaches in the Statistical Analysis of Ecotoxicity Data: A Guidance to Application. OECD environmental Health and Safety Publications Series on Testing and Assessment No.54. ENV/JM/MONO(2006)18

Appendix 1

DEFINITIONS

The following definitions are applicable to this test method (in this test all effect concentrations are expressed as a mass of test chemical per dry mass of the test soil):

Chemical is a substance or a mixture

NOEC (no observed effect concentration) is the test chemical concentration at which no effect is observed. In this test, the concentration corresponding to the NOEC, has no statistically significant effect ($p < 0.05$) within a given exposure period when compared with the control.

LOEC (lowest observed effect concentration) is the lowest test chemical concentration that has a statistically significant effect ($p < 0.05$) within a given exposure period when compared with the control.

ECx (effect concentration for x% effect) is the concentration that causes an x% of an effect on test organisms within a given exposure period when compared with a control. For example, an EC_{50} is a concentration estimated to cause an effect on a test end point in 50% of an exposed population over a defined exposure period.

Test Chemical is any substance or mixture tested using this test method.

Appendix 2

DETERMINATION OF THE MAXIMUM WATER HOLDING CAPACITY OF THE SOIL

The following method for determining the maximum water holding capacity of the soil is considered to be appropriate. It is described in Annex C of ISO DIS 11268-2 (Soil Quality - Effects of pollutants on earthworms (*Eisenia fetida*). Part 2: Determination of effects on reproduction (23)).

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

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

Where:

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

Appendix 3

DETERMINATION OF SOIL PH

The following method for determining the pH of a soil is based on the description given in ISO DIS 10390: Soil Quality – Determination of pH (16).

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

Appendix 4

REARING OF *HYPOASPIS (GEOLAELAPS) ACULEIFER* , FOOD MITES AND SYNCHRONISATION OF CULTURE

Rearing of Hypoaspis (Geolaelaps) aculeifer:

Cultures can be maintained in plastic vessels or glass jars filled with plaster of Paris / charcoal powder (9:1) mixture. The plaster can be kept moist by adding few drops of distilled or deionised water if required. Rearing temperatures are optimal between 20 ± 2°C, light / dark regime is not relevant for this species. Prey can be Typrophagus putrescentiae or Caloglyphus sp. mites (food mites should be handled with care since they could cause allergies in humans), but nematodes, enchytraeids and collembolans are also suited as prey items. Their source should be recorded. Population development can start with a single female because males develop in unfertilised eggs. Generations are largely overlapping. A female can live at least 100 days and can deposit approximately 100 eggs during its lifetime. A maximum oviposition rate is reached between 10 and 40 days (after becoming adults) and amounts to 2.2 eggs female^{-1} day^{-1}. Developmental time from egg to adult female is approximately 20 days at 20°C. More than one culture should be maintained and held beforehand.

Rearing of Typrophagus putrescentiae:

The mites are kept in a glass vessel filled with fine brewers yeast powder which is put in a plastic bucket filled with KNO_3-solution in order to avoid escaping. The food mites are placed on top of this powder. Afterwards, they are carefully mixed with the powder (which has to be replaced twice a week) using a spatula.

Synchronisation of culture:

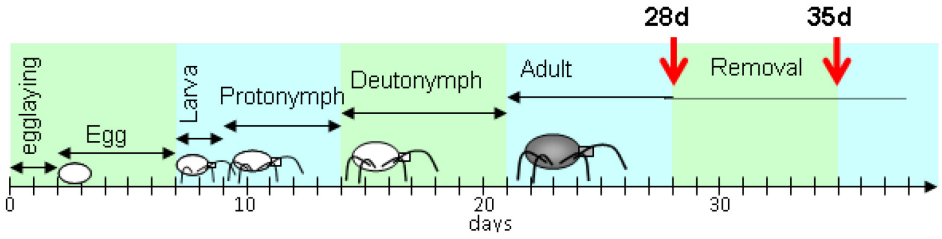
Specimens that are used in the test should be of similar age (ca. 7 days after reaching the adult stage). At a rearing temperature of 20 °C this is achieved by

Transfer females to a clean rearing vessel and add sufficient food

- Allow for two to three days of egg laying, remove females
- Take adult females for testing between the 28th and 35th day after start placing female adults in clean rearing vessels.

Adult females can be easily distinguished from males and other developmental stages by their larger size, bloated shape and their brown dorsal shield (males are slimmer and flat), immatures are white to cream-coloured. The development of the mites follows approximately the pattern described below at 20°C (figure): Egg 5d, Larva 2d, Protonymph 5d, Deutonymph 7d, preoviposition period of female 2d. Afterwards, the mites are adult.

Figure: Development of Hypoaspis (Geolaelaps) aculeifer at 20°C. (removal = females used for the test)



The adult test animals are removed from the synchronised culture and introduced into the test vessels between the 28th and the 35th day after the parental females have started egg laying (i.e. 7 – 14 days after they became adult). This ensures that the test animals have already passed their preoviposition period and have been mated by males that are also present in the culture vessel. Observations in laboratory cultures suggest, that females mate immediately or shortly after becoming adult if males are present (Ruf, Vaninnen, pers. obs.). The period of seven days is chosen to facilitate integration in laboratory routine and to buffer individual developmental variability among mites. The oviposition should be started with at least the same number of females that is eventually needed for the test (If for example 400 females are needed in the test, at least 400 females should be allowed to oviposit for two to three days. At least 1200 eggs should be the starting point for the synchronised population (sex ratio ca. 0.5, mortality ca. 0.2). To avoid cannibalism, it is more feasible to keep not more than 20-30 ovipositing females in one vessel.

Appendix 5

EXTRACTION METHODS

For micro-arthropods a heat extraction is an appropriate method to separate specimens from the soil / substrate (see figure below). The method is based on the activity of the organisms, so only mobile specimens will have the chance to be recorded. The principle of the heat extraction is to make conditions for the organisms gradually worse in the sample, so that they will leave the substrate and fall in a fixing liquid (e.g. ethanol). Crucial points are the duration of the extraction and the gradient of good to moderate to bad conditions for the organisms. The duration of extraction for ecotoxicological tests have to be as short as possible, because any population growth during the time of extraction would falsify the results. On the other hand the temperature and moisture conditions in the sample have to be always in a range that allows the mites to move. The heating of a soil sample leads to a desiccation of substrate. If the desiccation is too quick, some mites might also desiccated before they managed to escape.

Therefore the following procedure is proposed (24) (25):

Apparatus: Tullgren funnel or comparable methods like e.g. McFadyen (heating from above, sample is put over a funnel)

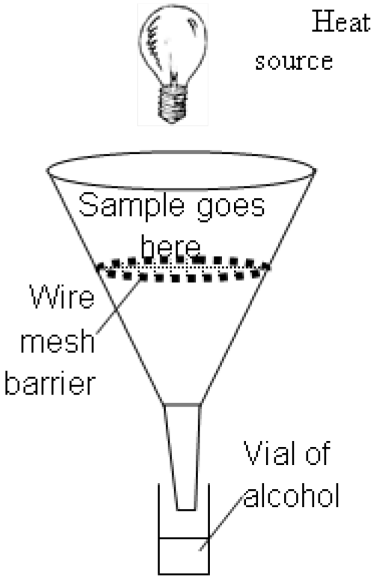
Heating regime: 25°C for 12 h, 35°C for 12 h, 45°C for 24 hours (in total 48 h). The temperature should be measured in the substrate.

Fixation liquid: 70% ethanol

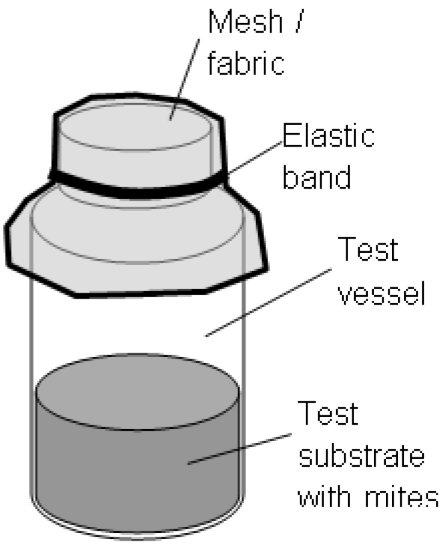
Details: Take glass vial that was used for the test. Remove lid and wrap a piece of mesh or fabric around the opening. The fabric should have a mesh size of 1.0 to 1.5 mm. Fix the fabric with an elastic band. Carefully turn the vial upside down and place it in the extraction apparatus. The fabric prevents substrate from trickling in the fixation liquid but allows mites to leave the sample. Start the heating regime after all vials are inserted. End the extraction after 48 hours. Remove fixation vials and count mites by means of a dissecting microscope.

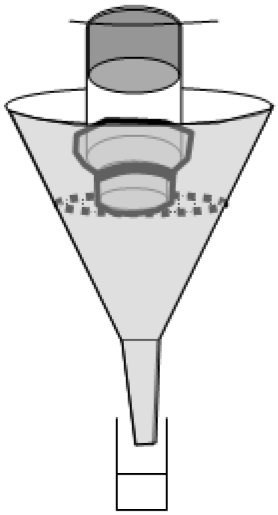
The extraction efficiency of the chosen method must have been proven once or twice a year using vessels containing a known number of juvenile and adult mites kept in untreated test substrate. Efficiency should be $\geq$ 90% on average combined for all developmental stages.

Tullgren-type extracting device



How to prepare the test vial after the test is finished, before extraction


Mesh /
fabric
Elastic
band
Test
vessel
Test
substrate
with mites



Appendix 6

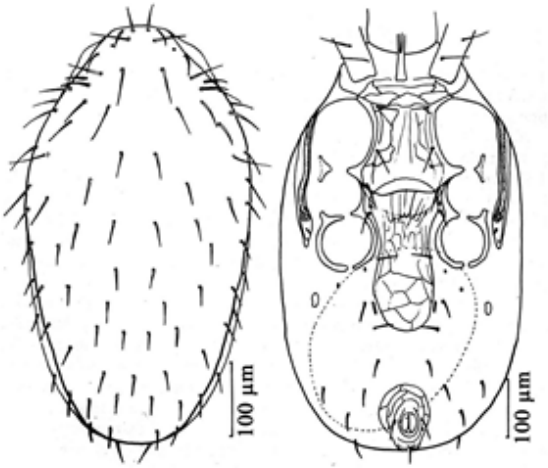
IDENTIFICATION OF HYPOASPIS (GEOLAELAPS) ACULEIFER

Subclass/order/suborder:	Family:	Genus/subgenus/species:
Acari/Parasitiformes/ Gamasida	Laelapidae	Hypoaspis (Geolaelaps) aculeifer

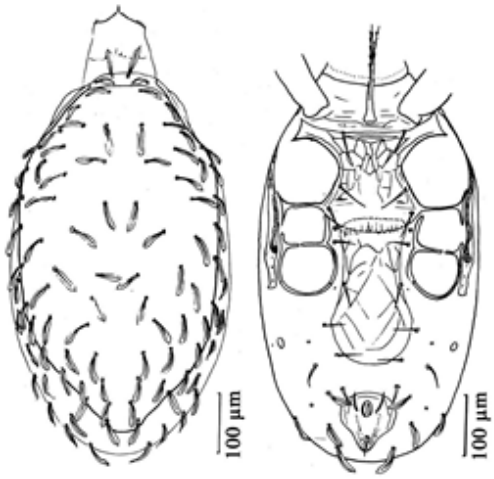
Author and Date:	F. Faraji, Ph.D. (MITOX), 23 January 2007

Literature used:	Karg, W. (1993). Die freilebenden Gamasina (Gamasides), Raubmilben. Tierwelt Deutschlands 59, 2nd revised edition: 1-523. Hughes, A.M. (1976). The mites of stored food and houses. Ministry of Agriculture, Fisheries and Food, Technical Bulletin 9: 400pp. Krantz, G.W. (1978). A manual of Acarology. Oregon State University Book Stores, Inc., 509 pp.

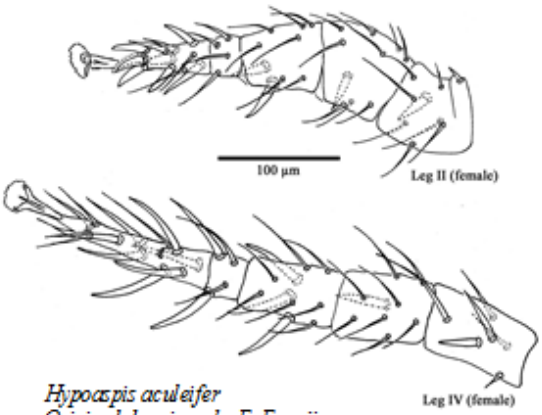
Deterministic characteristics:	Tectum with rounded denticulate edge; hypostomal grooves with more than 6 denticles; caudal dorsal setae of Z4 not very long; dorsal setae setiform; genital shield normal, not very enlarged and not reaching the anal shield; posterior half of dorsal shield without unpaired setae; legs II and IV with some thick macrosetae; dorsal seta Z5 about two times longer than J5; fixed digit of chelicera with 12-14 teeth and movable digit with 2 teeth; Idiosoma 520-685 μm long. Hypoaspis miles is also used in biological control and might get confused with H. aculeifer. The main difference is: H. miles belongs to subgenus Cosmolaelaps and has knife-like dorsal setae while H. aculeifer belongs to subgenus Geolaelaps and has setiform dorsal setae.



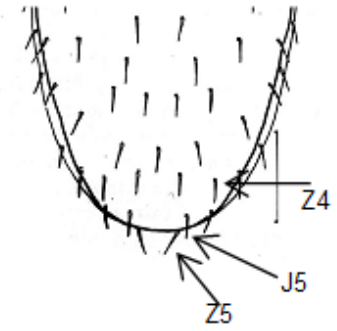
Hypoaspis aculeifer After Hughes, 1976



Hypoaspis miles After Hughes, 1976



Hypoaspis aculeifer
Original drawings by F. Faraji



Hypoaspis aculeifer,
dorsal shield with characteristic setae

Appendix 7

BASIC INFORMATION ON THE BIOLOGY OF *HYPOASPIS (GEOLAELAPS) ACULEIFER*

Hypoaspis aculeifer belongs to the family Lealapidae, order Acari (mites), class Arachnida, tribe Arthropoda. They are living in all kinds of soil and feed on other mites, nematodes, enchytraeids and collembolans (26). In case of food shortage they switch to cannibalism (27). Predatory mites are segmented in idiosoma and gnathosoma. A clear differentiation of the idiosoma in prosoma (head) and opisthosoma (abdomen) is missing. The gnathosoma (head shield) contains the instruments for feeding such as palps and chelicera. The chelicers are trifurcated and tusked with teeth of different shape. Beside ingestion the males are using their chelicers mainly to transfer the spermatophores to the females. A dorsal shield covers nearly completely the idiosoma. A big part of the female idiosoma is occupied by the reproductive organs, which are in particular distinct shortly before egg deposition. Ventrally, two shields can be found, the sternal and the genital shield. All legs are provided with bristles and thorns. The bristles are used to anchor when moving in or on top of the soil. The first pair of legs is used mainly as antenna. The second pair of legs is used not only for moving but also to clinch the prey. The thorns of the fourth pair of legs can serve as protection as well as 'moving motor' (28). Males are 0.55 – 0.65 mm long and have a weight of 10 – 15 µg. Females are 0.8 – 0.9 mm long and are weighing 50 – 60 µg (8) (28) (Fig 1).

Fig 1: Female, male, protonymph and larvae of H. aculeifer.

At 23°C, the mites become sexually mature after 16 days (females) and 18 days (males), respectively (6). The females carry over the sperms by the solenostom where they will be then transferred to the ovar. In the ovar the sperms mature and will be stored. Fertilisation takes place only after maturation of the sperms in the ovar. The fertilised or unfertilised eggs will be deposited by the females in clumps or separately, preferably in crevices or holes. Copulated females can bear juveniles of both sexes

whereas from eggs of uncopulated females only male juveniles are hatching. During development to the adult four phases of development (egg – larvae, larvae – protonymph, protonymph – deutonymph, deutonymph – adult) are passed through.

The egg is milky white, hyaline, elliptical and approximately 0.37 mm long with a solid mantle. According to (8), the larvae are between 0.42 – 0.45 mm in size. They have only three pairs of legs. In the head region palps and chelicers are developed. The chelicers, having some few small denticles, are used to hatch from the egg. After the first moult, 1 – 2 days after hatching, the protonymphs are developed. They are also white, the size is 0.45 – 0.62 mm (8) and they have four pairs of legs. On the chelicers the teeth are completely present. Beginning with that stadium the mites start to forage. For that reason the cuticula of the prey is pierced with the chelicers and a secretion for the extra intestinal digestion is emitted into the prey. The food mash can then be sucked by the mite. The chelicers can also be used to rip bigger particles out of food nuggets (28). After one further moult the deutonymphs are developed. They are 0.60 – 0.80 mm (8) in size and yellow to light brown in colour. Beginning with that phase they can be separated into females and males. After further ecdysis, during which time the animals are inactive and the brown shield is developing (approx. after 14 days) the mites are adult (28) (29) (30).Their life span is between 48 and 100 days at 25°C (27).

Appendix 8

SUMMARY AND TIME SCHEDULE OF THE MAIN ACTIONS TO BE TAKEN IN ORDER TO PERFORM THE HYPOASPIS TEST

Time (days) test start = day 0	***Activity / task***
Day -35 to -28	*Transfer females from stock culture to clean vessels to start synchronisation* *2 days later: removal of females* *twice or three times a week: supply with sufficient food*
Day -5 (+/- 2)	*Prepare artificial soil*
Day -4 (+/- 2)	*Determine WHC of artificial soil* *Dry over night* *Next day: weigh samples and calculate WHC*
Day -4 (+/- 2)	*Pre moisture artificial soil to achieve 20 - 30 % of WHC*
Day 0	*Start test: add test chemical to artificial soil* *Introduce 10 females to each replicate* *Weigh each replicate* *Set up abiotic controls for moisture content and pH, 2 replicates for each treatment* *Dry moisture controls over night* *Next day: weigh moisture controls* *Next day: measure pH of dried abiotic controls*
Day 3, 6, 9, 12 (approx.)	*Supply each replicate with sufficient amount of prey organisms* *Weigh each replicate and eventually add evaporated water*
Day 14	*Terminate test, set up extraction with all replicates plus extraction efficiency controls* *Dry water content controls over night* *Next day: weigh water content controls* *Next day: measure pH of dried controls*
Day 16	*Terminate extraction*
Day 16+	*Record number of adults and juveniles in extracted material* *Report results on template tables* *Report testing procedure in test protocol sheets*