PART C: METHODS FOR THE DETERMINATION OF ECOTOXICITY

GENERAL INTRODUCTION: PART C

The test methods described below are for the determination of some of the ecotoxicological properties listed in Annex VIII to Directive 79/831/EEC. Notifiers should be aware that methods for the determination of the following properties foreseen in Level 1 of Annex VIII are not included in the text:

— Prolonged toxicity study with *Daphnia magna*,

— Test on a higher plant,

— Prolonged toxicity study with fish.

When appropriate test methods for the determination of these properties are finalized they will be published in the form of a further adaptation to technical progress. In the interim, notifiers should use suitable, internationally recognized methods which should be identified to the competent authority.

C. 8

TOXICITY FOR EARTHWORMS

ARTIFICIAL SOIL TEST

1. **METHOD**

1.1. **Introduction**

In this laboratory test, the test substance is added to an artificial soil in which worms are placed for 14 days. After this period (and optionally after seven days) the lethal effect of the substance on the earthworms is examined. The test provides a method for relatively short-term screening of the effect of chemicals on earthworms, by dermal and alimentary uptake.

1.2. **Definition and unit**

LC_{50}: The concentration of a substance estimated as killing 50 % of the test animals during the test period.

1.3. **Reference substance**

A reference substance is used periodically as a means of demonstration that the sensitivity of the test system has not changed significantly.

Analytical grade chloroacetamide is recommended as the reference substance.

1.4. **Principle of the test**

Soil is a variable medium, so for this test a carefully defined artificial loam soil is used. Adult earthworms of the species *Eisenia foetida* (see note in Appendix) are kept in a defined artificial soil treated with different concentrations of the test substance. The content of the containers is spread on a tray 14 days (and optionally seven days) after the beginning of the test, and the earthworms surviving at each concentration counted.

1.5. **Quality criteria**

The test is designed to be as reproducible as possible with respect to the test substrate and organism. Mortality in the controls must not exceed 10 % at the end of the test, or the test is invalid.

1.6. **Description of the test method**

1.6.1. *Materials*

1.6.1.1. Test substrate

A defined artificial soil is used as a basic test substrate.

(a) Basic substrate (percentages are in terms of dry weight)

— 10 % sphagnum peat (as close to pH 5,5 to 6,0 as possible with no visible plant remains and finely ground),

— 20 % kaolinite clay with preferably more than 50 % kaolinite,

— About 69 % industrial quartz sand (dominant fine sand with more than 50 % of particle size 0,05 to 0,2 mm). If the substance is not sufficiently dispersible in water, 10 g per test container should be kept available for mixing with the test substance later on,

— About 1 % calcium carbonate ($CaCO_3$), pulverized, chemically pure, added to bring the pH to 6,0 ± 0,5.

(b) Test substrate

The test substrate contains the basic substrate, the test substance and deionized water.

Water content is about 25 to 42 % of the dry weight of the basic substrate. The water content of the substrate is determined by drying a sample to constant weight at 105 °C. The key criterion is that the artificial soil must be wetted to a point where there is no standing water. Care should be taken in mixing to obtain an even distribution of the test substance and the substrate. The way of introducing the test substance to the substrate has to be reported.

(c) Control substrate

The control substrate contains the basic substrate and water. If an additive agent is used, an additional control should contain the same quantity of the additive agent.

1.6.1.2. Test containers

Glass containers of about one litre capacity (adequately covered with plastic lids, dishes or plastic film with ventilation holes) filled with an amount of wet test or control substrate equivalent to 500 g dry weight of substrate.

1.6.2. *Test conditions*

Containers should be kept in climatic chambers at a temperature of 20 ± 2 °C with continuous light. Light intensity should be 400 to 800 lux.

The test period is 14 days, but mortality can be assessed optionally seven days after starting the test.

1.6.3. *Test procedure*

Test concentrations

Concentrations of the test substance are expressed as weight of substance per dry weight of basic substrate (mg/kg).

Range finding test

The range of concentrations just causing mortalities of 0 to 100 % may be determined in a range-finding test to provide information on the range of concentrations to be used in the definitive test.

The substance should be tested at the following concentrations: 1 000; 100; 10; 1; 0,1 mg substance/kilogram test substrate (dry weight).

If a full definitive test is to be carried out, one test batch per concentration and one for the untreated control, each with 10 worms, could be sufficient for the range-finding test.

Definitive test

The results of the range-finding test are used to choose at least five concentrations in a geometric series just spanning the range 0 to 100 % mortality and differing by a constant factor not exceeding 1,8.

Tests using these series of concentration should allow the LC_{50} value and its confidence limits to be estimated as precisely as possible.

In the definitive test at least four test batches per concentration and four untreated controls, each with 10 worms, are used. The results of these replicate batches are given as a mean and standard deviation.

When two consecutive concentrations, at a ratio of 1,8, give only 0 % and 100 % mortality, these two values are sufficient to indicate the range within which the LC_{50} falls.

Mixture of the basic test substrate and the test substance

The test substrate should, whenever possible, be made up without any additional agents other than water. Immediately before the start of the test, an emulsion or dispersion of the test substance in deionized water or other solvent is mixed with the basic test substrate, or sprayed evenly over it with a fine chromatographic or similar spray.

If insoluble in water, the test substance can be dissolved in as small a volume as possible of suitable organic solvent (e.g. hexane, acetone or chloroform).

Only agents which volatilize readily may be used to solubilize, disperse or emulsify the test substance. The test substrate must be ventilated before use. The amount of water evaporated must be replaced. The control should contain the same quantity of any additive agent.

If the test substance is not soluble, dispersible or emulsifiable in organic solvents, 10 g of a mixture of fine ground quartz sand and a quantity of test substance necessary to treat 500 g dry weight of artificial soil are mixed with 490 g of dry weight of test substrate.

For each test batch, an amount of wet test substrate equivalent to 500 g dry weight is placed in each glass container and 10 earthworms, which have been conditioned for 24 hours in a similar wet basic substrate and then washed quickly and surplus water absorbed on filter paper before use, are placed on the test substrate surface.

The containers are covered with perforated plastic lids, dishes or film to prevent the substrate drying and they are kept under the test conditions for 14 days.

The assessments should be made 14 days (and optionally seven days) after setting up the test. The substrate is spread on a plate made of glass or stainless steel. The earthworms are examined and the numbers of surviving earthworms determined. Earthworms are considered dead if they do not respond to a gentle mechanical stimulus to the front end.

When the examination is made at seven days, the container is refilled with the substrate and the surviving earthworms are replaced on the same test substrate surface.

1.6.4. *Test organisms*

Test organisms should be adult *Eisenia foetida* (see note in Appendix) (at least two months old with clitellum) wet weight 300 to 600 mg. (For breeding method see Appendix.)

2. DATA

2.1. Treatment and evaluation of results

The concentrations of the substance tested are reported with reference to the corresponding percentages of dead earthworms.

When the data are adequate the LC_{50} value and the confidence limits ($p = 0,05$) should be determined using standard methods (Litchfield and Wilcoxon, 1949, for equivalent method). The LC_{50} should be given as mg of test substance per kilogram of the test substrate (dry weight).

In those cases where the slope of the concentration curve is too steep to permit calculation of the LC_{50}, a graphical estimate of this value is sufficient.

When two consecutive concentrations at a ratio of 1,8 give only 0 % and 100 % mortality, the two values are sufficient to indicate the range within which the LC_{50} falls.

3. REPORTING

3.1. Test report

The test report shall, if possible, contain the following:

- statement that the test has been carried out in accordance with the abovementioned quality criteria,
- test carried out (range finding test and/or definitive test),
- exact description of the test conditions or statement that the test has been carried out in accordance with the method; any deviations have to be reported,
- exact description of how the test substance has been mixed into the basic test substrate,
- information about test organisms (species, age, mean and range in weight, keeping and breeding conditions, supplier),
- method used for determination of LC_{50},
- test results including all data used,
- description of observed symptoms or changes in behaviour of test organisms,
- mortality in the controls,
- LC_{50} or highest tested concentration without mortality and lowest tested concentration with a mortality of 100 %, 14 days (and optionally seven days) after setting up the test,
- plotting of the concentration/response curve,
- results obtained with the reference substance, whether in association with the present test or from previous quality control exercises.

4. REFERENCES

(1) OECD, Paris, 1981, *Test Guideline 207,* Decision of the Council C(81) 30 final.

(2) Edwards, C. A. and Lofty, J. R., 1977, *Biology of Earthworms,* Chapman and Hall, London, 331 pp.

(3) Bouche, M. B., 1972, *Lombriciens de France, Écologie et Systématique,* Institut National de la Recherche Agronomique, 671 pp.

(4) Litchfield, J. T. and Wilcoxon, F., A simplified method of evaluation dose effect experiments. *J. Pharm. Exp. Therap.*, vol. 96, 1949, p. 99.

(5) Commission of the European Communities, *Development of a standardized laboratory method for assessing the toxicity of chemical substances to earthworms,* Report EUR 8714 EN, 1983.

(6) Umweltbundesamt/Biologische Bundesanstalt für Land- und Forstwirtschaft, Berlin, 1984, Verfahrensvorschlag 'Toxizitätstest am Regenwurm *Eisenia foetida* in künstlichem Boden', in: Rudolph/Boje, *Ökotoxikologie,* ecomed, Landsberg, 1986.

Appendix

Breeding and keeping of the worms before testing

For breeding the animals, 30 to 50 adult worms, are put in a breeding box with fresh substrate and removed after 14 days. These animals may be used for further breeding batches. The earthworms hatched from the cocoons are used for testing when mature (under the prescribed conditions after two to three months).

Keeping and breeding conditions

Climatic chamber:	temperature 20 ± 2 °C preferably with continuous light (intensity 400 to 800 lux).
Breeding boxes:	suitable shallow containers of 10 to 20 l volume.
Substrate:	*Eisenia foetida* may be bred in various animal excrements. It is recommended to use as breeding medium a mixture of 50 % by volume peat and 50 % cow or horse dung. The medium should have a pH value of about 6 to 7 (regulated with calcium carbonate) and a low ionic conductivity (less than 6 mmhos or 0,5 % salt concentration).
	The substrate should be moist but not too wet.
	Other successful procedures may be used besides the method given above.

Note: Eisenia foetida exists in two races which some taxonomists have separated into species (Bouche, 1972). These are morphologically similar but one, *Eisenia foetida foetida,* has typically transverse striping or banding on the segments and the other, *Eisenia foetida andrei,* lacks this and has a variegated reddish colour. Where possible *Eisenia foetida andrei* should be used. Other species may be used if the necessary methodology is available.

C. 9

BIODEGRADATION

ZAHN — WELLENS TEST

1. METHOD

1.1. Introduction

The purpose of the method is the evaluation of the potential ultimate biodegradability of water-soluble, non-volatile organic substances when exposed to relatively high concentrations of micro-organisms in a static test.

Physico-chemical adsorption on the suspended solids may take place and this must be taken into account when interpreting results (see 3.2).

The substances to be studied are used in concentrations corresponding to DOC-values in the range of 50 to 400 mg/litre or COD-values in the range of 100 to 1 000 mg/litre (DOC = dissolved organic carbon; COD = chemical oxygen demand). These relatively high concentrations have the advantage of analytical reliability. Compounds with toxic properties may delay or inhibit the degradation process.

In this method, the measure of the concentration of dissolved organic carbon or the chemical oxygen demand is used to assess the ultimate biodegradability of the test substance.

A simultaneous use of a specific analytical method may allow the assessment of the primary biodegradation of the substance (disappearance of the parent chemical structure).

The method is applicable only to those organic test substances which, at the concentration used in the test:

— are soluble in water under the test conditions,

— have negligible vapour pressure under the test conditions,

— are not inhibitory to bacteria,

— are adsorbed within the test system only to a limited extent,

— are not lost by foaming from the test solution.

Information on the relative proportions of the major components of the test material will be useful in interpreting the results obtained, particularly in those cases where the results are low or marginal.

Information on the toxicity of the substance to micro-organisms is desirable for the interpretation of low results and in the selection of appropriate test concentrations.

1.2. Definitions and units

The amount of degradation attained at the end of the test is reported as the 'Biodegradability in the Zahn — Wellens test':

$$D_T(\%) = \left[1 - \frac{(C_T - C_B)}{(C_A - C_{BA})}\right] \times 100$$

where:

D_T = biodegradation (%) at time T,

C_A = DOC (or COD) values in the test mixture measured three hours after the beginning of the test (mg/l) (DOC = Dissolved Organic Carbon, COD = Chemical Oxygen Demand),

C_T = DOC or COD values in the test mixture at time of sampling (mg/l),

C_B = DOC or COD value of the blank at time of sampling (mg/l),

C_{BA} = DOC or COD value of the blank, measured three hours after the beginning of the test (mg/l).

The extent of degradation is rounded to the nearest full percent.

Percentage degradation is stated as the percentage DOC (or COD) removal of the tested substance.

The difference between the measured value after three hours and the calculated or preferably measured initial value may provide useful information on the elimination of the substance (see 3.2, Interpretation of results).

1.3. **Reference substances**

In some cases when investigating new substances reference substances may be useful; however, specific reference substances cannot yet be recommended.

1.4. **Principle of the test method**

Activated sludge, mineral nutrients and the test material as the sole carbon source in an aqueous solution are placed together in a one to four litre glass vessel equipped with an agitator and an aerator. The mixture is agitated and aerated at 20 to 25 °C under diffuse illumination or in a dark room for up to 28 days. The degradation process is monitored by determination of the DOC (or COD) values in the filtered solution at daily or other appropriate regular time intervals. The ratio of eliminated DOC (or COD) after each interval to the value three hours after the start is expressed as percentage biodegradation and serves as the measure of the extent of degradation at this time. The result is plotted *versus* time to give the biodegradation curve.

When a specific analytical method is used, changes in the concentration of the parent molecule due to biodegradation can be measured (primary biodegradability).

1.5. **Quality criteria**

The reproducibility of this test has been proven to be satisfactory in a ring test.

The sensitivity of the method is largely determined by the variability of the blank and, to a lesser extent, by the precision of the determination of dissolved organic carbon and the level of test compound in the liquor.

1.6. **Description of the test procedure**

1.6.1. *Preparations*

1.6.1.1. Reagents

Test water: drinking water with an organic-carbon content < 5 mg/litre. The concentration of calcium and magnesium ions together must not exceed 2,7 mmole/litre; otherwise adequate dilution with deionized or distilled water is required.

Sulphuric acid, analytical reagent (A.R.): 50 g/l.

Sodium hydroxide solution A.R.: 40 g/l.

Mineral nutrient solution: dissolve in one litre deionized water:

ammonium chloride, NH_4Cl, A.R.: 38,5 g,

sodium dihydrogenphosphate, $NaH_2PO_4.2H_2O$, A.R.: 33,4 g,

potassium dihydrogenphosphate, KH_2PO_4, A.R.: 8,5 g,

di-potassium mono-hydrogenphosphate, K_2HPO_4, A.R.: 21,75 g.

The mixture serves both as a nutrient and as buffering system.

1.6.1.2. Apparatus

Glass vessels with a volume of one to four litres (e.g. cylindrical vessels).

Agitator with a glass or metal stirrer on a suitable shaft (the stirrer should rotate about 5 to 10 cm above the bottom of the vessel). A magnetic stirrer with a 7 to 10 cm long rod can be used instead.

Glass tube of 2 to 4 mm inner diameter to introduce air. The opening of the tube should be about 1 cm above the bottom of the vessel.

Centrifuge (about 3 550 g).

pH-meter.

Dissolved-oxygen meter.

Paper filters.

Membrane filtration apparatus.

Membrane filters, pore size 0,45 µm. Membrane filters are suitable if it is assured that they neither release carbon nor absorb the substance in the filtration step.

Analytical equipment for determining organic carbon content and chemical oxygen demand.

1.6.1.3. Preparation of the inoculum

Activated sludge from a biological treatment plant is washed by (repeatedly) centrifuging or settling with test water (above).

The activated sludge must be in an appropriate condition. Such sludge is available from a properly working waste-water treatment plant. To get as many different species or strains of bacteria as possible, it may be preferred to mix inocula from different sources (e.g. different treatment plants, soil extracts, river waters, etc.). The mixture is to be treated as described above.

For checking the activity of the activated sludge see 'Functional control', below.

1.6.1.4. Preparation of the test solutions

To the test vessel add 500 ml of test water, 2,5 ml/litre mineral nutrient solution and activated sludge in an amount corresponding to 0,2 to 1,0 g/litre dry matter in the final mixture. Add sufficient stock solution of the substance to be tested so that a DOC concentration of 50 to 400 mg/litre results in the final mixture. The corresponding COD-values are 100 to 1 000 mg/litre. Make up with test water to a total volume of one to four litres. The total volume to be chosen is dependent on the number of samples to be taken for DOC or COD determinations and the volumes necessary for the analytical procedure.

Normally a volume of two litres can be regarded as satisfactory. At least one control vessel (blank) is set up to run in parallel with each test series; it contains only activated sludge and mineral nutrient solution made up with test water to the same total volume as in the test vessels.

1.6.2. *Performance of the test*

The test vessels are agitated with magnetic stirrers or screw propellers under diffuse illumination or in a dark room at 20 to 25 °C. Aeration is accomplished by compressed air cleaned by a cotton-wool strainer and a wash bottle if necessary. It must be ensured that the sludge does not settle and the oxygen concentration does not fall below 2 mg/litre.

The pH-value must be checked at regular intervals (e.g. daily) and adjusted to pH 7 to 8, if necessary.

Losses from evaporation are made up just before each sampling with deionized or distilled water in the required amounts. A good procedure is to mark the liquid level on the vessel before starting the test. New marks are made after each sampling (without aeration and stirring). The first samples are always taken three hours after the start of the test in order to detect adsorption of test material by the activated sludge.

The elimination of the test material is followed by DOC or COD determinations made daily or at some other regular interval. The samples from the test vessel and the blank are filtered through a carefully washed paper filter. The first 5 ml of test solution filtrate are discarded. Sludges difficult to filter may be removed previously by centrifugation for 10 minutes. DOC and COD determinations are made at least in duplicate. The test is run for up to 28 days.

Note: Samples remaining turbid are filtered through membrane filters. The membrane filters must not release or adsorb any organic material.

Functional control of activated sludge

A vessel containing a known substance should be run in parallel with each test series in order to check the functional capacity of the activated sludge. Diethyleneglycol has been found useful for this purpose.

Adaptation

If analyses are carried out at relatively short intervals (e.g. daily), adaptation can be clearly recognized from the degradation curve (see Figure 2). The test should therefore not be started immediately before the weekend.

If the adaptation occurs in the end of the period, the test can be prolonged until the degradation is finished.

Note: If a broader knowledge of the behaviour of the adapted sludge is needed, the same activated sludge is exposed once again to the same test material in accordance with the following procedure:

Switch of the agitator and the aerator and allow the activated sludge to settle. Draw off the supernatant liquid, fill up to two litres with test water, stir for 15 minutes and allow to settle again. After the supernatant liquid is drawn off again, use the remaining sludge to repeat the test with the same material in accordance with 1.6.1.4 and 1.6.2, above. The activated sludge can also be isolated by centrifuging instead of settling.

The adapted sludge may be mixed with fresh sludge to a concentration of 0,2 to 1 g dry weight/litre.

Analytical means

Normally samples are filtered through a carefully washed paper filter (for washing use deionized water).

Samples which remain turbid are filtered through membrane filters (0,45 µm).

The DOC concentration is determined in duplicate in the sample filtrates (the first 5 ml are discarded) by means of the TOC instrument. If the filtrate cannot be analysed on the same day, it must be stored in the refrigerator until the next day. Longer storage cannot be recommended.

The COD concentration is determined in the sample filtrates with a COD analytical set-up by the procedure described in reference (2), below.

2. DATA AND EVALUATION

DOC and/or COD concentrations are determined at least in duplicate in the samples according to 1.6.2, above. The degradation at time T is calculated according to the formula (with definitions) given unter 1.2, above.

The extent of degradation is rounded to the nearest full percent. The amount of degradation attained at the end of the test is reported as the 'Biodegradability in the Zahn — Wellens test'.

Note: If complete degradation is attained before the test time is over and this result is confirmed by a second analysis on the next day, the test can be concluded.

3. REPORTING

3.1. Test report

The test report shall, if possible, contain the following:

— the initial concentration of the substance,

— all other information and the experimental results concerning the tested substance, the reference substance if used, and the blank,

— the concentration after three hours,

— biodegradation curve with description,

— date and location where test organisms were sampled, status of adaptation, concentration used, etc.,

— scientific reasons for any changes of test procedure.

3.2. Interpretation of results

Removal of DOC (COD) which takes place gradually over days or weeks indicates that the test substance is being biodegraded.

However, physico-chemical adsorption can, in some cases, play a role and this is indicated when there is complete or partial removal from the outset, within the first three hours, and the difference between control and test supernatant liquors remains at an unexpectedly low level.

Futher tests are necessary if a distinction is to be drawn between biodegradation (or partial biodegradation) and adsorption.

This can be done in a number of ways, but the most convincing is to use the supernatant or sludge as inoculum in a base-set test (preferably a respirometric test).

Test substances giving high, non-adsorptive removal of DOC (COD) in this test should be regarded as potentially biodegradable. Partial, non-adsorptive removal indicates that the chemical is at least subject to some biodegradation. Low, or zero removals of DOC (COD) may be due to inhibition of microorganisms by the test substance and this may also be revealed by lysis and loss of sludge, giving turbid supernatants. The test should be repeated using a lower concentration of test substance.

The use of a compound-specific analytical method or of ^{14}C-labelled test substance may allow greater sensitivity. In the case of ^{14}C test compound, the recovery of the $^{14}CO_2$ will confirm that biodegradation has occurred.

When results are given in terms of primary biodegradation, an explanation should, if possible, be given on the chemical structure change that leads to the loss of response of the parent test substance.

The validation of the analytical method must be given together with the response found on the blank test medium.

4. REFERENCES

(1) OECD, Paris, 1981, *Test Guideline 302 B,* Decision of the Council C(81) 30 final.

(2) Annex V C.9 Degradation: Chemical Oxygen Demand, Commission Directive 84/449/EEC, *Official Journal of the European Communities,* No L 251, 19. 9. 1984.

Appendix

EVALUATION EXAMPLE

Organic compound: 4-Ethoxybenzoic acid
Theoretical test concentration: 600 mg/l
Theoretical DOC: 390 mg/l

Inoculum Sewage Treatment plant of . . .
Concentration: 1 gram dry material/litre
Adaptation status: not adapted

Analysis: DOC-determination

Amount of sample: 3 ml

Control substance: Diethyleneglycol

Toxicity of compound: No toxic effects below 1 000 mg/l
Test used: Fermentation tubes test

Test time	Control substance				Test substance		
	Blank DOC (1) mg/l	DOC (1) mg/l	DOC net mg/l	Degradation %	DOC (1) mg/l	DOC net mg/l	Degradation %
0	—	—	300,0	—	—	390,0	—
3 hours	4,0	298,0	294,0	2	371,6	367,6	6
1 day	6,1	288,3	282,2	6	373,3	367,2	6
2 days	5,0	281,2	276,2	8	360,0	355,0	9
5 days	6,3	270,5	264,2	12	193,8	187,5	52
6 days	7,4	253,3	245,9	18	143,9	136,5	65
7 days	11,3	212,5	201,2	33	104,5	93,2	76
8 days	7,8	142,5	134,7	55	58,9	51,1	87
9 days	7,0	35,0	28,0	91	18,1	11,1	97
10 days	18,0	37,0	19,0	94	20,0	2,0	99

(1) Mean values of triplicate determinations.

Figure 1

Examples of biodegradation curves

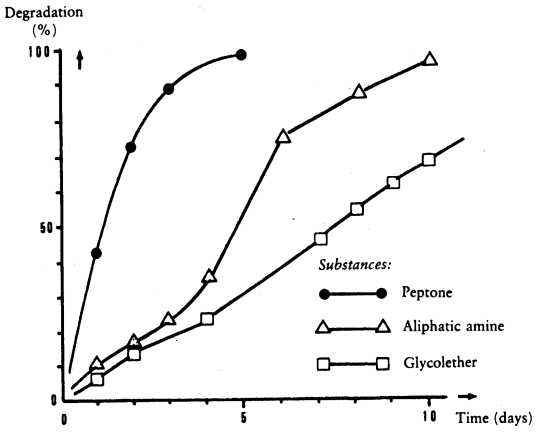
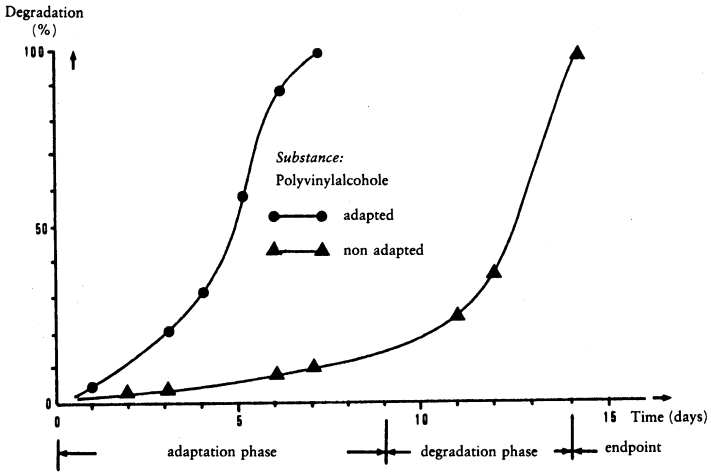


Figure 2

Examples of sludge adaptation



C. 10

BIODEGRADATION

ACTIVATED SLUDGE SIMULATION TESTS

1. METHOD

1.1. Introduction

1.1.1. *General remarks*

The method is applicable only to those organic substances which, at the concentration used in the test:

— are soluble in water to the extent necessary for the preparation of the test solutions,

— have negligible vapour pressure under the test conditions,

— are not inhibitory to bacteria.

Information on the relative proportions of the major components of the test material will be useful in interpreting the results obtained, particularly in those cases where the results are low or marginal.

Information on the toxicity of the substance to micro-organisms is desirable for the interpretation of the low results and in the selection of appropriate test concentrations.

1.1.2. *Determination of ultimate biodegradability (DOC/COD analysis)*

The purpose of the method is to determine the ultimate biodegradability by the measurement of the removal of the substance and any metabolites in an activated sludge plant model at a concentration corresponding to > 12 mg DOC/litre (or approximately 40 mg COD/litre); 20 mg DOC/litre seem to be optimal. (DOC = Dissolved Organic Carbon; COD = Chemical Oxygen Demand).

The organic carbon content (or the chemical oxygen demand) of the test material must be established.

1.1.3. *Determination of primary biodegradability (specific analysis)*

The purpose of the method is the determination of the primary biodegradability of a substance in an activated sludge plant model, at a concentration of about 20 mg/litre, using a specific analytical method (lower or higher concentration can be used if analytical method and consideration of toxicity permits). This allows the assessment of the primary biodegradability of the substance (disappearance of the parent chemical structure).

The purpose of this method is *not* the determination of the mineralization of the tested substance.

An adequate analytical method for the determination of the tested substance must be available.

1.2. Definitions and units

1.2.1. *DOC/COD analysis*

The degree of removal of the substance is given by:

$$DR = \frac{T - (E - E_o)}{T} \times 100\% \qquad [1\ (a)]$$

where:

DR = degree of removal in percent DOC (or COD) within the given mean retention time with respect to the test material,

T = concentration of the test material in the influent in mg DOC/litre (or mg COD/litre),

E = DOC (or COD) concentration in the effluent of the test unit in mg DOC/litre (or mg COD/litre),

E_o = DOC (or COD) concentration in the effluent of the blank unit in mg DOC/litre (or mg COD/litre).

The degradation is stated as the percentage DOC (or COD) removal within the given retention time with respect to the test material.

1.2.2. *Specific analysis*

The percentage elimination of the tested substance from the aqueous phase (R_W) within the given mean retention time is given by

$$R_W = \frac{C_I - C_O}{C_I} \times 100\% \qquad [1\,(b)]$$

where:

C_I = concentration of the substance in the influent of the test unit (mg substance/litre, determined by specific analysis),

C_O = concentration of the substance in the effluent of the test unit (mg substance/litre, determined by specific analysis).

1.3. **Reference substances**

In some cases when investigating a new substance, reference substances may be useful; however, specific reference substances cannot yet be recommended.

1.4. **Principle of the test methods**

For the determination of ultimate biodegradability, two activated sludge pilot units (OECD confirmatory test or porous pot units) are run in parallel. The test substance is added to the influent (synthetic or domestic sewage) of one of the units, while the other receives the sewage alone. For the determination of primary biodegradation with specific analysis in the influent and effluent, only one unit is used.

The DOC (or COD) concentrations are measured in the effluents, or the substance concentrations are determined by specific analysis.

The DOC due to test material is not measured but simply stated.

When DOC (or COD) measurements are performed, the difference in mean concentrations between the test and the control effluents is assumed to be due to undegraded test material.

When specific analyses are preformed, change in the concentration of the parent molecule can be measured (primary biodegradation).

The units may be operated following the 'coupled units mode', by a transinoculation procedure.

1.5. **Quality criteria**

The starting concentration of the substance depends on the type of analysis performed and its limitation.

1.6. **Description of the test method**

1.6.1. *Preparation*

1.6.1.1. Apparatus

A pair of units of the same type are needed except when specific analyses are performed. Two types of device may be used:

OECD confirmatory test

The equipment (Appendix 1) consists of a storage vessel (A) for synthetic sewage, dosing pump (B), aeration vessel (C), separator (D), air-lift pump (E), to recycle activated sludge, and vessel (F) for collecting the treated effluent.

Vessels (A) and (F) must be of glass or suitable plastic and hold at least 24 litres. Pump (B) must provide a constant flow of synthetic sewage to the aeration vessel; any suitable system may be used, providing that input flow and concentration are assured. During normal operation the height of separator (D) is so fixed that the volume

contained in the aeration vessel is three litres of mixed liquor. A sintered aeration cube (G) is suspended in vessel (C) at the apex of the cone. The quantity of air blown through the aerator may be monitored by means of a flow meter.

Air-lift pump (E) is set so that the activated sludge from the separator is continually and regularly recycled to aeration vessel (C).

'Porous pot'

The porous pot is constructed from sheets of porous polyethylene (2 mm thick, maximum pore size 95 µm), which are made into cylinders 14 cm in diameter with a conical base at 45° (Figures 1 and 2 of Appendix 2). The porous pot is contained in an impervious vessel of suitable plastic 15 cm in diameter with an outlet at a height of 17,2 cm on the cylindrical part, which determines the volume (3 litres) in the pot. There is a rigid supporting ring made of suitable plastic around the top of the inner vessel, so that there is an effluent space of 0,5 cm between the inner and outer vessels.

The porous pots may be mounted in the base of a thermostatically controlled water-bath. There is an air supply to the base of the inner vessel on which are placed suitable diffusers.

Vessels (A) and (E) must be of glass or suitable plastic and hold at least 24 litres. Pump (B) must provide a constant flow of synthetic sewage to the aeration vessel; any suitable system may be used, providing that input flow and concentration are assured.

Spare inner porous pots are required to replace any which may block in use; blocked pots are cleaned by 24-hour immersion in hypochlorite solution followed by thorough washing in tap water.

1.6.1.2. Filtration

Membrane filtration apparatus and membrane filters with a pore size of 0,45 µm. Membrane filters are suitable if it is assured that they neither release carbon nor adsorb the substance in the filtration step.

1.6.1.3. Sewage

Either suitable synthetic feed or domestic sewage may be used.

Example of synthetic feed

Dissolve in each litre of tap water:

Peptone:	160 mg,
Meat extract:	110 mg,
Urea:	30 mg,
NaCl:	7 mg,
$CaCl_2 . 2H_2O$:	4 mg,
$MgSO_4 . 7H_2O$:	2 mg,
K_2HPO_4:	28 mg.

Domestic sewage

This should be collected freshly each day from the overflow of the primary settlement tank of a treatment plant treating predominantly domestic sewage.

1.6.1.4. Stock solution of test material

A solution of test material, e.g. 1%, should be prepared for addition to the test unit. The concentration of the material must be determined, so that the appropriate volume to be added to the sewage or directly to the unit via a second pump to give the required test concentration is known.

1.6.1.5. Inoculum

Remark: When domestic sewage is used, there would be no point in using an inoculum of low bacterial concentration, but activated sludge may be used.

A variety of inocula may be used.

Three examples of suitable inoculum are given:

(a) Inoculum from secondary effluent

The inoculum should be obtained from a secondary effluent of good quality collected from a treatment plant dealing with predominantly domestic sewage. The effluent must be kept under aerobic conditions in the period between sampling and use. To prepare the inoculum, the sample is filtered through a coarse filter, the first 200 ml being discarded. The filtrate is kept aerobic until used. The inoculum must be used on the day of collection. At least 3 ml are to be used for inoculation.

(b) Composite inoculum

Inoculum from secondary effluent:

See description above.

Inoculum from soil:

100 g of garden soil (fertile, not sterile) are suspended in 1 000 ml chlorine-free drinking water. (Soils with an extremely large fraction of clay, sand or humus are unsuitable.) After stirring, the suspension is allowed to settle for 30 minutes. The supernatant is filtered through a coarse filter paper, the first 200 ml being discarded. The filtrate is aerated immediately and until use. The inoculum must be used on the day of collection.

Inoculum from a surface water:

A further partial inoculum is drawn from a mesosaprobic surface water. The sample is filtered through a coarse paper, the first 200 ml being discarded. The filtrate is kept aerobic until used. The inoculum must be used on the day of collection.

Equal volumes of the three partial inoculum samples are united, mixed well, and the final inoculum drawn from this mixture. At least 3 ml are to be used for inoculation.

(c) Inoculum from activated sludge

A volume (not more than 3 litres) of activated sludge (suspended solids content of up to 2,5 g/litre) taken from the aeration tank of a plant treating predominantly domestic sewage may be used as an inoculum.

1.6.2. *Procedure*

The test is performed at room temperature; this should be kept between 18 and 25 °C.

If it is appropriate, the test may be performed at a lower temperature (down to 10 °C); if the substance is degraded then no further work is normally required. If, however, the substance is not degraded, the test must be conducted at a steady temperature between 18 and 25 °C.

1.6.2.1. Running-in period: Sludge formation/stabilization of the units

The sludge growth/stabilization period is the period during which the concentration of the activated sludge suspended solids and the performance of the units progress to a steady state under the operating conditions used.

The running-in period is the period which lasts from the time the test substance is first added to the time when its removal reaches a plateau (relatively constant value). This period must not exceed six weeks.

The evaluation period is a three weeks period, three weeks from the time that the removal of the test substance reaches a relatively constant, and ususally high, value. For those substances which show little or no degradation in the first six weeks, the evaluation period is taken as the following three weeks.

Initially, fill the unit(s) needed for one test with the inoculum mixed with influent.

The aerator (and air lift (E) in the case of the OECD confirmatory test units) and dosing device (B) are then set in operation.

Influent without substance to be tested must pass through the aeration vessel (C) either at the rate of one litre per hour or a rate of one-half litre per hour; this gives a mean retention time of either three or six hours.

The rate of aeration should be regulated so that the content of vessel (C) is kept constantly in suspension while the dissolved oxygen content is at least 2 mg/litre.

Foaming must be prevented by appropriate means. Anti-foaming agents which inhibit the activated sludge must not be used.

The sludge which has accumulated around the top of the aeration vessel (C) (and, in the case of the OECD confirmatory test units, in the base of the settling vessel (D), and in the circulation circuit) must be returned to the mixed liquor at least once each day by brushing or some other appropriate means.

When sludge fails to settle, its density may be increased by addition of 2 ml portions of a 5 % solution of ferric chloride, repeated as necessary.

The effluent is collected in vessel (E or F) for 20 to 24 hours, and a sample is taken after thorough mixing. Vessel (E or F) must be carefully cleaned.

In order to monitor and control the efficiency of the process, the chemical oxygen demand (COD) or the dissolved organic carbon (DOC) of the filtrate of the accumulated effluent is measured at least twice weekly, as well as that of the filtered influent (using a membrane of pore size 0,45 µm, the first 20 ml (approximately) of the filtrate are discarded).

The reduction in COD or DOC should level off when a roughly regular daily degradation is obtained.

The dry matter content of the activated sludge in the aeration tank should be determined twice a week (in g/litre). The units may be operated in one of two ways: either the content of dry matter in the activated sludge should be determined twice a week, and, if it is more than 2,5 g/litre, the excess activated sludge must be discarded, or 500 ml of mixed liquor is wasted from each pot daily to give a mean sludge retention time of six days.

When the measured and estimated parameters (efficiency of the process (in COD or DOC removal), sludge concentration, sludge settleability, turbidity of the effluents, etc.) of the two units are sufficiently steady, the test substance may be introduced in the influent of one of the units, following 1.6.2.2.

Alternatively, the test substance may be added at the beginning of the sludge growth period (1.6.2.1), especially when sludge is added as the inoculum.

1.6.2.2. Test procedure

The operating conditions of the running-in period are maintained and sufficient stock solution (approximately 1 %) of the test material is added to the influent of the test unit so that the desired concentration of test material (approximately 10 to 20 mg DOC/litre or 40 mg COD/litre) in the sewage is obtained. This can be done by mixing the stock solution to the sewage daily or by means of a separate pumping system. This concentration may be reached progressively. If there are no toxic effects of the test substance on the activated sludge, higher concentrations can also be tested.

The blank unit is fed only with influent without added substances. Adequate volumes of the effluents are taken for analysis and filtered through membrane filters (0,45 µm) the first 20 ml (approximately) of filtrate being discarded.

The filtered samples have to be analysed on the same day, otherwise they must be preserved by any suitable method, for example, by using 0,05 ml of a 1 % mercuric chloride ($HgCl_2$) solution for each 10 ml of filtrate or by storing them at 2 to 4 °C up to 24 hours, or below – 18 °C for longer periods.

The running-in time, with addition of test substance, should not exceed six weeks and the evaluation period should not be shorter than three weeks, i.e. about 14 to 20 determinations should be available for calculation of the final result.

Coupled units mode

The coupling of the units is achieved by interchanging 1,5 litres of mixed liquor (including sludge) from the activated sludge aeration vessels between the two units once a day. In the case of strongly absorbing test materials, 1,5 litres of supernatant liquid only are drawn from the settling vessels and poured into the activated sludge vessel of the other unit.

1.6.2.3. Analysis

Two kinds of analyses may be performed in order to follow the behaviour of the substance:

DOC and COD

The DOC concentrations are performed in duplicate with the carbon analyser and/or the COD values according to reference (2).

Specific analysis

The concentrations of the tested substance are determined by a suitable analytical method. When possible, specific determination of the substance absorbed on sludge should be performed.

2. DATA AND EVALUATION

2.1. Coupled units mode

When using 'coupled units mode', the daily degrees of removal, DR are calculated according to 1.2.1.

These daily degrees of removal DR are corrected to DRc for the material transfer due to the transinoculation procedure with equation [2] for a three-hour or equation [3] for a six-hour mean retention time.

$$DRc = \frac{8}{7} DR - \frac{100}{7} \qquad [2]$$

$$DRc = \frac{4}{3} DR - \frac{100}{3} \qquad [3]$$

The mean of the series of DRc values is calculated and in addition the standard deviation according to equation [4]

$$s_{DRc} = \sqrt{\frac{\sum_{i=1}^{n} (\overline{DRc} - DRc_i)^2}{n - 1}} \qquad [4]$$

where:

s_{DRc} = standard deviation of the series of DRc values,

$\overline{DRc}$ = mean of DRc value,

n = number of determinations.

Outliers of the DRc series are eliminated according to a suitable statistical procedure, e.g. Nalimov (6), at the 95 % probability level and the mean and the standard deviation of the outlier-free DRc data set are recalculated.

The final result is then calculated with equation [5] as

$$DRc = \overline{DRc} \pm \frac{t_{n-1;\alpha}\, s_{DRc}}{\sqrt{n}} \qquad [5]$$

where:

$t_{n-1;\alpha}$ = table value of t for n value pairs of E and E_O and statistical confidence P ($P = 1 - \alpha$) whereby P is at 95 % (1).

The result is stated as the mean with tolerance limits at the 95 % probability level, the respective standard deviation and the number of data of the outlier-free DRc data set, and the number of outliers, e.g.

DRc = 98,6 ± 2,3 % DOC removal,

s = 4,65 % DOC removal,

n = 18,

x = number of outliers.

2.2. **Non-coupled units mode**

The performance of the units may be checked as follows:

$$\text{percentage removal of COD or DOC} = \frac{\text{COD or DOC of sewage — COD or DOC of effluent}}{\text{COD or DOC sewage}} \times 100$$

These daily removals may be plotted graphically to reveal any trends, e.g. to acclimatization.

2.2.1. *Using COD/DOC determinations*

The daily degree of removal DR is calculated according to 1.2.1.

The mean of the series of DR values is calculated; in addition, its standard deviation is calculated according to:

$$s_{DR} = \sqrt{\frac{\sum_{i=1}^{n} (\overline{DR} - DR_i)^2}{n - 1}} \qquad [6]$$

where:

s_{DR} = standard deviation of the series of DR_i values,

$\overline{DR}$ = mean of DR_i values,

n = number of determinations.

Outliers of the DR series are eliminated according to a suitable statistical procedure, e.g. Nalimov (6), at the 95 % probability level, and the mean and the standard deviation of the outliers-free DR set are recalculated.

The final result is then calculated with equation [7] as

$$DR = \overline{DR} \pm \frac{t_{n-1;\alpha}}{\sqrt{n}} s_{DR} \qquad [7]$$

where:

$t_{n-1;\alpha}$ = table value of t for n value pairs of E and E_O and statistical confidence P ($P = 1 - \alpha$) whereby P is set at 95 % (1).

The result is stated as the mean with tolerance limits at the 95 % probability level, the respective standard deviation and the number of data of the outlier free DR data set, and the number of outliers, e.g.

DR = (98,6 ± 2,3) % DOC removal,

s = 4,65 % DOC removal,

n = 18,

x = number of outliers.

2.2.2. *Using specific analysis*

The percentage of elimination of the tested substance from the aqueous phase (R_W) is calculated according to 1.2.2.

3. REPORTING

3.1. Test report

The test report shall, if possible, contain the following:

— the formsheet given in Appendix 3, showing the operating conditions for the test,

— which apparatus was chosen (OECD confirmatory test or porous pot),

— which operating mode was chosen: coupled units mode or not,

— which sewage: synthetic or domestic — in the case of domestic sewage, date and location of sample,

— which inoculum, with date and location of sample,

— a statement with description of the analytical method if specific analyses were performed,

— plot of COD or DOC removal *versus* time, including running-in and evaluation period,

— analytical recovery of the test substance as COD of DOC in the stock solution,

— if specific analyes were performed, plot of the percentage removal of the tested substance from the aqueous phase *versus* time (running-in and evaluation period),

— the mean removal of DOC or COD of test substance and standard deviation are calculated from the results of the evaluation period, i.e. when there is a steady removal of test material or period of steady operation,

— plot of activated sludge concentration *versus* time,

— any remark concerning the activated sludge (discard of excess sludge, presence of bulking, $FeCl_3$, etc.),

— concentration of the substance used in the test,

— any results concerning analysis done on the sludge,

— all information and experimental results concerning the test substance and the reference substance if used,

— scientific reasons for any changes of the procedure.

3.2. **Interpretation of results**

Low removal of the tested substance from the aqueous phase may be due to inhibition of micro-organisms by the test substance. This may also be revealed by lysis and loss of sludge, giving a turbid supernatant, and by a decrease of the COD (or DOC) removal efficiency of the pilot plant.

Physico-chemical adsorption can sometimes play a role. Differences between biological action on the molecule and phyisico-chemical adsorption may be revealed by analysis performed on the sludge after an adequate desorption.

Further tests are necessary if a distinction is to be drawn between biodegradation (or partial biogradation) and adsorption.

This can be done in a number of ways, but the most convincing is to use the supernatant as inoculum in a base-set test (respirometric test preferably).

If high DOC or COD removals are observed, then this is due to biodegradation while, at low removals, biodegradation is indistinguishable from elimination. For example, if a soluble compound exhibits a high adsorption constant of 98 % and the surplus sludge wastage rate is 10 % per day, an elimination of up to 40 % is possible; at a surplus sludge wastage rate of 30 % elimination due to adsorption on and removal with surplus sludge may amount to up to 65 % (4).

When using specific analysis, attention should be paid to the relationship between the structure of the substance and the specific analysis used. In this case, the phenomenon observed cannot be interpreted as a mineralization of the substance.

4. **REFERENCES**

(1) OECD, Paris, 1981, *Test Guideline 303 A,* Decision of the Council C(81) 30 final.

(2) Annex V C9 Degradation Test — Chemical Oxygen Demand, Commission Directive 84/449/EEC, *Official Journal of the European Communities,* No L 251, 19. 9. 1984.

(3) Painter, H. A., King, E. F., *WRC Porous-Pot method for assessing biodegradability,* Technical Report TR70, June 1978, Water Research Centre, United Kingdom.

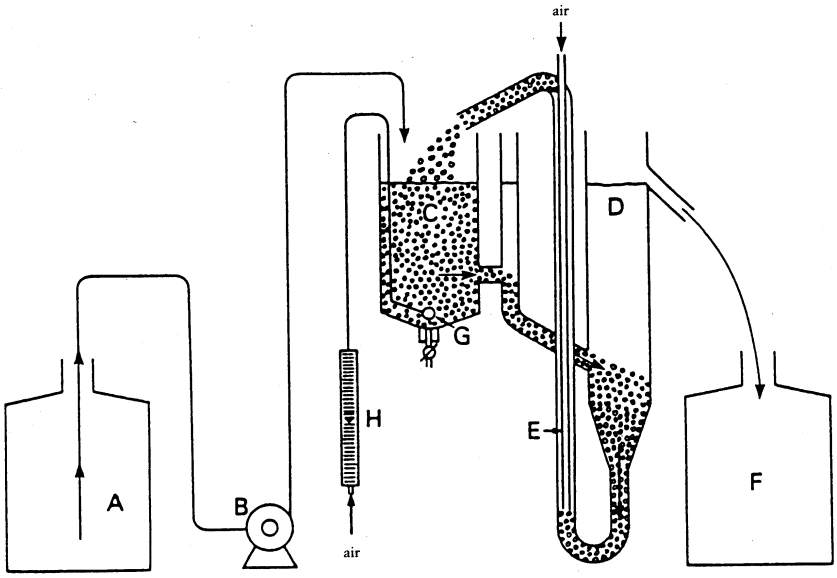
(4) Wierich, P., Gerike, P., The fate of soluble, recalcitrant, and adsorbing compounds in activated sludge plants, *Ecotoxicology and Environmental Safety,* vol. 5, No 2, June 1981, pp. 161 to 171.

(5) Council Directives 82/242/EEC and 82/243/EEC, *Official Journal of the European Communities,* No L 109, 22. 4. 1982, amending Council Directives 73/404/EEC and 73/405/EEC on biodegradability of detergents, *Official Journal of the European Communities,* No L 347, 17. 12. 1973.

(6) Streuli, H., Fehlerhafte Interpretation und Anwendung von Ausreißertests, insbesondere bei Ringversuchen zur Überprüfung analytisch-chemischer Untersuchungsmethoden, *Fresenius-Zeitschrift für Analytische Chemie,* 303 (1980), pp. 406 to 408.

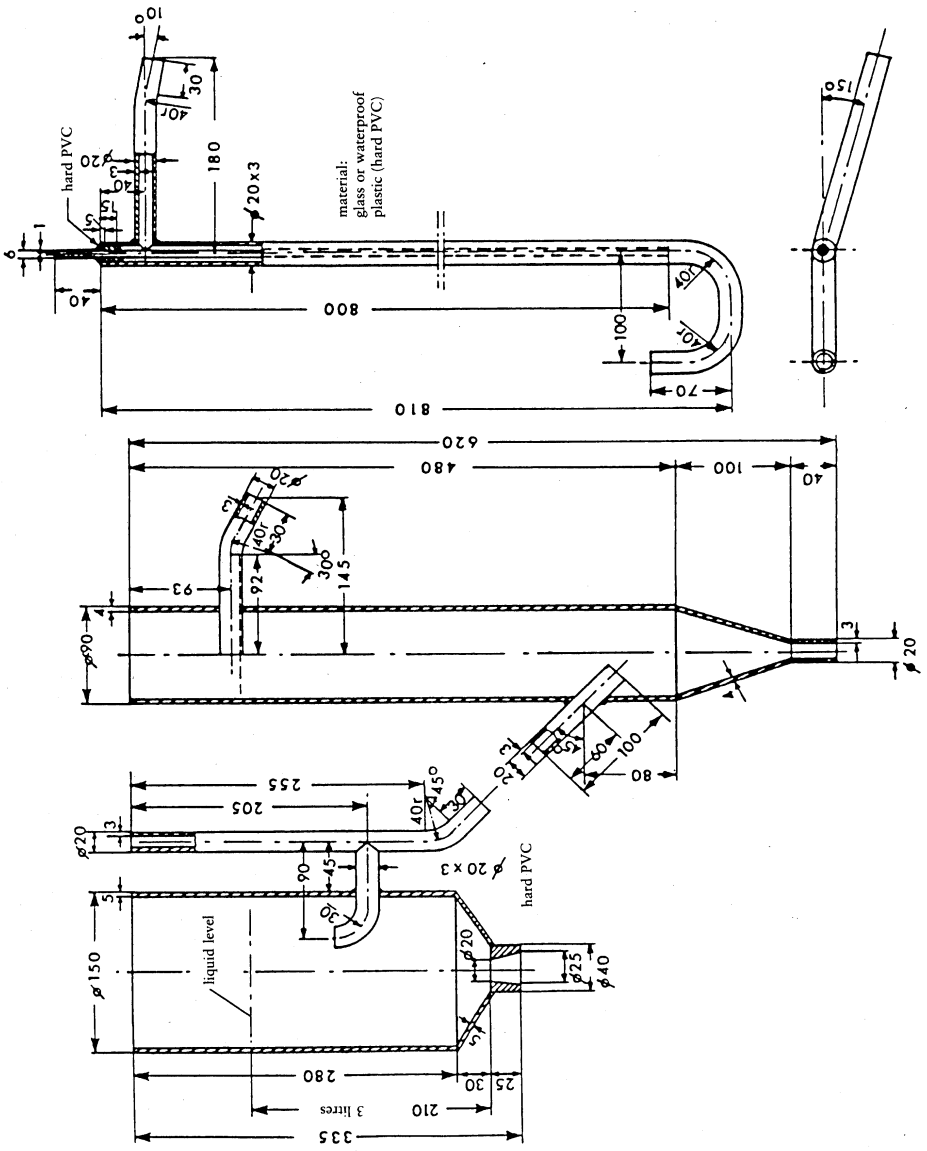
APPENDIX 1

Figure 1



Key: A = storage vessel;
B = dosing device;
C = aeration chamber (3 l capacity);
D = settling vessel;
E = air lift;
F = collector;
G = aerator;
H = air flow meter (optional).

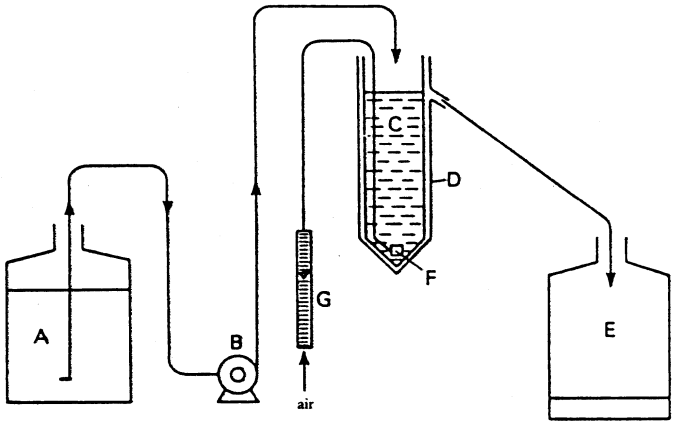
Figure 2


material:
glass or waterproof
plastic (hard PVC)
hard PVC
20x3
180
800
810
100
70
40
150
620
480
100
40
145
92
93
90
20
80
255
205
45°
90
45
liquid level
280
210
3 litres
335
25
30
ø150
ø25
ø40

APPENDIX 2

Figure 1

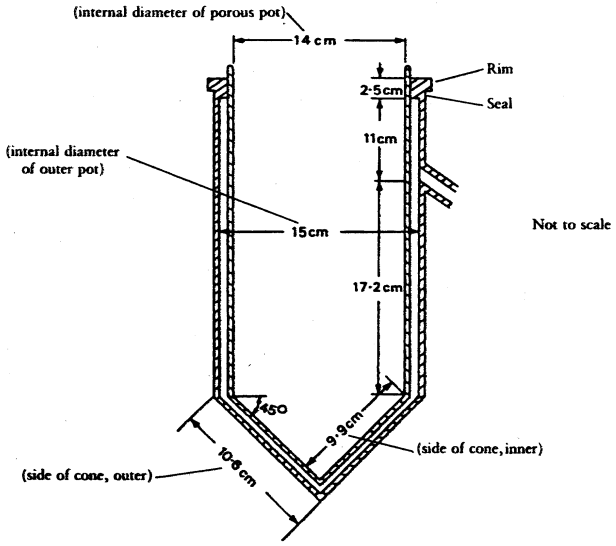
Equipment used for assessing biodegradability



Key: A = storage vessel;
B = dosing pump;
C = porous aeration vessel;
D = outer impermeable vessel;
E = effluent collection vessel;
F = diffuser-stone aerator;
G = rotameter (optional).

Figure 2

Details of three-litre porous-pot aeration vessel



Appendix 3

Operating conditions for the Activated Sludge Simulation Test

Check in each group

Apparatus

OECD confirmatory ☐
Porous pot ☐

Mode of operation

Single unit ☐
Coupled units ☐
Non-coupled units ☐

Transinoculation

None ☐
Activated sludge ☐
Supernatant ☐

Mean retention time

Three hours ☐
Six hours ☐

Base nutrient

Domestic sewage ☐
Synthetic sewage ☐

Inoculum

Secondary effluent ☐
Composite ☐
Activated sludge ☐

Test material addition

From the start ☐
Stepwise increase ☐
After sludge has formed ☐

Analysis

Specific ☐
COD ☐
DOC ☐

C. 11

BIODEGRADATION

ACTIVATED SLUDGE RESPIRATION INHIBITION TEST

1. METHOD

1.1. Introduction

The method described assesses the effect of a test substance on micro-organisms by measuring the respiration rate under defined conditions in the presence of different concentrations of the test substance.

The purpose of this method is to provide a rapid screening method whereby substances which may adversely affect aerobic microbial treatment plants can be identified, and to indicate suitable non-inhibitory concentrations of test substances to be used in biodegradability tests.

A range-finding test may precede a definitive test. It provides information about the range of concentrations to be used in the main test.

Two controls without test substance are included in the test design, one at the start and the other at the end of the test series. Each batch of activated sludge should also be checked using a reference substance.

This method is most readily applied to substances which, due to their water solubility and low volatility, are likely to remain in water.

For substances with limited solubility in the test media, it may not be possible to determine the EC_{50}.

Results based on oxygen uptake may lead to erroneous conclusions when the test substance has the propensity to uncouple oxidative phosphorylation.

It is useful to have the following information to perform the test:

— water solubility,

— vapour pressure,

— structural formula,

— purity of the test substance.

Recommendation

Activated sludge may contain potentially pathogenic organisms and should be handled with care.

1.2. Definitions and units

The respiration rate is the oxygen consumption of waste-water micro-organisms in aerobic sludge, expressed generally as mg O_2 per mg of sludge per hour.

In order to calculate the inhibitory effect of a test substance at a particular concentration, the respiration rate is expressed as a percentage of the mean of the two control respiration rates:

$$\left(1 - \frac{2R_s}{Rc_1 + Rc_2}\right) \times 100 = \text{per cent inhibition}$$

where:

R_s = oxygen-consumption rate at tested concentration of test substance,

Rc_1 = oxygen-consumption rate, control 1,

Rc_2 = oxygen-consumption rate, control 2.

EC_{50} in this method is the concentration of the test substance at which the respiration rate is 50 % of that shown by the control under conditions described in this method.

1.3. **Reference substances**

It is recommended that 3,5-dichlorophenol, as a known inhibitor of respiration, be used as a reference substance and tested for EC_{50} on each batch of activated sludge as a means of checking that the sensitivity of the sludge is not abnormal.

1.4. **Principle of the test method**

The respiration rate of an activated sludge fed with a standard amount of synthetic sewage feed is measured after a contact time of 30 minutes or three hours, or both. The respiration rate of the same activated sludge in the presence of various concentrations of the test substance under otherwise identical conditions is also measured. The inhibitory effect of the test substance at a particular concentration is expressed as a percentage of the mean respiration rates of two controls. An EC_{50} value is calculated from determinations at different concentrations.

1.5. **Quality criteria**

The test results are valid if:

— the two control respiration rates are within 15 % of each other,

— the EC_{50} (30 minutes and/or three hours) of 3,5-dichlorophenol is in the accepted range 5 to 30 mg/litre.

1.6. **Description of the test method**

1.6.1. *Reagents*

1.6.1.1. Solutions of the test substance

Solutions of the test substance are freshly prepared at the start of the study using a stock solution. A stock solution concentration of 0,5 g/litre is appropriate if the procedure recommended below is followed.

1.6.1.2. Solution of control substance

A solution of 3,5-dichlorophenol can for example be prepared by dissolving 0,5 g 3,5-dichlorophenol in 10 ml of 1M NaOH, diluting to approximately 30 ml with distilled water, adding under stirring 0,5M H_2SO_4 to the point of incipient precipitation — approximately 8 ml of 0,5M H_2SO_4 will be required — and finally diluting the mixture to one litre with distilled water. The pH should then be in the range 7 to 8.

1.6.1.3. Synthetic sewage

A synthetic sewage feed is made by dissolving the following amounts of substances in one litre of water:

— 16 g peptone,
— 11 g meat extract,
— 3 g urea,
— 0,7 g NaCl,
— 0,4 g $CaCl_2.2H_2O$,
— 0,2 g $MgSO_4.7H_2O$,
— 2,8 g K_2HPO_4.

Note 1: 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 the addition of dipotassium hydrogen phosphate.

Note 2: If the prepared medium is not used immediately, it shall be stored in the dark at 0 to 4 °C, for no longer than one week, in conditions which do not produce any change in its composition. The medium may also be sterilized prior to storage, or the peptone and meat extract may be added shortly before carrying out the test. Before use, it shall be mixed thoroughly and the pH adjusted.

1.6.2. *Apparatus*

Measuring apparatus: The precise design is not critical. However, there should be head space and the probe should fit tightly in the neck of the measuring flask.

Normal laboratory equipment and especially the following is necessary:

— measuring apparatus,

— aeration device,

— pH-electrode and measuring equipment,

— O_2-electrode.

1.6.3. *Preparation of the inoculum*

Activated sludge from a sewage treatment plant treating predominantly domestic sewage is used as the mircobial inoculum for the test.

If necessary, on return to the larboratory, coarse particles may be removed by settling for a short period, e.g. 15 minutes, and decanting the upper layer of finer solids for use. Alternatively, the sludge may be mixed using a blender for a few seconds.

In addition, if it is thought that inhibitory material is present, the sludge should be washed with tap water or an isotonic solution. After centrifuging, the supernatant is decanted (this procedure is repeated three times).

A small amount of the sludge is weighed and dried. From this result, the amount of wet sludge can be calculated which must be suspended in water in order to obtain an activated sludge with a mixed liquor suspended solids range between 2 and 4 g/litre. This level gives a concentration between 0,8 and 1,6 g/litre in the test medium if the procedure recommended below is followed.

If the sludge cannot be used on the day of collection, 50 ml of synthetic sewage is added to each litre of the activated sludge prepared as described above; this is then aerated overnight at 20 ± 2 °C. It is then kept aerated for use during the day. Before use the pH is checked and adjusted, if necessary, to pH 6 to 8. The mixed liquor suspended solids should be determined as described in the preceding paragraph.

If the same batch of sludge is required to be used on subsequent days (maximum four days), a further 50 ml of synthetic sewage feed is added per litre of sludge at the end of each working day.

1.6.4. *Performance of the test*

Duration/contact time:	30 minutes and/or three hours, during which aeration takes place
Water:	Drinking water (dechlorinated if necessary)
Air supply:	Clean, oil-free air. Air flow 0,5 to 1 litre/minute
Measuring apparatus:	Flat bottom flask such as a BOD-flask
Oxygen meter:	Suitable oxygen electrode, with a recorder
Nutrient solution:	Synthetic sewage (see above)
Test substance:	The test solution is freshly prepared at the start of the test
Reference substance:	e.g. 3,5-dichlorophenol (at least three concentrations)
Controls:	Inoculated sample without test substance
Temperature:	20 ± 2 °C.

A suggested experimental procedure which may be followed for both the test and reference substance for the three-hour contact period is given below:

Several vessels (e.g. one-litre beakers) are used.

At least five concentrations, spaced by a constant factor preferably not exceeding 3,2, should be used.

At time '0', 16 ml of the synthetic sewage feed are made up to 300 ml with water. 200 ml of microbial inoculum are added and the total mixture (500 ml) poured into a first vessel (first control C_1).

The test vessels should be aerated continuously such as to ensure that the dissolved O_2 does not fall below 2,5 mg/litre and that, immediately before the measurement of the respiration rate, the O_2 concentration is about 6,5 mg/litre.

At time '15 minutes' (15 minutes is an arbitrary, but convenient, interval) the above is repeated, except that 100 ml of the test substance stock solution are added to the 16 ml of synthetic sewage before adding water to 300 ml and microbial inoculum to make a volume of 500 ml. This mixture is then poured into a second vessel and aerated as above. This process is repeated at 15-minute intervals with different volumes of the test substance stock solution to give a series of vessels containing different concentrations of the test substance. Finally, a second control is prepared (C_2).

After three hours the pH is recorded, and a well-mixed sample of the contents of the first vessel is poured into the measuring apparatus and the respiration rate is measured over a period of up to 10 minutes.

This determination is repeated on the contents of each vessel at 15-minute intervals, in such a way that the contact time in each vessel is three hours.

The reference substance is tested on each batch of microbial inoculum in the same way.

A different regime (e.g. more than one oxygen meter) will be necessary when measurements are to be made after 30 minutes of contact.

If mesurement of the chemical oxygen consumption is required, further vessels are prepared containing test substance, synthetic sewage feed and water, but no activated sludge. Oxygen consumption is measured and recorded after an aeration time of 30 minutes and/or three hours (contact time).

2. DATA AND EVALUATION

The respiration rate is calculated from the recorder trace between approximately 6,5 mg O_2/litre and 2,5 mg O_2/litre, or over a 10-minute period when the respiration rate is low. The portion of the respiration curve over which the respiration rate is measured should be linear.

If the respiration rates of the two controls are not within 15 % of each other, or the EC_{50} (30 minutes and/or three hours) of the reference substance is not in the accepted range (5 to 30 mg/litre for 3,5-dichlorophenol), the test is invalid and must be repeated.

The per cent inhibition is calculated at each test concentration (see 1.2). The per cent inhibition is plotted against concentration on log-normal (or log-probability) paper, and an EC_{50} value derived.

95 % confidence limits for the EC_{50} values can be determined using standard procedures.

3. REPORTING

3.1. Test report

The test report shall, if possible, contain the following:

- test substance: chemical identification data,
- test system: source, concentration and any pre-treatment of the activated sludge,
- test conditions:
  - pH of the reaction mixture before the respiration measurement,
  - test temperature,
  - test duration,
  - reference substance and its measured EC_{50},
  - abiotic oxygen uptake (if any).
- results:
  - all measured data,
  - inhibition curve and method for calculation of EC_{50},
  - EC_{50} and, if possible, 95 % confidence limits, EC_{20} and EC_{80},
  - all observations and any deviations from this test method which could have influenced the result.

3.2. Interpretation of data

The EC_{50} value should be regarded merely as a guide to the likely toxicity of the test substance either to activated sludge sewage treatment or to waste-water microorganisms, since the complex interactions occurring in the environment cannot be accurately simulated in a laboratory test. In addition, test substances which may have an inhibitory effect on ammonia oxidation may also produce atypical inhibition curves. Accordingly, such curves should be interpreted with caution.

4. REFERENCES

(1) International Standard ISO 8192-1986.

(2) Broecker, B., Zahn, R., *Water Research* 11, 1977, p. 165.

(3) Brown, D., Hitz, H. R., Schaefer, L., *Chemosphere* 10, 1981, p. 245.

(4) ETAD (Ecological and Toxicological Association of Dyestuffs Manufacturing Industries), *Recommended Method No 103,* also described by:

(5) Robra, B., *Wasser/Abwasser* 117, 1976, p. 80.

(6) Schefer, W., *Textilveredlung* 6, 1977, p. 247.

(7) OECD, Paris, 1981, *Test Guideline 209,* Decision of the Council C(81) 30 final.

C. 12

BIODEGRADATION

MODIFIED SCAS TEST

1. METHOD

1.1. Introduction

The purpose of the method is the evaluation of the potential ultimate biodegradability of water-soluble, non-volatile organic substances when exposed to relatively high concentrations of micro-organisms over a long time period. The viability of the microorganisms is maintained over this period by daily addition of a settled sewage feed. (For weekend requirements, the sewage may be stored at 4 °C. Alternatively, the synthetic sewage of the OECD confirmatory test may be used.)

Physico-chemical adsorption on the suspended solids may take place and this must be taken into account when interpreting results (see 3.2).

Because of the long detention period of the liquid phase (36 hours), and the intermittent addition of nutrients, the test does not simulate those conditions experienced in a sewage treatment plant. The results obtained with various test substances indicate that the test has a high biodegradation potential.

The conditions provided by the test are highly favourable to the selection and/or adaptation of micro-organisms capable of degrading the test compound. (The procedure may also be used to produce acclimatized inocula for use in other tests.)

In this method, the measure of the concentration of dissolved organic carbon is used to assess the ultimate biodegradability of the test substances. It is preferable to determine DOC after acidification and purging rather than as the difference of $C_{total} - C_{inorganic}$.

The simultaneous use of a specific analytical method may allow the assessment of the primary degradation of the substance (disappearance of the parent chemical structure).

The method is applicable only to those organic test substances which, at the concentration used in the test:

— are soluble in water (at least 20 mg dissolved organic carbon/litre),

— have negligible vapour pressure,

— are not inhibitory to bacteria,

— do not significantly adsorb within the test system,

— are not lost by foaming from the test solution.

The organic carbon content of the test material must be established.

Information on the relative proportions of the major components of the test material will be useful in interpreting the results obtained, particularly in those cases where the results are low or marginal.

Information on the toxicity to microorganisms of the substance may be useful to the interpretation of low results and in the selection of an appropriate test concentration.

1.2. Definitions and units

C_T = concentration of test compound as organic carbon as present in or added to the settled sewage at the start of the aeration period (mg/litre),

C_t = concentration of dissolved organic carbon found in the supernatant liquor of the test at the end of the aeration period (mg/litre),

C_c = concentration of dissolved organic carbon found in the supernatant liquor of the control at the end of the aeration period (mg/litre).

The biodegradation is defined in this method as the disappearance of the organic carbon. The biodegradation can be expressed as:

1. The percentage removal D_{da} of the amount of substance added daily:

$$D_{da} = \frac{C_T - (C_t - C_c)}{C_T} \times 100 \qquad [1]$$

where D_{da} = degradation/daily addition.

2. The percentage removal D_{ssd} of the amount of substance present at the start of each day:

$$D_{ssd} = \frac{2C_T + C_{ti} - C_{ci} - 3C_{t(i+1)} + 3C_{c(i+1)}}{2C_T + C_{ti} - C_{ci}} \times 100 \qquad [2\,(a)]$$

$$\simeq \frac{2C_T - 2(C_t - C_c)}{2C_T + (C_t - C_c)} \times 100 \qquad [2\,(b)]$$

where D_{ssd} = degradation/substance start of day;

the indices i and (i + 1) refer to the day of measurement.

Equation 2(a) is recommended if effluent DOC varies from day to day, while equation 2(b) may be used when effluent DOC remains relatively constant from day to day.

1.3. **Reference substances**

In some cases, when investigating a new substance, reference substances may be useful; however, no specific reference substance is recommended here.

Data on several compounds evaluated in ring tests are provided (see Appendix 1) primarily so that calibration of the method may be performed from time to time and to permit comparison of results when another method is employed.

1.4. **Principle of the test method**

Activated sludge from a sewage treatment plant is placed in a semi-continuous activated sludge (SCAS) unit. The test compound and settled domestic sewage are added, and the mixture is aerated for 23 hours. The aeration is then stopped, the sludge allowed to settle and the supernatant liquor is removed.

The sludge remaining in the aeration chamber is then mixed with a further aliquot of test compound and sewage and the cycle is repeated.

Biodegradation is established by determination of the dissolved organic carbon content of the supernatant liquor. This value is compared with that found for the liquor obtained from a control tube dosed with settled sewage only.

When a specific analytical method is used, changes in the concentration of the parent molecule due to biodegradation can be measured (primary biodegradability).

1.5. **Quality criteria**

The reproducibility of this method based on removal of dissolved organic carbon has not yet been established. (When primary biodegradation is considered, very precise data are obtained for materials that are extensively degraded.)

The sensitivity of the method is largely determined by the variability of the blank and to a lesser extent by the precision of the determination of dissolved organic carbon and the level of test compound in the liquor at the start of each cycle.

1.6. **Description of the test procedure**

1.6.1. *Preparations*

A sufficient number of clean aeration units, alternatively, the original 1,5 litre SCAS test unit may be used, and air inlet tubes (Figure 1) for each test substance and controls are assembled. Compressed air supplied to the test units, cleaned by a cotton wool strainer, should be free of organic carbon and pre-saturated with water to reduce evaporation losses.

A sample of mixed liquor, containing 1 to 4 g suspended solids/litre, is obtained from an activated sludge plant treating predominantly domestic sewage. Approximately 150 ml of the mixed liquor are required for each aeration unit.

Stock solutions of the test substance are prepared in distilled water; the concentration normally required is 400 mg/litre as organic carbon which gives a test compound concentration of 20 mg/litre carbon at the start of each aeration cycle if no biodegradation is occurring.

Higher concentrations are allowed if the toxicity to microorganisms permits it.

The organic carbon content of the stock solutions is measured.

1.6.2. *Test conditions*

The test should be performed at 20 to 25 °C.

A high concentration of aerobic microorganisms is used (from 1 to 4 g/litre suspended solids), and the effective detention period is 36 hours. The carbonaceous material in the sewage feed is oxidized extensively, normally within eight hours after the start of each aeration cycle. Thereafter, the sludge respires endogenously for the remainder of the aeration period, during which time the only available substrate is the test compound unless this is also readily metabolized. These features, combined with daily re-inoculation of the test when domestic sewage is used as the medium, provide highly favourable conditions for both acclimatization and high degress of biodegradation.

1.6.3. *Performance of the test*

A sample of mixed liquor from a suitable predominantly domestic activated-sludge plant or laboratory unit is obtained and kept aerobic until used in the laboratory. Each aeration unit as well as the control unit are filled with 150 ml of mixed liquor (if the original SCAS test unit is used, multiply the given volumes by 10) and the aeration is started. After 23 hours, aeration is stopped and the sludge is allowed to settle for 45 minutes. The tap of each vessel is opened in turn, and 100 ml portions of the supernatant liquor are withdrawn. A sample of settled domestic sewage is obtained immediately before use, and 100 ml are added to the sludge remaining in each aeration unit. Aeration is started anew. At this stage no test materials are added, and the units are fed daily with domestic sewage only until a clear supernatant liquor is obtained on settling. This usually takes up to two weeks, by which time the dissolved organic carbon in the supernatant liquor at the end of each aeration cycle approaches a constant value.

At the end of this period, the individual settled sludges are mixed, and 50 ml of the resulting composite sludge are added to each unit.

95 ml of settled sewage and 5 ml of water are added to the control units, and 95 ml of the settled sewage plus 5 ml of the appropriate test compound stock solution (400 mg/litre) are added to the test units. Aeration is started again and continued for 23 hours. The sludge is then allowed to settle for 45 minutes and the supernatant drawn off and analysed for dissolved organic carbon content.

The above fill-and-draw procedure is repeated daily throughout the test.

Before settling, it may be necessary to clean the walls of the units to prevent the accumulation of solids above the level of the liquid. A separate scraper or brush is used for each unit to prevent cross contamination.

Ideally, the dissolved organic carbon in the supernatant liquors is determined daily, although less frequent analyses are permissible. Before analysis the liquors are filtered through washed 0,45 µm membrane filters or centrifuged. Membrane filters are suitable if it is assured that they neither release carbon nor absorb the substance in the filtration step. The temperature of the sample must not exceed 40 °C while it is in the centrifuge.

The length of the test for compounds showing little or no biodegradation is indeterminate, but experience suggests that this should be at least 12 weeks in general, but not longer than 26 weeks.

2. DATA AND EVALUATION

The dissolved organic carbon values in the supernatant liquors of the test units and the control units are plotted against time.

As biodegradation is achieved, the level found in the test will approach that found in the control. Once the difference between the two levels is found to be constant over three consecutive measurements, such number of further measurements as are sufficient to allow statistical treatment of the data are made and the percentage biodegradation of the test compound is calculated (D_{da} or D_{ssd}, see 1.2).

3. REPORTING

3.1. Test report

The test report shall, if possible, contain the following:

— all information on the kind of sewage, the type of unit used and the experimental results concerning the tested substance, the reference substance if used, and the blank,

— the temperature,

— removal curve with description, mode of calculation (see 1.2),

— date and location where the activated sludge and the sewage were sampled, status of adaptation, concentration, etc.,

— scientific reasons for any changes of test procedure,

— signature and date.

3.2. Interpretation of results

Since the substance to be tested by this method will not be readily biodegradable, any removal of DOC due solely to biodegradation will normally be gradual over days or weeks, except in such cases where acclimatization is sudden as indicated by an abrupt disappearance occurring after some weeks.

However, physico-chemical adsorption can sometimes play an important role; this is indicated when there is complete or partial removal of the added DOC at the outset. What happens subsequently depends on factors such as the degrees of adsorption and the concentration of suspended solids in the discarded effluent. Usually the difference between the concentration of DOC in the control and test supernatant liquors gradually increases from the initial low value and this difference then remains at the new value for the remainder of the experiment, unless acclimatization takes place.

If a distinction is to be drawn between biodegradation (or partial biodegradation) and adsorption, further tests are necessary. This can be done in a number of ways, but the most convincing is to use the supernatant liquor, or sludge, as inoculum in a base-set test (preferably a respirometric test).

Test substances giving high, non-adsorptive removal of DOC in this test should be regarded as potentially biodegradable. Partial, non-adsorptive removal indicates that the chemical is at least subject to some biodegradation.

Low, or zero removals of DOC may be due to inhibition of microorganisms by the test substance and this may also be revealed by lysis and loss of sludge, giving turbid supernatants. The test should be repeated using a lower concentration of test substance.

The use of a specific analytical method or of ^{14}C-labelled test substance may allow greater sensitivity. In the case of ^{14}C test compound, the recovery of the $^{14}CO_2$ will confirm that biodegradation has occurred.

When results are also given in terms of primary biodegradation, an explanation should, if possible, be given on the chemical structure change that leads to the loss of response of the parent test substance.

The validation of the analytical method must be given together with the response found on the blank test medium.

4. REFERENCES

(1) OECD, Paris, 1981, *Test Guideline 302 A*, Decision of the Council C(81) 30 final.

Appendix 1

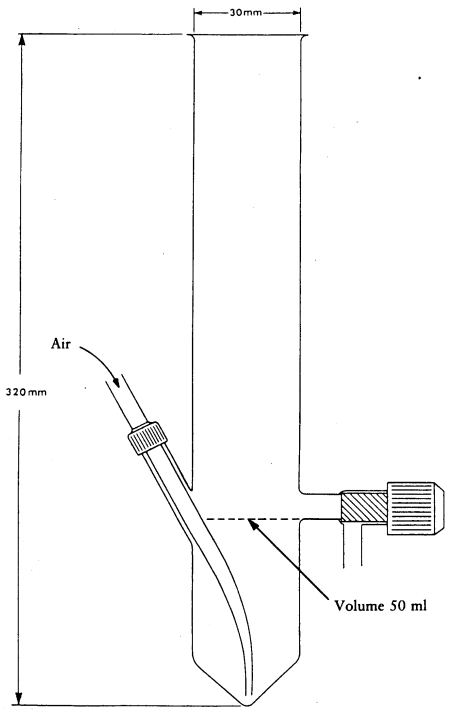
SCAS test: example of results

Substance	C_T (mg/l)	$C_t - C_c$ (mg/l)	Percentage biodegradition, D_{da}	Test duration (days)
4-acetyl aminobenzene sulphonate	17,2	2,0	85	40
Tetra propylene benzene sulphonate	17,3	8,4	51,4	40
4-nitrophenol	16,9	0,8	95,3	40
Diethylene glycol	16,5	0,2	98,8	40
Aniline	16,9	1,7	95,9	40
Cyclopentane tetra carboxylate	17,9	3,2	81,1	120

APPENDIX 2

Example of test apparatus

Figure 1



C.13 BIOCONCENTRATION : FLOW-THROUGH FISH TEST

1. METHOD

This Bioconcentration method is a replicate of the OECD TG 305 (1996).

1.1 INTRODUCTION

This method describes a procedure for characterising the bioconcentration potential of substances in fish under flow-through conditions. Although flow-through test regimes are much to be preferred, semi-static regimes are permissible, provided that the validity criteria are satisfied.

The method gives sufficient details for performing the test while allowing adequate freedom for adapting the experimental design to the conditions in particular laboratories and for varying characteristics of test substances.It is most validly applied to stable organic chemicals with log P_{ow} values between 1.5 and 6.0 (1) but may still be applied to superlipophilic substances (having log P_{ow}>6.0). The pre-estimate of the bioconcentration factor (BCF), sometimes denoted as K_B, for such superlipophilic substances will presumably be higher than the steady-state bioconcentration factor (BCF_{SS}) value expected to be obtained from laboratory experiments. Preestimates of the bioconcentration factor for organic chemicals with log P_{ow} values up to about 9.0 can be obtained by using the equation of Bintein at al (2). The parameters which characterise the bioconcentration potential include the uptake rate constant (k_1), the depuration rate constant (k_2) and the BCF_{ss}.

Radio-labelled test substances can facilitate the analysis of water and fish samples and may be used to determine whether degradate identification and quantification should be made. If total radioactive residues are measured (e.g. by combustion or tissue solubilisation), the BCF is based on the parent compound, any retained metabolites and also assimilated carbon. BCFs based on total radioactive residues may not, therefore, be directly comparable to a BCF derived by specific chemical analysis of the parent compound only.

Clean-up procedures may be employed in radiolabelled studies in order to determine BCF based on the parent compound, and the major metabolites may be characterised if deemed necessary. It is also possible to combine a fish metabolism study with a bioconcentration study by analysis and identification of the residues in tissues.

1.2 DEFINITIONS AND UNITS

Bioconcentration/Bioaccumulation is the increase in concentration of the test substance in or on an organism (specified tissues thereof) relative to the concentration of test substance in the surrounding medium.

The bioconcentration factor (BCF or K_B) at any time during the uptake phase of this accumulation test is the concentration of test substance in/on the fish or specified tissues thereof (C_f as µg/g (ppm)) divided by the concentration of the chemical in the surrounding medium (C_w as µg/ml (ppm)).

The steady-state bioconcentration factor (BCF_{ss} or K_B) does not change significantly over a prolonged period of time, the concentration of the test substance in the surrounding medium being constant during this period of time.

A plateau or steady-state is reached in the plot of test substance in fish (C_f) against time when the curve becomes parallel to the time axis and three successive analyses of C_f made on samples taken at intervals of at least two days are within ± 20% of each other, and there are no significant differences among the three sampling periods. When pooled samples are analysed at least four successive analyses are required. For test substances which are taken up slowly the intervals would more appropriately be seven days.

Bioconcentration factors calculated directly from kinetic rate constants (k_1/k_2) are termed kinetic concentration factor, BCF_K.

The octanol-water partition coefficient (P_{ow}) is the ratio of a chemical's solubility in n-octanol and water at equilibrium (Method A.8) also expressed as K_{ow}. The logarithm of P_{ow} is used as an indication of a chemical's potential for bioconcentration by aquatic organisms.

The exposure or uptake phase is the time during which the fish are exposed to the test chemical.

The uptake rate constant (k_1) is the numerical value defining the rate of increase in the concentration of test substance in/on test fish (or specified tissues thereof) when the fish are exposed to that chemical (k_1 is expressed in day^{-1}).

The post-exposure or depuration (loss) phase is the time, following the transfer of the test fish from a medium containing test substance to a medium free of that substance, during which the depuration (or the net loss) of the substance from the test fish (or specified tissue thereof) is studied.

The depuration (loss) rate constant (k_2) is the numerical value defining the rate of reduction in the concentration of the test substance in the test fish (or specified tissues thereof) following the transfer of the test fish from a medium containing the test substance to a medium free of that substance (k_2 is expressed in day^{-1}).

1.3 PRINCIPLE OF THE TEST METHOD

The test consists of two phases: the exposure (uptake) and post-exposure (depuration) phases. During the uptake phase, separate groups of fish of one species are exposed to at least two concentrations of the test substance. They are then transferred to a medium free of the test substance for the depuration phase. A depuration phase is always necessary unless uptake of the substance during the uptake phase has been insignificant (e.g. the BCF is less than 10). The concentration of the test substance in/on the fish (or specified tissue thereof) is followed through both phases of the test. In addition to the two test concentrations, a control group of fish is held under identical conditions except for the absence of the test substance, to relate possible adverse effects observed in the bioconcentration test to a matching control group and to obtain background concentrations of test substance.

The uptake phase is run for 28 days unless it is demonstrated that equilibrium has been reached earlier. A prediction of the length of the uptake phase and the time to steady-state can be made from equation in Annex 3. The depuration period is then begun by transferring the fish to the same medium but without the test substance in another clean vessel. Where possible the bioconcentration factor is calculated preferably both as the ratio (BCF_{ss}) of concentration of the fish (C_f) and in the water (C_w) at apparent steady-state and as a kinetic bioconcentration factor, BCF_K as the ratio of the rate constants of uptake (k_1) and depuration (k_2) assuming first-order kinetics. If first-order kinetics are obviously not obeyed, more complex models should be employed (Annex 5).

If a steady-state is not achieved within 28 days, the uptake phase should be extended until steady-state is reached, or 60 days, whichever comes first; the depuration phase is then begun.

The uptake rate constant, the depuration (loss) rate constant (or constants, where more complex models are involved), the bioconcentration factor, and where possible, the confidence limits of each of these parameters are calculated from the model that best describes the measured concentrations of test substance in fish and water.

The BCF is expressed as a function of the total wet weight of the fish. However, for special purposes, specified tissues or organs (e.g. muscle, liver), may be used if the fish are sufficiently large or the fish may be divided into edible (fillet) and non-edible (viscera) fractions. Since, for many organic substances, there is a clear relationship between the potential for bioconcentration and lipophilicity, there is also a corresponding relationship between the lipid content of the test fish and the observed bioconcentration of such substances. Thus, to reduce this source of variability in test results for those substances with high lipophilicity (i.e. with log P_{ow}>3), bioconcentration should be expressed in relation to lipid content in addition to whole body weight.

The lipid content should be determined on the same biological material as is used to determine the concentration of the test substance, when feasible.

1.4 INFORMATION ON THE TEST SUBSTANCE

Before carrying out the test for bioconcentration, the following information for the test substance should be known:

a) solubility in water
b) octanol-water partition coefficient P_{ow} (denoted also as K_{ow}, determined by an HPLC method in A.8)
c) hydrolysis
d) phototransformation in water determined under solar or simulated solar irradiation and under the irridiation conditions of the test for bioconcentration (3)
e) surface tension (i.e. for substances where the log P_{ow} cannot be determined)
f) vapour pressure
g) ready biodegradability (where appropriate)

Other information required is the toxicity to the fish species to be used in the test, preferably the asymptotic LC_{50} (i.e. time-independent). An appropriate analytical method, of known accuracy, precision and sensitivity, for the quantification of the test substance in the test solutions and in biological material must be available, together with details of sample preparation and storage. Analytical detection limit of test substance in both water and fish tissues should also be known. When ^{14}C labelled test substance is used, the percentage of radioactivity associated with impurities should be known.

1.5 VALIDITY OF THE TEST

The following conditions should apply for a test to be valid:

-the temperature variation is less than $\pm$ 2°C;

-the concentration of dissolved oxygen does not fall below 60% saturation;

-the concentration of the test substance in the chambers is maintained within ± 20% of the mean of the measured values during the uptake phase;

-the mortality or other adverse effects/disease in both control and treated fish is less than 10% at the end of the test; where the test is extended over several weeks or months, death or other adverse effects in both sets of fish should be less than 5% per month and not exceed 30% in all.

1.6 REFERENCE COMPOUNDS

The use of reference compounds of known bioconcentration potential would be useful in checking the experimental procedure, when required. However, specific substances cannot yet be recommended.

1.7 DESCRIPTION OF THE TEST METHOD

1.7.1 Apparatus

Care should be taken to avoid the use of materials, for all parts of the equipment, that can dissolve, sorb or leach and have an adverse effect on the fish. Standard rectangular or cylindrical tanks, made of chemically inert material and of a suitable capacity in compliance with loading rate can be used. The use of soft plastic tubing should be minimised. Teflon (R), stainless steel and/or glass tubing are preferably used. Experience has shown that for substances with high adsorption coefficients, such as the synthetic pyrethroids, silanized glass may be required. In these situations the equipment will have to be discarded after use.

1.7.2 Water

Natural water is generally used in the test and should be obtained from an uncontaminated and uniform quality source. The dilution water must be of a quality that will allow the survival of the chosen fish species for the duration of the acclimation and test periods without them showing any abnormal appearance or behaviour. Ideally, it should be demonstrated that the test species can survive, grow and reproduce in the dilution water (e.g. in laboratory culture or a life-cycle toxicity test). The water should be characterised at least by pH, hardness, total solids, total organic carbon and, preferably also ammonium, nitrite and alkalinity and, for marine species, salinity. The parameters which are important for optimal fish well-being are fully known, but Annex 1 gives recommended maximum concentrations of a number of parameters for fresh and marine test waters.

The water should be of constant quality during the period of a test. The pH value should be within the range 6.0 to 8.5, but during a given test it should be within a range of ± 0.5 pH units. In order to ensure that the dilution water will not unduly influence the test result (for example, by complexation of the test substance) or adversely affect the performance of the stock of fish, samples should be taken at intervals for analysis. Determination of heavy metals (e.g. Cu, Pb, Zn, Hg, Cd, Ni), major anions and cations (e.g. Ca, Mg, Na, K, Cl, SO_4), pesticides (e.g. total organophosphorous and total organochlorine pesticides), total organic carbon and suspended solids should be made, for example, every three months where a dilution water is known to be relatively constant in quality. If water quality has been demonstrated to be constant over at least one year, determinations can be less frequent and intervals extended (e.g. every six months).

The natural particle content as well as the total organic carbon (TOC) of the dilution water should be as low as possible to avoid adsorption of the test substance to organic matter which may reduce its bioavailability (4). The maximum acceptable value is 5 mg/l for particulate matter (dry matter, not passing a 0.45 μm filter) and 2 mg/l for total organic carbon (see Annex 1). If necessary, the water should be filtered before use. The contribution to the organic carbon content in water from the test fish (excreta) and from the food residues should be as low as possible. Throughout the test, the concentration of organic carbon in the test vessel should not exceed the concentration of organic carbon originating from the test substance and, if used, the solubilising agent by more than 10 mg/l (± 20%).

1.7.3 Test Solutions

A stock solution of the test substance is prepared at a suitable concentration. The stock solution should preferably be prepared by simply mixing or agitating the test substance in the dilution water. The use of solvents or dispersants (solubilising agents) is not recommended; however this may occur in some cases in order to produce a suitably concentrated stock solution. Solvents which may be used are ethanol, methanol, ethylene glycol monomethyl ether, ethylene glycol dimethyl ether, dimethylformamide and triethylene glycol. Dispersants which may be used are Cremophor RH40, Tween 80, methylcellulose 0.01% and HCO-40. Care should be taken when using readily biodegradable agents as these can cause problems with bacterial growth in flow-through tests. The test substance may be radio-labelled and should be of the highest purity (e.g. preferably >98%).

For flow-through tests, a system which continuously dispenses and dilutes a stock solution of the test substance (e.g. metering pump, proportional diluter, saturator system) is required to deliver the test concentrations to the test chambers. At least five volume replacements through each test chamber per day are preferably allowed. The flow-through mode is to be preferred, but where this is not possible (e.g. when the test organisms are adversely affected) a semi-static technique may be used provided that the validity criteria are satisfied. The flow rates of stock solutions and dilution water should be checked both 48 h before and then at least daily during the test. In this check the determination of the flow-rate through each test chamber is included and ensured that it does not vary by more than 20% either within or between chambers.

1.7.4 Selection of species

Important criteria in the selection of species are that they are readily available, can be obtained in convenient sizes and can be satisfactorily maintained in the laboratory. Other criteria for selecting fish species include recreational, commercial, ecological importance as well as comparable sensitivity, past successful use etc.

Recommended test species are given in Annex 2. Other species may be used but the test procedure may have to be adapted to provide suitable test conditions. The rationale for the selection of the species and the experimental method should be reported in this case.

1.7.5 Holding of fish

Acclimate the stock population of fish for at least two weeks in water at the test temperature and feed throughout on a sufficient diet and of the same type to be used during the test.

Following a 48-hour settling-in period, mortalities are recorded and the following criteria applied:

- mortalities of greater than 10% of population in seven days: reject the entire batch;
- mortalities of between 5 and 10% of population in seven days: acclimate for seven additional days;
- mortalities of less than 5% of population in seven days: accept the batch - if more than 5% mortality during second seven days reject the entire batch.

Ensure that fish used in tests are free from observable diseases and abnormalities. Discard any diseased fish. Fish should not receive treatment for disease in the two weeks preceding the test, or during the test.

1.8 PERFORMANCE OF THE TEST

1.8.1 Preliminary Test

It may be useful to conduct a preliminary experiment in order to optimise the test conditions of the definitive test, e.g. selection of test substance concentration(s), duration of the uptake and depuration phases.

1.8.2 Conditions of Exposure

1.8.2.1 Duration of Uptake Phase

A prediction of the duration of the uptake phase can be obtained from practical experience (e.g. from a previous study or an accumulation related chemical) or from certain empirical relationships utilising knowledge of either the aqueous solubility or the octanol/water partition coefficient of the test substance (see Annex 3).

The uptake phase should be run for 28 days unless it can be demonstrated that equilibrium has been reached earlier. If the steady-state has not been reached by 28 days, the uptake phase should be extended, taking further measurements, until steady-state is reached or 60 days, whichever is shorter.

1.8.2.2 Duration of the Depuration Phase

A period of half the duration of the uptake phase is usually sufficient for an appropriate (e.g. 95%) reduction in the body burden of the substance to occur (see Annex 3 for explanation of the estimation). If the time required to reach 95% loss is impractically long, exceeding for example twice the normal duration of the uptake phase (i.e. more than 56 days) a shorter period may be used (i.e. until the concentration of test substance is less than 10% of steady-state concentration). However, for substances having more complex patterns of uptake and depuration than are represented by a one-compartment fish model, yielding first order kinetics, allow longer depuration phases for determination of loss rate constants. The period may, however, be governed by the period over which the concentration of test substance in the fish remains above the analytical detection limit.

1.8.2.3 Numbers of Test Fish

Select the numbers of fish per test concentration such that minimum of four fish per sample are available at each sampling. If greater statistical power is required, more fish per sample will be necessary.

If adult fish are used, report whether male or female, or both are used in the experiment. If both sexes are used, differences in lipid content between sexes should be documented to be non-significant before the start of the exposure; pooling all male and all female fish may be necessary.

In any one test, fish of similar weight are selected, such that the smallest are no smaller than two-thirds of the weight of the largest. All should be of the same year-class and come from the same source. Since weight and age of a fish appear sometimes to have a significant effect on BCF values (1) these details are recorded accurately. It is recommended that a sub-sample of the stock of fish is weighed before the test in order to estimate the mean weight.

1.8.2.4 Loading

High water-to-fish ratios are used in order to minimise the reduction in C_W caused by the addition of the fish at the start of the test and also to avoid decreases in dissolved oxygen concentration. It is important that the loading rate is appropriate for the test species used. In any case, a loading rate of 0.1-1.0 g of fish (wet weight) per litre of water per day is normally recommended. High loading rates can be used if it is shown that the required concentration of test substance can be maintained within ± 20% limits, and that the concentration of dissolved oxygen does not fall below 60% saturation.

In choosing appropriate loading regimes, account of the normal habitat of the fish species is taken. For example, bottom-living fish may demand a larger bottom area of the aquarium for the same volume of water than pelagic fish species.

1.8.2.5 Feeding

During the acclimation and test periods, fish are fed with an appropriate diet of known lipid and total protein content, in an amount sufficient to keep them in a healthy condition and to maintain body weight. Fish are fed daily throughout the acclimation and test periods at a level of approximately 1 to 2% of body weight per day; this keeps the lipid concentration in most species of fish at a relatively constant level during the test. The amount of feed should be re-calculated, for example, once per week, in order to maintain consistent body weight and lipid content. For this calculation, the weight of the fish in each test chamber can be estimated from the weight of the fish sampled most recently in that chamber. Do not weigh the fish remaining in the chamber.

Uneaten food and faeces are siphoned daily from the test chambers shortly after feeding (30 minutes to 1 hour). Chambers are kept as clean as possible throughout the test so that the concentration of organic matter is kept as low as possible, since the presence of organic carbon may limit the bioavailability of the test substance (1).

Since many feeds are derived from fishmeal, the feed should be analysed for the test substance. It is also desirable to analyse the feed for pesticides and heavy metals.

1.8.2.6 Light and Temperature

The photoperiod is usually 12 to 16 hours and the temperature (± 2°C) should be appropriate for the test species (see Annex 2). The type and characteristics of illumination should be known. Caution should be given to the possible phototransformation of the test substance under the irradiation conditions of the study. Appropriate illumination sholud be used avoiding exposure of the fish to unnatural photoproducts. In some cases it may be appropriate to use a filter to screen out UV irradiation below 290 nm.

1.8.2.7 Test Concentrations

Fish are exposed under flow-through conditions to at least two concentrations of the test substance in water. Normally, the higher (or highest) concentration of the test substance are selected to be about 1% of its acute asymptotic LC_{50}, and to be at least ten-fold higher than its detection limit in water by the analytical method used.

The highest test concentration can also be determined by dividing the acute 96h LC_{50} by an appropriate acute/chronic ratio (appropriate ratios for some chemicals can be about 3 up to 100). If possible, choose the other concentration(s) such that it differs from the one above by a factor of ten. If this is not possible because of the 1% of LC_{50} criterion and the analytical limit, a lower factor than ten can be used or the use of ^{14}C labelled test substance should be considered. No concentration used should be above the solubility of the test substance.

Where a solubilising agent is used its concentration should not be greater than 0.1 ml/l and should be the same in all test vessels. Its contribution, together with the test substance, to the overall content of organic carbon in the test water should be known. However, every effort should be made to avoid the use of such materials.

1.8.2.8 Controls

One dilution water control or if relevant, one control containing the solubilising agent should be run in addition to the test series, provided that it has been established that the agent has no effects on the fish. If not, both controls should be set up.

1.8.3 Frequency of Water Quality Measurements

During the test, dissolved oxygen, TOC, pH and temperature should be measured in all vessels. Total hardness and salinity, if relevant, should be measured in the controls and one vessel at the higher (or highest) concentration. As a minimum, dissolved oxygen and salinity, if relevant, should be measured three times - at the beginning, around the middle and end of the uptake period - and once a week in the depuration period. TOC should be measured at the beginning of the test (24 h and 48 h prior to test initiation of uptake phase) before addition of the fish and at least once a week, during both uptake and depuration phases. Temperature should be measured daily, pH at the beginning and end of each period and hardness once each test. Temperature should preferably be monitored continuously in at least one vessel.

1.8.4 Sampling and Analysis of Fish and Water

1.8.4.1 Fish and Water Sampling schedule

Water from the test chambers for the determination of test substance concentration is sampled before addition of the fish and during both uptake and depuration phases. As a minimum, the water is sampled at the same time as the fish and before feeding. During the uptake phase, the concentrations of test substance are determined in order to check compliance with the validity criteria.

Fish is sampled on at least five occasions during the uptake phase and at least on four occasions during the depuration phase. Since on some occasions it will be difficult to calculate a reasonably precise estimate of the BCF value based on this number of samples, especially when other than simple first-order depuration kinetics are indicated, it may be advisable to take samples at a higher frequency in both periods (see Annex 4). The extra samples are stored and analysed only if the results of the first round of analyses prove inadequate for the calculation of the BCF with the desired precision.

An example of an acceptable sampling schedule is given in Annex 4. Other schedules can readily be calculated using other assumed values of P_{ow} to calculate the exposure time for 95% uptake.

Sampling is continued during the uptake phase until a steady-state has been established or for 28 days, whichever is the shorter. If the steady-state has not been reached within 28 days sampling continues until a steady-state has been attained or for 60 days, whichever is shorter. Before beginning the depuration phase the fish are transfered to clean tanks.

1.8.4.2 Sampling and Sample Preparation

Water samples for analysis are obtained e.g. by siphoning through inert tubing from a central point in the test chamber. Since neither filtration nor centrifuging appears always to separate the non-bioavailable fraction of the test substance from that which is bioavailable (especially for super-lipophilic chemicals i.e. those chemicals with a log P_{ow}>5) (1) (5), samples may not be subjected to those treatments.

Instead, measures should be taken to keep the tanks as clean as possible and the content of total organic carbon should be monitored during both the uptake and depuration phases.

An appropriate number of fish (normally a minimum of four) is removed from the test chambers at each sampling time. The sampled fish are rinsed quickly with water, blot “dry”, killed instantly using the most appropriate and humane method, and then weighed.

It is preferable to analyse fish and water immediately after sampling in order to prevent degradation or other losses and to calculate approximate uptake and depuration rates as the test proceeds. Immediate analysis also avoids delay in determining when a plateau has been reached.

Failing immediate analysis, samples are stored by an appropriate method. Before the beginning of the study, information on the proper method of storage for the particular test substance - for example, deep-freezing, holding at 4 ^{0}C, duration of storage, extraction, etc. are obtained.

1.8.4.3 Quality of Analytical method

Since the whole procedure is governed essentially by the accuracy, precision and sensitivity of the analytical method used for the test substance, check experimentally that the precision and reproducibility of the chemical analysis, as well as recovery of the test substance from both water and fish are satisfactory for the particular method. Also, check that the test substance is not detectable in the dilution water used.

If necessary, the values of C_w and C_f obtained from the test are corrected for the recoveries and background values of controls. Fish and water samples are handled throughout in such a manner as to minimise contamination and loss (e.g. resulting from adsorption by the sampling device).

1.8.4.4 Analysis of Fish Sample

If radiolabelled materials are used in the test, it is possible to analyse for total radio label (i.e. parent and metabolites) or, the samples may be cleaned up so that parent compound can be analysed separately. Also, the major metabolites may be characterised at steady-state or at the end of the uptake phase, whichever is the sooner. If the BCF in terms of total radiolebelled residues is ≥1000% , it may be advisable, and for certain categories of chemicals such as pesticides strongly recommended, to identify and quantify degradates representing ≥10% of total residues in fish tissues at steady state. If degradates representing ≥10% of total radiolabelled residues in the fish tissue are identified and quantified, then it is also recommended to identify and quantify degradates in the test water.

The concentration of the test substance should usually be determined for each weighed individual fish. If this is not possible, pooling of the samples on each sampling occasion may be done but pooling does restrict the statistical procedures which can be applied to the data. If a specific statistical procedure and power are important considerations, then an adequate number of fish to accommodate the desired pooling procedure and power should be included in the test (6) (7).

BCF should be expressed both as a function of total wet weight and, for high lipophilic substances, as a function of the lipid content. Lipid content of the fish is determined on each sampling occasion if possible. Suitable methods should be used for determination of lipid content (ref. 8 and 2 of Annex 3). Chloroform/methanol extraction technique may be recommended as standard method (9). The various methods do not give identical values (10), so it is important to give details of the method used. When possible, the analysis for lipid should be made on the same extract as that produced for analysis for the test substance, since the lipids often have to be removed from the extract before it can be analysed chromatographically. The lipid content of the fish (as mg/kg wet weight) at the end of the experiment should not differ from that at the start by more ± 25%. The tissue percent solids should also be reported to allow conversion of lipid concentration from a wet to a dry basis.

2. DATA

2.1 TREATMENT OF RESULTS

The uptake curve of the test substance is obtained by plotting its concentration in/on fish (or specified tissues) in the uptake phase against time on arithmetic scales. If the curve has reached a plateau, that is, become approximately asymptotic to the time axis, the steady-state BCF_{ss} is calculated from:

$$\frac{C_f \ as\ steady-state\ (mean)}{C_w \ as\ steady-state\ (mean)}$$

When no steady-state is reached, it may be possible to calculate a BCF_{ss} of sufficient precision for hazard assessment from a "steady-state" at 80% ($1.6/k_2$) or 95% ($3.0/k_2$) of equilibrium.

Also the concentration factor (BCF_K) is determined, as the ratio k_1/k_2, the two first-order kinetic constants. The depuration rate constant (k_2) is usually determined from the depuration curve (i.e. a plot of the decrease in test substance concentration in the fish with time). The uptake rate constant (k_1) is then calculated given k_2 and a value of C_f which is derived from the uptake curve (see also Annex 5). The preferred method for obtaining BCF_K and the rate constants, k_1 and k_2, is to use non-linear parameter estimation methods on a computer (11). Otherwise, graphical methods may be used to calculate k_1 and k_2. If the depuration curve is obviously not first-order, then more complex models should be employed (see references in Annex 3) and advice from a biostatistician sought.

2.2 INTERPRETATION OF RESULTS

The results should be interpreted with caution where measured concentrations of test solutions occur at levels near the detection limit of the analytical method.

Clearly defined uptake and loss curves are an indication of good quality bioconcentration data. The variation in uptake/depuration constants between the two test concentrations should be less than 20%. Observed significant differences in uptake/depuration rates between the two applied test concentrations should be recorded and possible explanations given. Generally the confidence limit of BCFs from well-designed studies approach ± 20%.

3. REPORTING

The test report must include the following information:

3.1 TEST SUBSTANCE:

- physical nature and, where relevant, physicochemical properties;
- chemical identification data (including the organic carbon content, if appropriate);
- if radio labelled, the precise position of the labelled atom(s) and the percentage of radioactivity associated with impurities;

3.2 TEST SPECIES

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

3.3 TEST CONDITIONS :

- test procedure used (e.g. flow-through or semi-static);
- type and characteristics of illumination used and photoperiod(s);

— test design (e.g. number and size of test chambers, water volume replacement rate, number of replicates, number of fish per replicate, number of test concentrations, length of uptake and depuration phases, sampling frequency for fish and water samples;

— method of preparation of stock solutions and frequency of renewal (the solubilizing agent, its concentration and its contribution to the organic carbon content of test water must be given, when used);

— the nominal test concentrations, the means of the measured values and their standard deviations in the test vessels and the method by which these were attained;

— source of the dilution water, description of any pre-treatment, results of any demonstration of the ability of test fish to live in the water, and water characteristics: pH, hardness, temperature, dissolved oxygen concentration, residual chlorine levels (if measured), total organic carbon, suspended solids, salinity of the test medium (if appropriate) and any other measurements made;

— water quality within test vessels, pH, hardness, TOC, temperature and dissolved oxygen concentration;

— detailed information on feeding (e.g. type of food, source, composition - at least lipid and protein content if possible, amount given and frequency);

— information on the treatment of fish and water samples, including details of preparation, storage, extraction and analytical procedures (and precision) for the test substance and lipid content (if measured).

3.4 RESULTS :

— results from any preliminary study performed;

— mortality of the control fish and the fish in each exposure chamber and any observed abnormal behaviour;

— the lipid content of the fish (if determination on testing occasion);

— curves (including all measured data) showing the uptake and depuration of the test chemical in the fish, the time to steady-state;

— C_f and C_w (with standard deviation and range, if appropriate) for all sampling times (C_f expressed in µg/g wet weight (ppm) of whole body or specified tissues thereof e.g. lipid, and C_w in µg/ml (ppm). C_w values for the control series (background should also be reported);

— the steady-state bioconcentration factor (BCF_{ss}) and/or kinetic concentration factor (BCF_K) and if applicable, 95% confidence limits for the uptake and depuration (loss) rate constants (all expressed in relation to the whole body and the total lipid content, if measured, of the animal or specified tissues thereof), confidence limits and standard deviation (as available) and methods of computation/data analysis for each concentration of test substance used;

— where radio-labelled substances are used, and if it is required, the accumulation of any detected metabolites may be presented;

— anything unusual about the test, any deviation from these procedures, and any other relevant information;

Minimise results as “not detected at the limit of detection” by pre-test method development and experimental design, since such results cannot be used for rate constant calculations.

4. REFERENCES

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ANNEX 1

CHEMICAL CHARACTERISTICS OF AN ACCEPTABLE DILUTION WATER

	SUBSTANCE	LIMIT CONCENTRATION
1	Particulate Matter	5 mg/l
2	Total Organic Carbon	2 mg/l
3	Un-ionised ammonia	1 µg/l
4	Residual chlorine	10 µg/l
5	Total organophosphorous pesticides	50 ng/l
6	Total organochlorine pesticides plus polychlorinated biphenyls	50 ng/l
7	Total organic chlorine	25 ng/l
8	Aluminium	1µg/l
9	Arsenic	1µg/l
10	Chromium	1µg/l
11	Cobalt	1µg/l
12	Copper	1µg/l
13	Iron	1µg/l
14	Lead	1µg/l
15	Nickel	1µg/l
16	Zinc	1µg/l
17	Cadmium	100 ng/l
18	Mercury	100 ng/l
19	Silver	100 ng/l

ANNEX 2

FISH SPECIES RECOMMENDED FOR TESTING

	Recommended Species	Recommended range of test temperature (^{o}C)	Recommended total length of test animal (cm)
1	**Danio rerio**[(1)] (Teleostei, Cyprinidae) (Hamilton-Buchanan) Zebra - fish	20 - 25	3.0 ± 0.5
2	**Pimephales promelas** (Teleostei, Cyprinidae) (Rafinesque) Fathead minnow	20 - 25	5.0 ± 2.0
3	**Cyprinus carpio** (Teleostei, Cyprinidae) (Linnaeus) Common Carp	20 - 25	5.0 ± 3.0
4	**Oryzias latipes** (Teleostei, Poeciliidae) (Temminck and Schlegel) Ricefish	20 - 25	4.0 ± 1.0
5	**Poecilia reticulata** (Teleostei, Poeciliidae) (Peters) Guppy	20 - 25	3.0 ± 1.0
6	**Lepomis macrochirus** (Teleostei, Centrarchidae) (Rafinesque) Bluegill	20 - 25	5.0 ± 2.0
7	**Oncorhynchus mykiss** (Teleostei, Salmonidae) (Walbaum) Rainbow trout	13 - 17	8.0 ± 4.0
8	**Gasterosteus aculeatus** (Teleostei, Gasterosteidae) (Linnaeus) Three-spined stickleback	18 - 20	3.0 ± 1.0

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Various estuarine and marine species have been used in different countries, for example:

Spot	*Leiostomus xanthurus*
Sheepshead minnow	*Cyprinodon variegatus*
Silverside	*Menidia beryllina*
Shiner perch	*Cymatogaster aggregata*
English sole	*Parophrys vetulus*
Staghorn sculpin	*Leptocottus armatus*
Three-spined stikleback	*Gasterosteus aculeatus*
Sea bass	*Dicentracus labrax*
Bleak	*Alburnus alburnus*

COLLECTION

The fresh water fish listed in the table above are easy to rear and/or are widely available throughout the year, whereas the availability of marine and estuarine species is partially confined to the respective countries. They are capable of being bred and cultivated either in fish farms or in the laboratory, under disease-and parasite-controlled conditions, so that the test animal will be healthy and of known parentage. These fish are available in many parts of the world.

ANNEX 3

PREDICTION OF THE DURATION OF THE UPTAKE AND DEPURATION PHASES

1. Prediction of the duration of the uptake phase

Before performing the test, an estimate of k_2 and hence some percentage of the time needed to reach steady-state may be obtained from empirical relationships between k_2 and the n-octanol/water partition coefficient (P_{ow}) or k_2 and the aqueous solubility (s).

An estimate of k_2 (day^{-1}) may be obtained, for example from the following empirical relationship (1):

$$\log_{10} k_2 = - 0.414 \log_{10}(P_{ow}) + 1.47 (r^2=0.95) \qquad \text{[equation 1]}$$

For other relationships see Ref. (2).

If the partition coefficient (P_{ow}) is not known, an estimate can be made (3) from a knowledge of the aqueous solubility (s) of the substance using:

$$\log_{10} (P_{ow}) = 0.862 \log_{10}(s) + 0.710 \ (r^2 = 0.994) \text{[equation 2]}$$

where s = solubility (moles/l) : (n=36)

These relationships apply only to chemicals with log P_{ow} values between 2 and 6.5 (4).

The time to reach some percentage of steady-state may be obtained, by applying the k_2-estimate, from the general kinetic equation describing uptake and depuration (first-order kinetics):

$$\frac{dC_f}{dt} = k_1 . c_w - k_2 . c_f$$

or if C_w is constant:

$$c_f = \frac{k_1}{k_2} . c_w (1 - e^{-k_2 t}) \qquad \text{[equation 3]}$$

When steady-state is approached (t->∞), equation 3 may be reduced (5) (6) to:

$$c_f = \frac{k_1}{k_2} . c_w \qquad \text{or} \qquad C_f / C_w = k_1 / k_2 = \text{BCF}$$

Then $k_1 / k_2 . C_w$ is an approach to the concentration in the fish at "steady-state" ($C_{f,s}$).

Equation 3 may be transcribed to:

$$C_f = C_{f,s} (1 - e^{-k_2 t}) \qquad \text{or} \qquad \frac{c_f}{c_{fs}} = 1 - e^{-k_2 t} \qquad \text{[equation 4]}$$

Applying equation 4, the time to reach some percentage of steady-state may be predicted when k_2 is pre-estimated using equation 1 or 2.

As a guideline, the statistically optimal duration of the uptake phase for the production of statistically acceptable data (BCF_K) is that period which is required for the curve of the logarithm of the concentration of the test substance in fish plotted against linear time to reach its mid-point, or $1.6/k_2$, or 80% of steady-state but not more than $3.0/k_2$ or 95% of steady-state (7).

The time to reach 80% of steady-state is (equation 4):

$0.80 = 1 - e^{-k_2 t_{80}}$ or $t_{80} = \frac{1.6}{k_2}$ [equation 5]

Similarly 95 percent of steady-state is: $t_{95} = \frac{3.0}{k_2}$ [equation 6]

For example, the duration of the uptake phase (up) for a test substance with log P_{ow} = 4 would be (using equations 1,5,6):

$\log_{10} k_2 = - 0.414.(4) + 1.47$ $k_2 = 0.652$ days^{-1}

up (80 pct) = 1.6/0.652, i.e. 2.45 days (59 hours)
or up (95 pct) = 3.0/0.652, i.e. 4.60 days (110 hours)

Similarly, for a test substance with s = 10^{-5} mol/l (log(s) = -5.0), the duration of up would be (using equations 1,2,5,6):

	$\log_{10} (P_{ow})$	=	- 0.862 (-5.0) + 0.710 = 5.02
	$\log_{10} K_2$	=	- 0.414 (5.02) + 1.47
	k_2	=	0.246 days $^{-1}$
	up (80 pct)	=	1.6/0.246, i.e. 6.5 days (156 hours)
or	up (95 pct)	=	3.0/0.246, i.e. 12.2 days (293 hours)

Alternatively, the expression :

t_{eq} = 6.54 x 10^{-3} P_{ow} + 55.31 (hours)

may be used to calculate the time for effective steady-state to be reached (4).

2. <u>Prediction of the duration of the depuration phase</u>

A prediction of the time needed to reduce the body burden to some percentage of the initial concentration may also be obtained from the general equation describing uptake and depuration (first order kinetics) (1) (8).

For the depuration phase, C_w is assumed to be zero. The equation may be reduced to:

$\frac{dC_f}{dt} = -k_2 C_f$ or $C_f = C_{f,o} . e^{-k_2 t}$

where $C_{f,o}$ is the concentration at the start of the depuration period. 50 percent depuration will then be reached at the time (t_{50}):

$\frac{C_f}{C_{f,o}} = \frac{1}{2} = e^{-k_2 t_{50}}$ or $t_{50} = \frac{0.693}{k_2}$

Similarly 95 percent depuration will be reached at:

$t_{95} = \frac{3.0}{k_2}$

If 80% uptake is used for the first period ($1.6/k_2$) and 95% loss in the depuration phase ($3.0/k_2$), then the depuration phase is approximately twice the duration of the uptake phase.

It is important to note, however, that the estimations are based on the assumption that uptake and depuration patterns will follow first order kinetics. If first order kinetics are obviously not obeyed, more complex models should be employed (e.g. ref (1)).

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ANNEX 4

THEORETICAL EXAMPLE OF SAMPLING SCHEDULE FOR BIOCONCENTRATION TESTS

OF SUBSTANCES WITH log P_{ow} = 4.

Fish Sampling	Sample Time Schedule		No. of water samples	No. of fish per sample
	Minimal required frequency (days)	Additional sampling		
Uptake phase	-1 0		2* 2	add 45-80 fish
1st	0.3	0.4	2 (2)	4 (4)
2nd	0.6	0.9	2 (2)	4 (4)
3rd	1.2	1.7	2 (2)	4 (4)
4th	2.4	3.3	2 (2)	4 (4)
5th	4.7		2	6
Depuration phase				**Transfer fish to water free of test chemical**
6th	5.0	5.3		4 (4)
7th	5.9	7.0		4 (4)
8th	9.3	11.2		4 (4)
9th	14.0	17.5		6 (4)

* Sample water after minimum of 3 'chamber-volume' have been delivered.

Values in brackets are numbers of samples (water, fish) to be taken if additional sampling is carried out.

Note: Pre-test estimate of k_2 for log P_{ow} of 4.0 is 0.652 $days^{-1}$. The total duration of the experiment is set to 3 x up = 3 x 4.6 days, i.e. 14 days. For the estimation of 'up' refer to Annex 3.

ANNEX 5

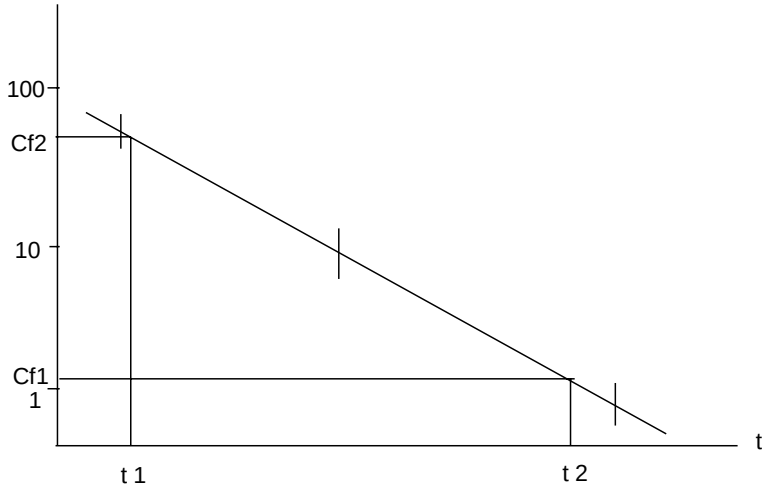
MODEL DISCRIMINATION

Most bioconcentration data have been assumed to be 'reasonably' well described by a simple two-compartment/ two-parameter model, as indicated by the rectilinear curve which approximates to the points for concentrations in fish, during the depuration phase, when these are plotted on semi-log paper. (Where these points cannot be described by a rectilinear curve then more complex models should be employed, see, for example, Spacie and Hamelink, Ref 1 in Annex 3).

GRAPHICAL METHOD FOR DETERMINATION OF DEPURATION (LOSS) RATE CONSTANT k_2

Plot the concentration of the test substance found in each sample of fish against sampling time on semi-log paper. The slope of that line is k_2.

$$k_2 = \frac{1n(C_{f1}/C_{f2})}{t_2 - t_1}$$



Note that deviations from straight line may indicate a more complex depuration pattern than first order kinetics. A graphical method may be applied for resolving types of depuration deviating from first order kinetics.

GRAPHICAL METHOD FOR DETERMINATION OF UPTAKE RATE CONSTANT k_1

Given K_2, calculate k_1 as follows:

$$k_1 = \frac{c_f k_2}{c_w x(1 - e^{-k_2 t})} \quad \text{[equation 1]}$$

The value of C_f is read from the midpoint of the smooth uptake curve produced by the data when log concentration is plotted versus time (on an arithmetical scale).

COMPUTER METHOD FOR CALCULATION OF UPTAKE AND DEPURATION (LOSS) RATE CONSTANTS

The preferred means for obtaining the bioconcentration factor and k_1 and k_2 rate constants is to use non-linear parameter estimation methods on a computer. These programs find values for k_1 and k_2 given a set of sequential time concentration data and the model:

$$c_f = c_w \cdot \frac{k_1}{k_2} x(1 - e^{-k_2 t}) \qquad 0 < t < t_c \qquad \text{[equation 2]}$$

$$c_f = c_w \cdot \frac{k_1}{k_2} x(e^{-k_2 (t - t_c)} - e^{-k_2 t}) \qquad t > t_c \qquad \text{[equation 3]}$$

where t_c = time at the end of the uptake phase.

This approach provides standard deviation estimates of k_1 and k_2.

As k_2 in most cases can be estimated from the depuration curve with relatively high precision, and because a strong correlation exists between the two parameters k_1 and k_2 if estimated simultaneously, it may be advisable first to calculate k_2 from the depuration data only, and subsequently calculate k_1 from the uptake data using non-linear regression.

C.14. FISH JUVENILE GROWTH TEST

1. **METHOD**

This growth toxicity test method is a replicate of the OECD TG 215 (2000).

1.1 INTRODUCTION

This test is designed to assess the effects of prolonged exposure to chemicals on the growth of juvenile fish. It is based on a method, developed and ring-tested (1)(2) within the European Union, for assessing the effects of chemicals on the growth of juvenile rainbow trout *(Oncorynchus mykiss*) under flow-through conditions. Other well documented species may be used. For example, experience has been gained from growth tests with zebrafish (*Danio rerio*) [1] (3)(4) and ricefish (medaka, *Oryzias latipes*) (5)(6)(7).

See also General Introduction Part C.

1.2 DEFINITIONS

Lowest Observed Effect Concentration (LOEC): is the lowest tested concentration of a test substance at which the substance is observed to have a significant effect (at $p < 0.05$) when compared with the control. However, all test concentrations above the LOEC must have a harmful effect equal to or greater than those observed at the LOEC.

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

EC_x: in this Test Method is the concentration of the test substance which causes a x % variation in growth rate of the fish when compared with controls.

Loading Rate: is the wet weight of fish per volume of water.

Stocking Density: is the number of fish per volume of water.

Individual fish specific growth rate: expresses the growth rate of one individual based on its initial weight.

Tank-average specific growth rate: expresses the mean growth rate of a tank population at one concentration.

Pseudo specific growth rate: expresses the individual growth rate compared to the mean initial weight of the tank population.

[1] Meyer, A., Bierman, C.H. and Orti, G. (1993). The phylogenetic position of the zebrafish (*Danio rerio*), a model system in developmental biology: an invitation to the comparative method. Proc. R. Soc. Lond. B. 252, 231-236.

1.3 PRINCIPLE OF THE TEST METHOD

Juvenile fish in exponential growth phase are placed, after being weighted, in test chambers and are exposed to a range of sublethal concentrations of the test substance dissolved in water preferably under flow-through, or, if not possible, under appropriate semi-static (static-renewal) conditions. The test duration is 28 days. Fish are fed daily. The food ration is based on initial fish weights and may be recalculated after 14 days. At the end of the test, the fish are weighed again. Effects on growth rates are analysed using a regression model in order to estimate the concentration that would cause a x % variation in growth rate, i.e. EC_x (e.g. EC_{10}, EC_{20}, or EC_{30}). Alternatively, the data may be compared with control values in order to determine the lowest observed effect concentration (LOEC) and hence the no observed effect concentration (NOEC).

1.4 INFORMATION ON THE TEST SUBSTANCE

Results of an acute toxicity test (see Test Method C. 1.) preferably performed with the species chosen for this test, should be available. This implies that the water solubility and the vapour pressure of the test substance are known and a reliable analytical method is available for the quantification of the substance in the test solutions with known and reported accuracy and limit of detection is available.

Useful information includes the structural formula, purity of the substance, stability in water and light, pK_a, P_{ow} and results of a test for ready biodegradability (see Test Method C. 4).

1.5 VALIDITY OF THE TEST

For the test to be valid the following conditions apply:

— the mortality in the control(s) must not exceed 10 % at the end of the test;

— the mean weight of fish in the control(s) must have increased enough to permit the detection of the minimum variation of growth rate considered as significant. A ring-test (2) has shown that for rainbow trout the mean weight of fish in the controls must have increased by at least the half (i.e. 50 %) of their mean initial weight over 28 days; e.g. initial weight: 1 g/fish (= 100 %), final weight after 28 days: $\geq$ 1.5 g/fish ($\geq$ 150 %);

— the dissolved oxygen concentration must have been at least 60 % of the air saturation value (ASV) throughout the test;

— the water temperature must not differ by more than ± 1 °C between test chambers at any one time during the test and should be maintained within a range of 2 °C within the temperature ranges specified for the test species (Annex 1).

1.6 DESCRIPTION OF THE TEST METHOD

1.6.1 **Apparatus**

Normal laboratory equipment and especially the following:

a) oxygen and pH meters;

b) equipment for determination of water hardness and alkalinity;

c) adequate apparatus for temperature control and preferably continuous monitoring;

d) tanks made of chemically inert material and of suitable capacity in relation to the recommended loading and stocking density (see section 1.8.5 and Annex 1);

e) suitably accurate balance (i.e. accurate to ± 0.5 %).

1.6.2 **Water**

Any water in which the test species shows suitable long-term survival and growth may be used as a test water. It should be of constant quality during the period of the test. The pH of the water should be within the range 6.5 to 8.5, but during a given test it should be within a range of ± 0.5 pH units. Hardness above 140 mg/l (as $CaCO_3$) is recommended. In order to ensure that the dilution water will not unduly influence the test result (for example by complexion of test substance), samples should be taken at intervals for analysis. Measurements of heavy metals (e.g. Cu, Pb, Zn, Hg, Cd and Ni), major anions and cations (e.g. Ca, Mg, Na, K, Cl and SO_4), pesticides (e.g. total organophosphorus and total organochlorine pesticides), total organic carbon and suspended solids should be made, for example, every three months where a dilution water is known to be relatively constant in quality. If water quality has been demonstrated to be constant over at least one year, determinations can be less frequent and intervals extended (e.g. every 6 months). Some chemical characteristics of an acceptable dilution water are listed in Annex 2.

1.6.3 **Test Solutions**

Test solutions of the chosen concentrations are prepared by dilution of a stock solution.

The stock solution should preferably be prepared by simply mixing or agitating the test substance in the diluition water by using mechanical means (e.g. stirring or ultrasonication). Saturation columns (solubility columns) can be used for achieving a suitable concentrated stock solution.

The use of solvents or dispersants (solubilising agents) may be required in some cases in order to produce a suitably concentrated stock solution. Examples of suitable solvents are acetone, ethanol, methanol, dimethylsulfoxide, dimethylformamide and triethyleneglycol. Examples of suitable dispersants are Cremophor RH40, Tween 80, Methylcellulose 0.01 % and HCO-40. Care should be taken when using readily biodegradable agents (e.g. acetone) and/or highly volatile compounds as these can cause problems with bacterial built-up in flow-through tests. When a solubilising agent is used it must have no significant effects on the fish growth nor visible adverse effects on the juvenile as revealed by a solvent-only control.

For flow-through tests, a system which continually dispenses and dilutes a stock solution of the test substance (e.g. metering pump, proportional diluter, saturator system) is required to deliver a series of concentrations to the test chambers. The flow rates of stock solutions and dilution water should be checked at intervals, preferably daily, during the test and should not vary by more than 10 % throughout the test. A ring-test (2) has shown that, for rainbow trout, a frequency of water removal during the test of 6 litres/g of fish/day is acceptable (see section 1.8.2.2).

For semi-static (renewal) tests, the frequency of medium renewal will depend on the stability of the test substance, but a daily water renewal is recommended. If, from preliminary stability tests (see section 1.4), the test substance concentration is not stable (i.e. outside the range 80-120 % of nominal or falling below 80 % of the measured initial concentration) over the renewal period, consideration should be given to the use of a flow-through test.

1.6.4 **Selection of species**

Rainbow trout (*Oncorhynchus mykiss*) is the recommended species for this test since most experience has been gained from ring-test with this species (1)(2). However, other well documented species can be used but the test procedure may have to be adapted to provide suitable test conditions. For example, experience is also available with zebrafish (*Danio rerio*) (3)(4) and ricefish (medaka, *Oryzias latipes*) (5)(6)(7). The rationale for the selection of the species and the experimental method should be reported in this case.

1.6.5 **Holding of fish**

The test fish shall be selected from a population of a single stock, preferably from the same spawning, which has been held for at least two weeks prior to the test under conditions of water quality and illumination similar to those used in the test. They should be fed a minimum ration of 2 % body weight per day and preferably 4 % body weight per day throughout the holding period and during the test.

Following a 48 h setting-in period, mortalities are recorded and the following criteria applied:

— mortalities of greater than 10 % of population in seven days: reject the entire batch;

— mortalities of between 5 % and 10 % of population: acclimation for seven additional days; if more than 5 % mortality during second seven days, reject the entire batch;

— mortalities of less than 5 % of population in seven days: accept the batch.

Fish should not receive treatment for disease in the two weeks preceding the test, or during the test.

1.7 TEST DESIGN

The 'test design' relates to the selection of the number and spacing of the test concentrations, the number of tanks at each concentration level and the number of fish per tank. Ideally, the test design should be chosen with regard to:

a) the objective of the study;

b) the method of statistical analysis that will be used;

c) the availability and cost of experimental resources.

The statement of the objective should, if possible, specify the statistical power at which a given size of difference (e.g. in growth rate) is required to be detected or, alternatively, the precision with which the EC_x (e.g. with x = 10, 20, or 30, and preferably not less than 10) is required to be estimated. Without this, a firm prescription of the size of the study cannot be given.

It is important to recognise that a design which is optimal (makes best use of resources) for use with one method of statistical analysis is not necessarily optimal for another. The recommended design for the estimation of a LOEC/NOEC would not therefore be the same as that recommended for analysis by regression.

In most of cases, regression analysis is preferable to the analysis of variance, for reasons discussed by Stephan and Rogers (8). However, when no suitable regression model is found ($r^2 < 0.9$) NOEC/LOEC should be used.

1.7.1 **Design for analysis by regression**

The important considerations in the design of a test to be analysed by regression are:

a) The effect concentration (e.g. $EC_{10,20,30}$) and the concentration range over which the effect of the test substance is of interest, should necessarily be spanned by the concentrations included in the test. The precision with which estimates of effect concentrations can be made, will be best when the effect concentration is in the middle of the range of concentrations tested. A preliminary range-finding test may be helpful in selecting appropriate test concentrations.

b) To enable satisfactory statistical modelling, the test should include at least one control tank and five additional tanks at different concentrations. Where appropriate, when a solubilising agent is used, one control containing the solubilising agent at the highest tested concentration should be run in addition to the test series (see sections 1.8.3 and 1.8.4).

c) An appropriate geometric series or logarithmic series (9) (see Annex 3) may be used. Logarithmic spacing of test concentration is to be preferred.

d) If more than six tanks are available, the additional tanks should either be used to provide replication or distributed across the range of concentrations in order to enable closer spacing of the levels. Either of these measures are equally desirable.

1.7.2 **Design for estimation of a NOEC/LOEC using Analysis of Variance (ANOVA)**

There should preferably be replicate tanks at each concentration, and statistical analysis should be at the tank level (10). Without replicate tanks, no allowance can be made for variability between tanks beyond that due to individual fish. However, experience has shown (11) that between-tank variability was very small compared with within-tank (i.e. between-fish) variability in the case examined. Therefore a relatively acceptable alternative is to perform statistical analysis at the level of individual fish.

Conventionally, at least five test concentrations in a geometric series with a factor preferably not exceeding 3.2 are used.

Generally, when tests are performed with replicate tanks, the number of replicate control tanks and therefore the number of fish should be the double of the number in each of the test concentrations, which should be of equal size (12)(13)(14). On the opposite, in absence of replicate tanks, the number of fish in the control group should be the same as the number in each test concentration.

If the ANOVA is to be based on tanks rather than individual fish (which would entail either individual marking of the fish or the use of 'pseudo' specific growth rates (see section 2.1.2)), there is a need for enough replication of tanks to enable the standard deviation of 'tanks-within-concentrations' to be determined. This means that the

degrees of freedom for error in the analysis of variance should be at least 5 (10). If only the controls are replicated, there is a danger that the error variability will be biased because it may increase with the mean value of the growth rate in question. Since growth rate is likely to decrease with increasing concentration, this will tend to lead to an overestimate of the variability.

1.8 PROCEDURE

1.8.1 **Selection and weighing of test fish**

It is important to minimise variation in weight of the fish at the beginning of the test. Suitable size ranges for the different species recommended for use in this test are given in Annex 1. For the whole batch of fish used in the test, the range in individual weights at the start of the test should ideally be kept to within ± 10 % of the arithmetic mean weight and, in any case, should not exceed 25 %. It is recommended to weight a subsample of fish before the test in order to estimate the mean weigh.

Food should be withheld from the stock population for 24 h prior to the start of the test. Fish should then be chosen at random. Using a general anaesthetic (e.g. an aqueous solution of 100 mg/l tricaine methane sulphonate (MS 222) neutralised by the addition of two parts of sodium bicarbonate per part of MS 222), fish should be weighted individually as wet weights (blotted dry) to the precision given in Annex 1. Those fish with weights within the intended range should be retained and then should be randomly distributed between the test vessels. The total wet weight of fish in each test vessel should be recorded. The use of anaesthetics likewise handling of fish (including blotting and weighing) may cause stress and injuries to the juvenile fish, in particular for those species of small size. Therefore handling of juvenile fish must be done with the utmost care to avoid stressing and injuring test animals.

The fish are weighed again on day 28 of the test (see section 1.8.6). However, if it is deemed necessary to recalculate the food ration, fish can be weighed again on day 14 of the test (see section 1.8.2.3). Other method as photographic method could be used to determine changes in fish size from which food rations could be adjusted.

1.8.2 **Conditions of exposure**

1.8.2.1 *Duration*

The test duration is ≥ 28 days.

1.8.2.2 *Loading rates and stocking densities*

It is important that the loading rate and stocking density is appropriate for the test species used (see Annex 1). If the stocking density is too high, then overcrowding stress will occur leading to reduced growth rates and possibly to disease. If it is too low, territorial behaviour may be induced which could also affect growth. In any case, the loading rate should be low enough in order that a dissolved oxygen concentration of at least 60 % ASV can be maintained without aeration. A ring-test (2) has shown that, for rainbow trout, a loading rate of 16 trouts of 3-5 g in a 40-litre volume is acceptable. Recommended frequency of water removal during the test is 6 litres/g of fish/day.

1.8.2.3 *Feeding*

The fish should be fed with an appropriate food (Annex 1) at a sufficient rate to induce acceptable growth rate. Care should be taken to avoid microbial growth and water turbidity. For rainbow trout, a rate of 4 % of their body weight per day is likely to satisfy these conditions (2)(15)(16)(17). The daily ration may be divided into two equal portions and given to the fish in two feeds per day, separated by at least 5 h. The ration is based on the initial total fish weight for each test vessel. If the fish are weighted again on day 14, the ration is then recalculated. Food should be withheld from the fish 24 h prior to weighing.

Uneaten food and fecal material should be removed from the test vessels each day by carefully cleaning the bottom of each tank using a suction.

1.8.2.4 *Light and temperature*

The photoperiod and water temperature should be appropriate for the test species (Annex 1).

1.8.3 **Test concentrations**

Normally five concentrations of the test substance are required, regardless of the test design (see section 1.7.2). Prior knowledge of the toxicity of the test substance (e.g. from an acute test and/or from range-finding studies) should help in selecting appropriate test concentrations. Justification should be given if fewer than five concentrations are used. The highest tested concentration should not exceed the substance solubility limit in water.

Where a solubilising agent is used to assist in stock solution preparation, its final concentration should not be greater than 0.1 ml/l and should preferably be the same in all test vessels (see section 1.6.3). However, every effort should be made to avoid use of such materials.

1.8.4 **Controls**

The number of dilution-water controls depends on the test design (see sections 1.7-1.7.2). If a solubilising agent is used, then the same number of solubilising-agent controls as dilution-water controls should also be included.

1.8.5 **Frequency of analytical determinations and measurements**

During the test, the concentrations of test substance are determined at regular intervals (see below).

In flow-through tests, the flow rates of diluent and toxicant stock solution should be checked at intervals, preferably daily, and should not vary by more than 10 % throughout the test. Where the test substance concentrations are expected to be within ± 20 % of the nominal values (i.e. within the range 80-120 %; see sections 1.6.2 and 1.6.3), it is recommended that, as a minimum, the highest and lowest test concentrations be analysed at the start of the test and at weekly intervals thereafter. For the test where the concentration of the test substance is not expected to remain within ± 20 % of nominal (on the basis of stability data of the test substance), it is necessary to analyse all test concentrations, but following the same regime.

In semi-static (renewal) tests where the concentration of the test substance is expected to remain within ± 20 % of the nominal values, it is recommended that, as a minimum, the highest and lowest test concentrations be analysed when freshly prepared and immediately prior to renewal at the start of the study and weekly thereafter. For tests where the concentration of the test substance is not expected to remain within ± 20 % of nominal, all test concentrations must be analysed following the same regime as for more stable substances.

It is recommended that results be based on measured concentrations. However, if evidence is available to demonstrate that the concentration of the test substance in solution has been satisfactorily maintained within ± 20 % of the nominal or measured initial concentration throughout the test, then the results can be based on nominal or measured values.

Samples may need to be filtered (e.g. using a 0.45 μm pore size) or centrifuged. Centrifugation is the recommended procedure. However, if the test material does not adsorb to filters, filtration may also be acceptable.

During the test, dissolved oxygen, pH and temperature should be measured in all test vessels. Total hardness, alkalinity and salinity (if relevant) should be measured in the controls and one vessel at the highest concentration. As a minimum, dissolved oxygen and salinity (if relevant) should be measured three times (at the beginning, middle and end of the test). In semi-static tests, it is recommended that dissolved oxygen be measured more frequently, preferably before and after each water renewal or at least once a week. pH should be measured at the beginning and end of each water renewal in static renewal test and at least weekly in flow-through tests. Hardness and alkalinity should be measured once each test. Temperature should preferably be monitored continuously in at least one test vessel.

1.8.6 **Observations**

Weight: At the end of the test all surviving fish must be weighed as wet weights (blotted dry) either in groups by test vessel or individually. Weighing of animals by test vessel is preferred to individual weights which require that fish be individually marked. In the case of the measurement of individual weights for determination of individual fish specific growth rate, the marking technique selected should avoid stressing the animals (alternatives to freeze marking may be appropriate, e.g. the use of colored fine fishing line).

The fish should be examined daily during the test period and any external abnormalities (such as hemorrhage, discoloration) and abnormal behaviour noted. Any mortalities should be recorded and the dead fish removed as soon as possible. Dead fish are not replaced, the loading rate and stocking density being sufficient to avoid effects on growth through changes in number of fish per tank. However, the feeding rate will need to be adjusted.

2. DATA AND REPORTING

2.1 TREATMENT OF RESULTS

It is recommended that a statistician be involved in both the design and analysis of the test since this test method allows for considerable variation in experimental design as for example, in the number of test chambers, number of test concentrations, number of fish, etc. In view of the options available in test design, specific guidance on statistical procedure is not given here.

Growth rates should not be calculated for test vessels where the mortality exceeds 10 %. However, mortality rate should be indicated for all test concentrations.

Whichever method is used to analyse the data, the central concept is the specific growth rate r between time t_1 and time t_2. This can be defined in several ways depending on whether fish are individually marked or not or whether a tank average is required.

$$r_1 = \frac{\log_e w_2 - \log_e w_1}{t_2 - t_1} \times 100$$

$$r_2 = \frac{\overline{\log_e w_2} - \overline{\log_e w_1}}{t_2 - t_1} \times 100$$

$$r_3 = \frac{\log_e w_2 - \overline{\log_e w_1}}{t_2 - t_1} \times 100$$

where,

r_1 = individual fish specific growth rate

r_2 = tank-average specific growth rate

r_3 = 'pseudo' specific growth rate

w_1, w_2 = weights of a particular fish at times t_1 and t_2, respectively

$\log_e w_1$ = logarithm of the weight of an individual fish at the start of the study period

$\log_e w_2$ = logarithm of the weight of an individual fish at the end of the study period

$\overline{\log_e w_1}$ = average of the logarithms of the values w_1 for the fish in the tank at the start of the study period

$\overline{\log_e w_2}$ = average of the logarithms of the values w_2 for the fish in the tank at the end of the study period

t_1, t_2 = time (days) at start and end of study period

r_1, r_2, r_3 can be calculated for the 0-28 days period and, where appropriate (i.e. when measurement at day 14 has been done) for the 0-14 and 14-28 days periods.

2.1.1 **Analysis of results by regression (concentration-response modelling)**

This method of analysis fits a suitable mathematical relationship between the specific growth rate and concentration, and hence enables the estimation of the 'EC_x' i.e. any required EC value. Using this method the calculation of r for individual fish (r_1) is not necessary and instead, the analysis can be based on the tank-average value of r (r_2). This last method is preferred. It is also more appropriate in case of the use of smallest species.

The tank-average specific growth rates (r_2) should be plotted graphically against concentration, in order to inspect the concentration response relationship.

For expressing the relationship between r_2 and concentration, an appropriate model should be chosen and its choice must be supported by appropriate reasoning.

If the numbers of fish surviving in each tank are unequal, then the process of model fitting, whether simple or non-linear, should be weighted to allow for unequal sizes of groups.

The method of fitting the model must enable an estimate of, for example, the EC_{20} and of its dispersion (either standard error or confidence interval) to be derived. The graph of the fitted model should be shown in relation to the data so that the adequacy of the fit of the model can be seen (8)(18)(19)(20).

2.1.2 **Analysis of results for the estimation of the LOEC**

If the test has included replication of tanks at all concentration levels, the estimation of the LOEC could be based on an analysis of variance (ANOVA) of the tank-average specific growth rate (see section 2.1), followed by a suitable method (e.g. Dunnett's or Williams' test (12)(13)(14)(21)) of comparing the average r for each concentration with the average r for the controls to identify the lowest concentration for which this difference is significant at a 0.05 probability level. If the required assumptions for parametric methods are not met - non-normal distribution (e.g. Shapiro-Wilk's test) or heterogeneous variance (Bartlett's test), consideration should be given to transforming the data to homogenise variances prior to performing the ANOVA, or to carrying out a weighted ANOVA.

If the test has not included replication of tanks at each concentration, an ANOVA based on tanks will be insensitive or impossible. In this situation, an acceptable compromise is to base the ANOVA on the 'pseudo' specific growth rate r_3 for individual fish.

The average r_3 for each test concentration may then be compared with the average r_3 for the controls. The LOEC can then be identified as before. It must be recognised that this method provides no allowance for, nor protection against, variability between tanks, beyond that which is accounted for by the variability between individual fish. However, experience has shown (8) that between-tank variability was very small compared with within-tank (i.e. between fish) variability. If individual fish are not included in the analysis, the method of outlier identification and justification for its use must be provided.

2.2 INTERPRETATION OF RESULTS

The results should be interpreted with caution where measured toxicant concentrations in test solutions occur at levels near the detection limit of the analytical method or, in semi static tests, when the concentration of the test substance decreases between freshly prepared solution and before renewal.

2.3 TEST REPORT

The test report must include the following information:

2.3.1 **Test substance:**

— physical nature and relevant physical-chemical properties;

— chemical identification data including purity and analytical method for quantification of the test substance where appropriate.

2.3.2 **Test species:**

— scientific name, possibly

— strain, size, supplier, any pre-treatment, etc.

2.3.3 **Test conditions:**

— test procedure used (e.g. semi-static/renewal, flow-through, loading, stocking density, etc.);

— test design (e.g. number of test vessels, test concentrations and replicates, number of fish per vessel);

— method of preparation of stock solutions and frequency of renewal (the solubilising agent and its concentration must be given, when used);

— the nominal test concentrations, the means of the measured values and their standard deviations in the test vessels and the method by which these were attained and evidence that the measurements refer to the concentrations of the test substance in true solution;

— dilution water characteristics: pH, hardness, alkalinity, temperature, dissolved oxygen concentration, residual chlorine levels (if measured), total organic carbon, suspended solids, salinity of the test medium (if measured) and any other measurements made;

— water quality within test vessels: pH, hardness, temperature and dissolved oxygen concentration;

— detailed information on feeding, (e.g. type of food(s), source, amount given and frequency).

2.3.4 **Results:**

— evidence that controls met the validity criterion for survival, and data on mortalities occurring in any of the test concentrations;

— statistical analytical techniques used, statistics based on replicates or fish, treatment of data and justification of techniques used;

— tabulated data on individual and mean fish weights on days 0, 14 (if measured) and 28 values of tank-average or pseudo specific growth rates (as appropriate) for the periods 0-28 days or possibly 0-14 and 14-28;

— results of the statistical analysis (i.e. regression analysis or ANOVA) preferably in tabular and graphical form and the LOEC ($p = 0.05$) and the NOEC or EC_x with, when possible, standard errors, as appropriate;

— incidence of any unusual reactions by the fish and any visible effects produced by the test substance.

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ANNEX 1

FISH SPECIES RECOMMENDED FOR TESTING AND SUITABLE TEST CONDITIONS

Species	Recommended test temperature range (^{o}C)	Photoperiod (hours)	Recommended range for initial fish weight (g)	Required measurement precision	Loading rate (g/l)	Stocking density (per litre)	Food	Test duration (days)
Recommended species:								
Oncorhynchus mykiss rainbow trout	12.5 – 16.0	12 – 16	1 – 5	to nearest 100 mg	1.2 – 2.0	4	Dry propietary salmonid fry food	≥ 28
Other well documented species:								
Danio rerio zebrafish	21 – 25	12 – 16	0.050 – 0.100	to nearest 1 mg	0.2 – 1.0	5 – 10	Live food (*Brachionus Artemia*)	≥ 28
Oryzias latipes ricefish (Medaka)	21 – 25	12 – 16	0.050 – 0.100	to nearest 1 mg	0.2 – 1.0	5 – 20	Live food (*Brachionus Artemia*)	≥ 28

ANNEX 2

SOME CHEMICAL CHARACTERISTICS OF AN ACCEPTABLE DILUTION WATER

SUBSTANCE	CONCENTRATIONS
Particulate matter	< 20 mg/l
Total organic carbon	< 2 mg/l
Unionised ammonia	< 1 µg/l
Residual chlorine	< 10 µg/l
Total organophosphorus pesticides	< 50 ng/l
Total organochlorine pesticides plus polychlorinated biphenyls	< 50 ng/l
Total organic chlorine	< 25 ng/l

ANNEX 3

LOGARITHMIC SERIES OF CONCENTRATIONS SUITABLE FOR TOXICITY TEST (9)

Column (Number of concentrations between 100 and 10, or between 10 and 1)*						
1	**2**	**3**	**4**	**5**	**6**	**7**
100	100	100	100	100	100	100
32	46	56	63	68	72	75
10	22	32	40	46	52	56
3.2	10	18	25	32	37	42
1.0	4.6	10	16	22	27	32
	2.2	5.6	10	15	19	24
	1.0	3.2	6.3	10	14	18
		1.8	4.0	6.8	10	13
		1.0	2.5	4.6	7.2	10
			1.6	3.2	5.2	7.5
			1.0	2.2	3.7	5.6
				1.5	2.7	4.2
				1.0	1.9	3.2
					1.4	2.4
					1.0	1.8
						1.3
						1.0

* A series of five (or more) successive concentrations may be chosen from a column. Mid-points between concentrations in column (x) are found in column (2x + 1). The values listed can represent concentrations expressed as percentage per volume or weight (mg/l or µg/l). Values can be multiplied or divided by any power of 10 as appropriate. Column 1 might be used if there was considerable uncertainty on the toxicity level.

C.15. FISH, SHORT-TERM TOXICITY TEST ON *EMBRYO* AND SAC-FRY STAGES

1. METHOD

This short-term toxicity test method is a replicate of the OECD TG 212 (1998).

1.1 INTRODUCTION

This short-term toxicity test on Fish Embryo and Sac-Fry stages is a short-term test in which the life stages from the newly fertilized egg to the end of the sac-fry stage are exposed. No feeding is provided in the embryo and sac-fry test, and the test should thus be terminated while the sac-fry are still nourished from the yolk-sac.

The test is intended to define lethal, and to a limited extent, sublethal effects of chemicals on the specific stages and species tested. This test would provide useful information in that is could (a) form a bridge between lethal and sublethal tests, (b) be used as a screening test for either a Full Early Life Stage test or for chronic toxicity tests and (c) be used for testing species where husbandry techniques are not sufficiently advanced to cover the period of change from endogenous to exogenous feeding.

It should be borne in mind that only tests incorporating all stages of the life-cycle of fish are generally liable to give an accurate estimate of the chronic toxicity of chemicals to fish, and that any reduced exposure with respect to life stages may reduce the sensitivity and thus underestimate the chronic toxicity. It is therefore expected that the embryo and sac-fry test would be less sensitive than a Full Early Life Stage test, particularly with respect to chemicals with high lipophilicity (log P_{ow} > 4) and chemicals with a specific mode of toxic action. However smaller differences in sensitivity between the two tests would be expected for chemicals with a non-specific, narcotic mode of action (1).

Prior to the publication of this test, most experience with this embryo and sac-fry test has been with the freshwater fish *Danio rerio* Hamilton-Buchanan (Teleostei, Cyprinidae – common name zebrafish). More detailed guidance on test performance for this species is therefore given in Annex 1. This does not preclude the use of other species for which experience is also available (Table 1).

1.2 DEFINITIONS

Lowest Observed Effect Concentration (LOEC): is the lowest tested concentration of a test substance at which the substance is observed to have a significant effect (at $p < 0.05$) when compared with the control. However, all test concentrations above the LOEC must have a harmful effect equal to or greater than those observed at the LOEC.

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

1.3 PRINCIPLE OF THE TEST

The embryo and sac-fry stages of fish are exposed to a range of concentrations of the test substance dissolved in water. Within the protocol a choice is possible between a semi-static and a flow-through procedure. The choice depends on the nature of the test substance. The test is begun by placing fertilised eggs in the test chambers and is terminated just before the yolk-sac of any larvae in any of the test chambers has been completely absorbed or before mortalities by starvation start in controls. Lethal and sub-lethal effects are assessed and compared with control values to determine the lowest observed effect concentration and hence the no observed effect concentration. Alternatively, they may be analysed using a regression model in order to estimate the concentration that would cause a given percentage effect (i.e. LC/ECx, where x is a defined % effect).

1.4 INFORMATION ON THE TEST SUBSTANCE

Results of an acute toxicity test (see Method C. 1) preferably performed with the species chosen for this test, should be available. The results may be useful in selecting an appropiate range of test concentrations in the early life stages test. Water solubility (including solubility in the test water) and the vapour pressure of the test substance should be known. A reliable analytical method for the quantification of the substance in the test solutions with known and reported accuracy and limit of detection should be available.

Information on the test substance which is useful in establishing the test conditions includes the structural formula, purity of the substance, stability in light, stability under the conditions of the test, pKa, P_{ow} and results of a test for ready biodegradability (see Method C. 4).

1.5 VALIDITY OF THE TEST

For a test to be valid, the following conditions apply:

— overall survival of fertilised eggs in the controls and where relevant, in the solvent-only vessels must be greater than or equal to the limits defined in Annexes 2 and 3;

— the dissolved oxygen concentration must be between 60 and 100 % of the air saturation value (ASV) throughout the test;

— the water temperature must not differ by more than ± 1.5 °C between test chambers or between successive days at any time during the test and should be within the temperature ranges specified for the test species (Annexes 2 and 3).

1.6 DESCRIPTION OF THE TEST METHOD

1.6.1 **Test chambers**

Any glass or other chemically inert vessels can be used. The dimensions of the vessels should be large enough to allow compliance with the loading rate (see section 1.7.1.2). It is recommended that test chambers be randomly positioned in the test area. A randomised block design with each treatment being present in each block is preferable to a completely randomised design when there are systematic effects in the laboratory that can be controlled using blocking. Blocking, if used, should be taken account of in the subsequent data analysis. The test chambers should be shielded from unwanted disturbance.

1.6.2 **Selection of fish species**

Recommended fish species are given in Table 1A. This does not preclude the use of other species (examples are given in Table 1B), but the test procedure may have to be adapted to provide suitable test conditions. The rationale for the selection of the species and the experimental method should be reported in this case.

1.6.3 **Holding of the brood fish**

Details on holding the brood stock under satisfactory conditions may be found in OECD TG 210 [1] and in references (2)(3)(4)(5)(6).

1.6.4 **Handling of embryos and larvae**

Embryos and larvae may be exposed, within the main vessel, in smaller vessels fitted with mesh sides or ends to permit a flow of test solution through the vessel. Non-turbulent flow through these small vessels may be induced by suspending them from an arm arranged to move the vessel up and down but always keeping the organisms submerged; a siphon-flush system can also be used. Fertilised eggs of salmonid fishes can be supported on racks or meshes with apertures sufficiently large to allow larvae to drop through after hatching. The use of pasteur pipettes is appropriate to remove the embryos and larvaes in the semi-static tests with complete daily renewal (see paragraph 1.6.6)

Where egg containers, grids or meshes have been used to hold eggs within the main test vessel, these restraints should be removed after the larvae hatch [1], except that meshes should be retained to prevent the escape of the fish. If there is a need to transfer the larvae, they should not be exposed to the air and nets should not be used to release fish from egg containers (such a caution may not be necessary for some less fragile species, e.g. the carp). The timing of this transfer varies with the species and transfer may not always be necessary. For the semi-static technique, beakers or shallow containers may be used, and, if necessary, equipped with a mesh screen slightly elevated above the bottom of the beaker. If the volume of these containers is sufficient to comply with loading requirements, (see 1.7.1.2) no transfer of embryo or larvae may be necessary.

1.6.5 **Water**

Any water which conforms to the chemical characteristics of an acceptable dilution water as listed in Annex 4 and in which the test species shows control survival at least as good as that described in Annexes 2 and 3 is suitable as a test water. It should be of constant quality during the period of the test. The pH should remain within a range of ± 0.5 pH units. In order to ensure that the dilution water will not unduly influence the test result (for example by complexation of test substance), or adversely affect the performance of the brood stock, samples should be taken at intervals for analysis. Measurements of heavy metals (e.g. Cu, Pb, Zn, Hg, Cd and Ni), major anions and cations (e.g. Ca, Mg, Na, K, Cl and SO_4), pesticides (e.g. total organophosphorus and total organochlorine pesticides), total organic carbon and suspended solids should be made, for example, every three months, where a dilution water is known to be relatively constant in quality. If water quality has been demonstrated to be constant over at least one year, determinations can be less frequent and intervals extended (e.g. every six months).

[1] OECD, Paris, 1992, Test Guideline 210, " Fish, Early-life Stage Toxicity Test".

1.6.6 **Test Solutions**

Test solutions of the chosen concentrations are prepared by dilution of a stock solution.

The stock solution should preferably be prepared by simply mixing or agitating the test substance in the dilution water by using mechanical means (e.g. stirring and ultrasonication). Saturation colums (solubility columns) can be used for achieving a suitable concentrated stock solution. As far as possible, the use of solvents or dispersants (solubilising agents) should be avoided; however, such coupounds may be required in some cases in order to produced a suitably concentrated stock solution. Examples of suitable solvents are acetone, ethanol, methanol, dimethylformamide and triethyleneglycol. Examples of suitable dispersants are Cremophor RH40, Tween 80, methylcellulose 0.01 % and HCO-40. Care should be taken when using readily biodegradable agents (e.g. acetone) and/or highly volatile as these can cause problems with bacterial built-up in flow-through tests. When a solubilising agent is used it must have no significant effect on survival nor visible adverse effect on the early-life stages as revealed by a solvent-only control. However, every effort should be made to avoid the use of such materials.

For the semi-static technique, two different renewal procedures may be followed; either (i) new test solutions are prepared in clean vessels and surviving eggs and larvae gently transferred into the new vessels in a small volume of old solution, avoiding exposure to air, or (ii) the test organisms are retained in the vessels whilst a proportion (at least three-quarters) of the test water is changed. The frequency of medium renewal will depend on the stability of the test substance, but a daily water renewal is recommended. If, from preliminary stability tests (see section 1.4), the test substance concentration is not stable (i.e.outside the range 80- 120 % of nominal or falling below 80 % of the measured initial concentration) over the renewal period, consideration should be given to the use of a flow-through test. In any case, care should be taken to avoid stressing the larvae during the water renewal operation.

For flow-through tests, a system which continually dispenses and dilutes a stock solution of the test substance (e.g. metering pump, proportional diluter, saturator system) is required to deliver a series of concentrations to the test chambers. The flow rates of stock solutions and dilution water should be checked at intervals, preferably daily, and should not vary by more than 10 % throughout the test. A flow rate equivalent to at least five test chamber volumes per 24 hours has been found suitable (2).

1.7 PROCEDURE

Useful information on the performance of fish embryo and sac-fry toxicity tests is available in the literature, some examples of which are included in the literature section of this text (7)(8)(9).

1.7.1 **Conditions of exposure**

1.7.1.1 *Duration*

The test should start preferably within 30 minutes after the eggs have been fertilised. The embryos are immersed in the test solution before, or as soon as possible after, commencement of the blastodisc cleavage stage and in any case before the onset of the gastrula stage. For eggs obtained from commercial supplier, it may not be possible to start the test immediately after fertilisation. As the sensitivity of the test may be seriously influenced by delaying the start of the test, the test should be initiated within 8 hours after fertilisation. As larvae are not fed during the exposure period, the test should be terminated just before the yolk sac of any larvae in any of the test chambers has been completely absorbed or before mortalities by starvation start in controls. The duration will depend upon the species used. Some recommended durations are given in Annexes 2 and 3.

1.7.1.2 *Loading*

The number of fertilised eggs at the start of the test should be sufficient to meet statistical requirements. They should be randomly distributed among treatments, and at least 30 fertilised eggs, divided equally (or as equally as possible since it can be difficult to obtain equal batches when using some species) between at least three replicate test chambers, should be used per concentration. The loading rate (biomass per volume of test solution) should be low enough in order that a dissolved oxygen concentration of at least 60 % ASV can be maintained without aeration. For flow-through tests, a loading rate not exceeding 0.5 g/l per 24 hours and not exceeding 5 g/l of solution at any time has bean recommended (2).

1.7.1.3 *Light and temperature*

The photoperiod and test water temperature should be appropriate for the test species (Annex 2 and 3). For the purpose of temperature monitoring, it may be appropriate to use an additional test vessel.

1.7.2 **Test concentrations**

Normally, five concentrations of the test substance spaced by a constant factor not exceeding 3.2 are required. The curve relating LC_{50} to period of exposure in the acute study should be considered when selecting the range of test concentrations. The use of fewer than five concentrations, for example in limit tests, and a narrower concentration interval may be appropriate in some circumstances. Justification should be provided if fewer than five concentrations are used. Concentrations of the substance higher than the 96 hour LC_{50} or 100 mg/l, whichever is the lower, need not be tested. Substances should not be tested above their solubility limit in in the test water.

When a solubilising agent is used to aid preparation of test solutions (see section 1.6.6), its final concentration in the test vessels should not be greater than 0.1 ml/l and should be the same in all test vessels.

1.7.3 **Controls**

One dilution-water control (replicated as appropriate) and also, if relevant, one control containing the solubilising-agent (replicated as appropriate) should be run in addition to the test series.

1.7.4 **Frequency of analytical determinations and measurements**

During the test, the concentrations of the test substance are determined at regular intervals.

In semi-static tests where the concentration of the test substance is expected to remain within ± 20 % of the nominal (i.e. within the range 80 - 120 %; see section 1.4 and 1.6.6), it is recommended that, as a minimum, the highest and lowest test concentrations be analysed when freshly prepared and immediately prior to renewal on at least three occasions spaced evenly over the test (i.e. analyses should be made on a sample from the same solution - when freshly prepared and at renewal).

For tests where the concentration of the test substance is not expected to remain within ± 20 % of nominal (on the basis of stability data of the substance), it is necessary to analyse all test concentrations ,when freshly prepared and at renewal, but following the same regime (i.e. on at least three occasions spaced evenly over the test). Determination of test substance concentrations prior to renewal need only be performed on one replicate vessel at each test concentration. Determinations should be made no more than seven days apart. It is recommended that results be based on measured concentrations. However, if evidence is available to demonstrate that the concentration of the test substance in solution has been satisfactorily maintained within ± 20 % of the nominal or measured initial concentration throughout the test, then results can be based on nominal or measured initial values.

For flow-through tests, a similar sampling regime to that described for semi-static tests is appropriate (but measurement of 'old' solutions is not applicable in this case). However, if the test duration is more than seven days, it may be advisable to increase the number of sampling occasions during the first week (e.g. three sets of measurements) to ensure that the test concentrations are remaining stable.

Samples may need to be centrifuged or filtered (e.g. using a 0.45 μm pore size). However, since neither centrifuging nor filtration appears always to separate the non-bioavailable fraction of the test substance from that which is bioavailable, samples may not be subjected to those treatments.

During the test, dissolved oxygen, pH and temperature should be measured in all test vessels. Total hardness and salinity (if relevant) should be measured in the controls and one vessel at the highest concentration. As a minimum, dissolved oxygen and salinity (if relevant) should be measured three times (at the beginning, middle and end of the test). In semi-static tests, it is recommended that dissolved oxygen be measured more frequently, preferably before and after each water renewal or at least once at week. pH should be measured at the beginning and end of each water renewal in semi-static test and at least weekly in flow-through tests. Hardness should be measured once each test. Temperature should be measured daily and it should preferably be monitored continuously in at least one test vessel.

1.7.5 **Observations**

1.7.5.1 *Stage of embryonic development*

The embryonic stage (i.e. gastrula stage) at the beginning of exposure to the test substance should be verified as precisely as possible. This can be done using a representative sample of eggs suitably preserved and cleared. The literature may also be consulted for the description and illustration of embryonic stages (2)(5)(10)(11).

1.7.5.2 *Hatching and survival*

Observations on hatching and survival should be made at least once daily and numbers recorded. It may be desirable to make more frequent observations at the beginning of the test (e.g. each 30 minutes during the first three hours), since in some cases, survival times can be more relevant than only the number of deaths (e.g. when there are acute toxic effects). Dead embryos and larvae should be removed as soon as observed since they can decompose rapidly. Extreme care should be taken when removing dead individuals not to knock or physically damage adjacent eggs/larvae, these being extremely delicate and sensitive. Criteria for death vary according to life stage:

— **for eggs**: particularly in the early stages, a marked loss of translucency and change in colouration, caused by coagulation and/or precipitation of protein, leading to a white opaque appearance;

— **for embryos**: absence of body movement and/or absence of heart beat and/or opaque discoloration in species whose embryos are normally translucent;

— **for larvae**: immobility and/or absence of respiratory movement and/or absence of heart-beat and/or white opaque colouration of central nervous system and/or lack of reaction mechanical stimulus.

1.7.5.3 *Abnormal appearance*

The number of larvae showing abnormality of body form and/or pigmentation, and the stage of yolk-sac absorption, should be recorded at adequate intervals depending on the duration of the test and the nature of the abnormality described. It should be noted that abnormal embryos and larvae occur naturally and can be of the order of several per cent in the control(s) in some species. Abnormal animals should only be removed from the test vessels on death.

1.7.5.4 *Abnormal behaviour*

Abnormalities, e.g. hyperventilation, uncoordinated swimming, and atypical quiescence should be recorded at adequate intervals depending on the duration of the test. These effects, although difficult to quantify, can, when observed, aid in the interpretation of mortality data i.e. provide information on the mode of toxic action of the substance.

1.7.5.5 *Length*

At the end of the test, measurement of individual lengths is recommended; standard, fork or total length may be used. If however, caudal fin rot or fin erosion occurs, standard lengths should be used. Generally, in a well-run test, the coefficient of variation for length among replicates in the controls should be ≤ 20 %.

1.7.5.6 *Weight*

At the end of the test, individual weights can be measured; dry weights (24 hours at 60 °C) are preferable to wet weights (blotted dry). Generally, in a well-run test, the coefficient of variation for weight among replicates in the controls should be ≤ 20 %.

These observations will result in some or all of the following data being available for statistical analysis:

- cumulative mortality;
- numbers of healthy larvae at end of test;
- time to start of hatching and end of hatching (i.e. 90 % hatching in each replicate);
- numbers of larvae hatching each day;
- length (and weight) of surviving animals at end of the test;
- numbers of larvae that are deformed or of abnormal appearance;
- numbers of larvae exhibiting abnormal behaviour.

2. DATA AND REPORTING

2.1 TREATMENT OF RESULTS

It is recommended that a statistician be involved in both the design and analysis of the test since the method allows for considerable variation in experimental design as, for example, in the number of test chambers, number of test concentrations, starting number of fertilised eggs and in the parameters measured. In view of the options available in test design, specific guidance on statistical procedures is not given here.

If LOEC/NOECs are to be estimated, it will be necessary for variations to be analysed within each set of replicates using analysis of variance (ANOVA) or contingency table procedures. In order to make a multiple comparison between the results at the individual concentrations and those for the controls, Dunnett's method may be found useful (12)(13). Other useful examples are also available (14)(15). The size of the effect detectable using ANOVA or other procedures (i.e. the power of the test) should be calculated and reported. It should be noted that not all the observations listed in section 1.7.5.6 are suitable for statistical analysis using ANOVA. For example, cumulative mortality and numbers of healthy larvae at the end of the test could be analysed using probit methods.

If LC/EC_xs are to be estimated, (a) suitable curve(s), such as the logistic curve, should be fitted to the data of interest using a statistical method such as least squares or non-linear least squares. The curve(s) should be parameterised so that the LC/EC_x of interest and its standard error can be estimated directly. This will greatly ease the calculation of the confidence limits around the LC/EC_x. Unless there are good reasons to prefer different confidence levels, two-sided 95 % confidence should be quoted. The fitting procedure should preferably provide a means for assessing the significance of the lack of fit. Graphical methods for fitting curves can be used. Regression analysis is suitable for all observations listed in section 1.7.5.6.

2.2 INTERPRETATION OF RESULTS

The results should be interpreted with caution where measured toxicant concentrations in test solutions occur at levels near the detection limit of the analytical method. The interpretation of results for concentrations above the water solubility of the substance should also be made with care.

2.3 THE TEST REPORT

The test report must include the following information:

2.3.1 **Test substance:**

— physical nature and relevant physical-chemical properties;

— chemical identification data, including purity and analytical method for quantification of the tests substance where appropriate.

2.3.2 **Test species:**

— scientific name, strain, numbers of parental fish (i.e. how many females were used for providing the required numbers of eggs in the test), source and method of collection of the fertilised eggs and subsequent handling.

2.3.3 **Test conditions:**

— test procedure used (e.g. semi-static or flow-through, time period from fertilisation to start the test, loading, etc);

— photoperiod(s);

— test design (e.g. number of test chambers and replicates, number of embryos per replicate);

— method of preparation of stock solutions and frequency of renewal (the solubilising agent and its concentration must be given, when used);

— the nominal test concentrations, the measured values, their means and their standard deviations in the test vessels and the method by which these were attained and, if the test substance is soluble in water at concentrations below those tested, evidence should be provided that the measurements refer to the concentrations of the test substance in solution;

— dilution water characteristics: pH, hardness, temperature, dissolved oxygen concentration, residual chlorine levels (if measured), total organic carbon, suspended solids, salinity of the test medium (if measured) and any other measurements made;

— water quality within test vessels: pH, hardness, temperature and dissolved oxygen concentration.

2.3.4 **Results:**

— results from any preliminary studies on the stability of the test substance;

— evidence that controls met the overall survival acceptability standard of the test species (Annexes 2 and 3);

— data on mortality/survival at embryo and larval stages and overall mortality/survival;

— days to hatch and numbers hatched;

— data for length (and weight);

— incidence and description of morphological abnormalities, if any;

— incidence and description of behavioural effects, if any;

— statistical analysis and treatment of data;

— for tests analysed using ANOVA, the lowest observed effect concentration (LOEC) at p=0.05 and the no observed effect concentration (NOEC) for each response assessed, including a description of the statistical procedures used and an indication of what size of effect could be detected;

— for tests analysed using regression techniques, the LC/EC_x and confidence intervals and a graph of the fitted model used for its calculation;

— explanation for any deviation from this testing method.

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TABLE 1A: FISH SPECIES RECOMMENDED FOR TESTING

FRESHWATER
Oncorhynchus mykiss Rainbow trout (9)(16)
Danio rerio Zebrafish (7)(17)(18)
Cyprinus caprio Common carp (8)(19)
Oryzias latipes Japanese ricefish/Medaka (20)(21)
Pimephales promelas Fathead minnow (8)(22)

TABLE 1B: EXAMPLES OF OTHER WELL-DOCUMENTED SPECIES WHICH HAVE ALSO BEEN USED

FRESHWATER	**SALTWATER**
Carassius auratus Goldfish (8)	*Menidia peninsulae* Tidewater silverside (23)(24)(25)
Lepomis macrochirus Bluegill (8)	*Clupea harengus* Herring (24)(25)
	Gadus morhua Cod (24)(25)
	Cyprinodon variegatus Sheepshead minnow (23)(24)(25)

ANNEX 1

GUIDANCE ON PERFORMANCE OF A TOXICITY TEST ON EMBRYOS AND SAC-FRY OF ZEBRAFISH *(Brachydanio rerio)*

INTRODUCTION

The zebrafish originates from the Coromandel coast of India where it inhabits fast-flowing streams. It is a common aquarium fish of the carp family, and information about procedures for its care and culture can be found in standard reference books on tropical fish. Its biology and use in fishery research have been reviewed by Laale (1).

The fish rarely exceeds 45 mm in length. The body is cylindrical with 7-9 dark-blue horizontal silvery stripes. These stripes run into the caudal and anal fins. The back is olive-green. Males are slimmer than females. Females are more silvery and the abdomen is distended, particularly prior to spawning.

Adult fishes are able to tolerate large fluctuations in temperature, pH and hardness. However, in order to get healthy fish which produce eggs of good quality, optimal conditions should be provided.

During spawning the male pursues and butts the female, and as the eggs are expelled they are fertiliZed. The eggs, which are transparent and non-adhesive, fall to the bottom where they may be eaten by the parents. Spawning is influenced by light. If the morning light is adequate, the fish usually spawns in the early hours following daybreak.

A female can produce batches of several hundreds of eggs at weekly intervals.

CONDITIONS OF PARENTAL FISH, REPRODUCTION AND EARLY-LIFE STAGES

Select a suitable number of healthy fish and keep these in a suitable water (e.g. Annex 4) for at least 2 weeks prior to the intended spawning. The group of fish should be allowed to breed at least once before producing the batch of eggs used in the test. The density of fish during this period should not exceed 1 gramme of fish per litre. Regular changes of water or the use of purification systems will enable the density to be higher. The temperature in the holding tanks should be maintained at $25 \pm 2\ ^{o}C$. The fish should be provided with a varied diet, which may consist of, for example, appropriate commercial dry food, live newly hatched Arthemia, chironomids, Daphnia, white worms (Enchytraeids).

Two procedures are outlined below, which in practice have led to a sufficient batch of healthy, fertilized eggs for a test to be run:

i. Eight females and 16 males are placed in a tank containing 50 litres of dilution water, shielded from direct light and left as undisturbed as possible for at least 48 hours. A spawning tray is placed at the bottom of the aquarium in the afternoon the day before start of the test. The spawning tray consists of a frame (plexi-glass or other suitable material), 5-7 cm high with a 2-5 mm coarse net attached at the top and a 10-30 µm fine net at the bottom. A number of 'spawning-trees', consisting of untwisted nylon rope, are attached to the coarse net of the frame. After the fish have been left in dark for 12 hours, a faint light is turned on which will initiate the spawning. Two to four hours after spawning, the spawning tray is removed and the eggs collected. The spawning tray will prevent the fish from eating the eggs and at the same time permit an easy collection of the eggs. The group of fish should have spawned at least once before the spawning from which eggs are used for testing.

ii. Five to 10 male and female fish are housed individually at least 2 weeks prior to the intended spawning. After 5-10 days, the abdomens of the females will be distended and their genital papillae visible. Male fish lack papillae. Spawning is performed in spawning tanks equipped with a false mesh bottom (as above). The tank is filled with dilution water, so that the depth of water above the mesh is 5-10 cm. One female and two males are placed in the tank the day before the intended spawning. The water temperature is gradually increased one degree higher than the acclimatisation temperature. The light is turned off and the tank is left as undisturbed as possible. In the morning a faint light is turned on which will initiate spawning. After 2-4 hours, the fish are removed and the eggs collected. If larger batches of eggs are needed than can be obtained from one female, a sufficient number of spawning tanks may be set-up in parallel. By recording the reproduction success of the individual females prior to the test (size of batch and quality), those females with highest reproduction success may be selected for breeding.

The eggs should be transferred to the test vessels by means of glass tubes (inner diameter not less than 4 mm) provided with a flexible suction bulb. The amount of water accompanying the eggs on their transfer should be as small as possible. The eggs are heavier than water and sink out of the tube. Care should be taken to prevent eggs (and larvae) coming into contact with the air. Microscopic examination of sample(s) of the batch(es) should be carried out to ensure that there are no irregularities in the first developmental stages. Disinfection of the eggs is not allowed.

The mortality rate of the eggs is highest within the first 24 hours after fertilisation. A mortality of 5-40 percent is often seen during this period. Eggs degenerate as a result of unsuccessful fertilization or development failures. The quality of the batch of eggs seems to depend on the female fish, as some females consistently produce good quality eggs, others never will. Also the development rate and the rate of hatching vary from one batch to another. The successfully fertilized eggs and the yolk sac larvae survive well, normally above 90 percent. At 25 °C the eggs will hatch 3-5 days after fertilization and the yolk sac will be absorbed approximately 13 days after fertilization.

The embryonic development has been well defined by Hisaoka and Battle (2). Due to the transparency of the eggs and post-hatch larvae, the development of the fish may be followed and the presence of malformations may be observed. Approximately 4 hours after spawning, the non-fertilized eggs may be distinguished from the fertilized (3). For this examination, eggs and larvae are placed in test vessels of small volume and studied under a microscope.

The test conditions, which apply to the early life stages, are listed in Annex 2. Optimal values for pH values and hardness of the dilution water are 7.8 and 250 mg $CaCO_3$/l respectively.

CALCULATIONS AND STATISTICS

A two-stage approach is proposed. First, the data on mortality, abnormal development and hatching-time are analysed statistically. Then, for those concentrations at which no adverse effects on any of these parameters have been detected, the body length is statistically evaluated. This approach is advisable since the toxicant may selectively kill smaller fish, delay hatching-time and induce gross malformations, thus leading to biased length measurements. Furthermore, there will be roughly the same number of fish to be measured per treatment, ensuring the validity of the test statistics.

LC_{50} AND EC_{50} DETERMINATIONS

The percentage of surviving eggs and larvae is calculated and corrected for mortality in the controls in accordance with Abbott's formula (4):

$$P = 100 - (\frac{C - P'}{C} x 100)$$

where,

P = corrected % survival
P′ = % survival observed in the test concentration
C = % survival in the control

If possible, the LC_{50} is determined by a suitable method at the end of the test.

If the inclusion of morphological abnormalities in the EC_{50} statistic is desired, guidance can be found in Stephan (5).

ESTIMATION OF LOEC AND NOEC

An objective of the egg and sac-fry test is to compare the non-zero concentrations with the control, i.e. to determine the LOEC. Therefore multiple comparison procedures should be utilised (6)(7)(8)(9)(10).

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ANNEX 2

TEST CONDITIONS, DURATION AND SURVIVAL CRITERIA FOR RECOMMENDED SPECIES

SPECIES	TEMP (^{0}C)	SALINITY (0/00)	PHOTO-PERIOD (hrs)	DURATION OF STAGES (days)		TYPICAL DURATION OF TEST	SURVIVAL OF CONTROL, (MINIMUM %)	
				Embryo	Sac-fry		Hatching success	Post-hatch
FRESHWATER								
Brachydanio rerio Zebrafish	25 ± 1	–	12 – 16	3 – 5	8 – 10	As soon as possible after fertilisation (early gastrula stage) to 5 days post-hatch (8-10 days)	80	90
Oncorhynchus mykiss Rainbow trout	10 ± 1 [(1)] 12 ± 1 [(2)]	–	0 [(a)]	30 – 35	25 – 30	As soon as possible after fertilisation (early gastrula stage) to 20 days post-hatch (50-55 days)	66	70
Cyprinus carpio Common carp	21 – 25	–	12 – 16	5	> 4	As soon as possible after fertilisation (early gastrula stage) to 4 days post-hatch (8 - 9 days)	80	75
Oryzias latipes Japanese ricefish/Medaka	24 ± 1 [(1)] 23 ± 1 [(2)]	–	12 – 16	8 – 11	4 – 8	As soon as possible after fertilisation (early gastrula stage) to 5 days post-hatch (13 - 16 days)	80	80
Pimephales promelas Fathead minnow	25 ± 2	–	16	4 – 5	5	As soon as possible after fertilisation (early gastrula stage) to 4 days post-hatch (8 - 9 days)	60	70

[(1)]For embryos [(2)]For larvae [(a)]Darkness for embryo and larvae until one week after hatching except when they are being inspected. Then subdued lighting throughout the test

ANNEX 3

TEST CONDITIONS, DURATION AND SURVIVAL CRITERIA FOR OTHER WELL DOCUMENTED SPECIES

SPECIES	TEMP (°C)	SALINITY (0/00)	PHOTO-PERIOD (hrs)	DURATION OF STAGES (days)		TYPICAL DURATION OF EMBRYO AND SAC-FRY TEST	SURVIVAL OF CONTROL (MINIMUM %)	
				EMBRYO	SAC-FRY TEST		Hatching success	Post-hatch
FRESHWATER								
Carassius auratus Goldfish	24 ± 1	–	–	3 – 4	> 4	As soon as possible after fertilisation (early gastrula stage) to 4 days post-hatch (7 days)	–	80
Leopomis macrochirus Blugill sunfish	21 ± 1	–	16	3	> 4	As soon as possible after fertilisation (early gastrula stage) to 4 days post-hatch (7 days)	–	75
SALTWATER								
Menidia peninsulae Tidewater silverside	22 - 25	15 – 22	12	1.5	10	As soon as possible after fertilisation (early gastrula stage) to 5 days post-hatch (6-7 days)	80	60
Clupea harengus Herring	10 ± 1	8 – 15	12	20 – 25	3 – 5	As soon as possible after fertilisation (early gastrula stage) to 3 days post-hatch (23-27 days)	60	80
Gadus morhua Cod	5 ± 1	5 – 30	12	14 – 16	3 – 5	As soon as possible after fertilisation (early gastrula stage) to 3 days post-hatch (18 days)	60	80
Cyprinodon variegatus Sheepshead minnow	25 ± 1	15 – 30	12	–	–	As soon as possible after fertilisation (early gastrula stage) to 4/7 days post-hatch (28 days)	> 75	80

ANNEX 4

SOME CHEMICAL CHARACTERISTICS OF AN ACCEPTABLE DILUTION WATER

SUBSTANCE	CONCENTRATIONS
Particulate matter	< 20 mg/l
Total organic carbon	< 2 mg/l
Unionised ammonia	< 1 µg/l
Residual chlorine	< 10 µg/l
Total organophosphorus pesticides	< 50 ng/l
Total organochlorine pesticides plus polychlorinated biphenyls	< 50 ng/l
Total organic chlorine	< 25 ng/l

C.16. HONEYBEES - ACUTE ORAL TOXICITY TEST

1. METHOD

This acute toxicity test method is a replicate of the OECD TG 213 (1998).

1.1 INTRODUCTION

This toxicity test is a laboratory method, designed to assess the oral acute toxicity of plant protection products and other chemicals, to adult worker honeybees.

In the assessment and evaluation of toxic characteristics of substances, determination of acute oral toxicity in honeybees may be required, e.g. when exposure of bees to a given chemical is likely. The acute oral toxicity test is carried out to determine the inherent toxicity of pesticides and other chemicals to bees. The results of this test should be used to define the need for further evaluation. In particular, this method can be used in step-wise programmes for evaluating the hazards of pesticides to bees, based on sequential progression from laboratory toxicity tests to semi-field and field experiments (1). Pesticides can be tested as active substances (a.s.) or as formulated products.

A toxic standard should be used to verify the sensitivity of the bees and the precision of the test procedure.

1.2 DEFINITIONS

Acute oral toxicity: is the adverse effects occurring within a maximum period of 96h of an oral administration of a single dose of test substance.

Dose: is the amount of test substance consumed. Dose is expressed as mass (µg) of test substance per test animal (µg/bee). The real dose for each bee can not be calculated as the bees are fed collectively, but an average dose can be estimated (totally consumed test substance/number of test bees in one cage).

LD_{50} (Median Lethal Dose) oral: is a statistically derived single dose of a substance that can cause death in 50 % of animals when administered by the oral route. The LD_{50} value is expressed in µg of test substance per bee. For pesticides, the test substance may be either an active substance (a.s.) or a formulated product containing one or more than one active substance.

Mortality: an animal is recorded as dead when it is completely immobile.

1.3 PRINCIPLE OF THE TEST METHOD

Adult worker honeybees (*Apis mellifera*) are exposed to a range of doses of the test substance dispersed in sucrose solution. The bees are then fed the same diet, free of the test substance. Mortality is recorded daily during at least 48 h and compared with control values. If the mortality rate is increasing between 24 h and 48 h whilst control mortality remains at an accepted level, i.e. $\leq$ 10 %, it is appropriate to extend the duration of the test to a maximum of 96 h. The results are analysed in order to calculate the LD_{50} at 24 h and 48 h and, in case the study is prolonged, at 72 h and 96 h.

1.4 VALIDITY OF THE TEST

For a test to be valid, the following conditions apply:

— the average mortality for the total number of controls must not exceed 10 % at the end of the test;

— the LD_{50} of the toxic standard meets the specified range.

1.5 DESCRIPTION OF THE TEST METHOD

1.5.1 Collection of bees

Young adult worker bees of the same race should be used, i.e. bees of the same age, feeding status, etc. Bees should be obtained from adequately fed, healthy, as far as possible disease-free and queen-right colonies with known history and physiological status. They could be collected in the morning of use or in the evening before test and kept under test conditions to the next day. Bees collected from frames without brood are suitable. Collection in early spring or late autumn should be avoided as the bees have a changed physiology during this time. If tests must be conduced in early spring or late autumn, bees can be emerged in an incubator and reared for one week with “bee bread” (pollen collected from the comb) and sucrose solution. Bees treated with chemical substances, such as antibiotics, anti-varroa products, etc., should not be used for toxicity test for four weeks from the time of the end of the last treatment.

1.5.2 Housing and feeding conditions

Easy to clean and well-ventilated cages are used. Any appropriate material can be used, e.g. stainless steel, wire mesh, plastic or disposable wooden cages, etc. Groups of ten bees per cage are preferred. The size of test cages should be appropriate to the number of bees, i.e. providing adequate space.

The bees should be held in the dark in an experimental room at a temperature of 25 ± 2 °C. The relative humidity, normally around 50-70 %, should be recorded throughout the test. Handling procedures, including treatment and observations may be conducted under (day) light. Sucrose solution in water with a final concentration of 500 g/l (50 % w/v) is used as food. After given test doses, food should be provided *ad libitum*. The feeding system should allow recording food intake for each cage (see section 1.6.3.1). A glass tube (ca 50 mm long and 10 mm wide with the open end narrowed to about 2 mm diameter) can be used.

1.5.3 Preparation of bees

The collected bees are randomly allocated to test cages, which are randomly placed in the experimental room.

The bees may be starved for up to 2 h before the initiation of the test. It is recommended that the bees are deprived of food prior to treatment so that all bees are equal in terms of their gut contents at the start of the test. Moribund bees should be rejected and replaced by healthy bees before starting the test.

1.5.4 Preparation of doses

Where the test substance is a water miscible compound this may be dispersed directly in 50 % sucrose solution. For technical products and substances of low water solubility, vehicles such as organic solvent, emulsifiers or dispersants of low toxicity to bees may be used (e.g. acetone, dimethylformamide, dimethylsulfoxide). The concentration of the vehicle depends on the solubility of the test substance and it should be the same for all concentrations tested. However, a concentration of the vehicle of 1 % is generally appropriate and should not be exceeded.

Appropriate control solutions should be prepared, i.e. where a solvent or a dispersant is used to solubilise the test substance, two separate control groups should be used: a solution in water, and a sucrose solution with the solvent/carrier at the concentration used in dosing solutions.

1.6 PROCEDURE

1.6.1 **Test and control groups**

The number of doses and replicates tested should meet the statistical requirements for determination of LD_{50} with 95 % confidence limits. Normally, five doses in a geometric series, with a factor not exceeding 2.2, and covering the range for LD_{50}, are required for the test. However, the dilution factor and the number of concentrations for dosage have to be determined in relation to the slope of the toxicity curve (dose versus mortality) and with consideration taken to the statistical method which is chosen for analysis of the results. A range-finding test enables the choice of the appropriate concentrations for dosage.

A minimum of three replicate test groups, each of ten bees, should be dosed with each test concentration. A minimum of three control batches, each of ten bees, should be run in addition to the test series. Control batches should also be included for the solvents/carriers used (see section 1.5.4).

1.6.2 **Toxic standard**

A toxic standard should be included in the test series. At least three doses should be selected to cover the expected LD_{50} value. A minimum of three replicate cages, each containing ten bees, should be used with each test dose. The preferred toxic standard is dimethoate, for which the reported oral LD_{50}-24 h is in the range 0.10-0.35 µg a.s./bee (2). However, other toxic standards would be acceptable where sufficient data can be provided to verify the expected dose response (e.g. parathion).

1.6.3 **Exposure**

1.6.3.1 *Administration of doses*

Each test group of bees must be provided with 100-200 µl of 50 % sucrose solution in water, containing the test substance at the appropriate concentration. A larger volume is required for products of low solubility, low toxicity or low concentration in the formulation, as higher proportions in the sucrose solution have to be used. The amount of treated diet consumed per group should be monitored. Once consumed (usually within 3-4 h), the feeder should be removed from the cage and replaced with one containing sucrose solution alone. The sucrose solutions are then provided *ad libitum*. For some compounds, at higher concentrations rejection of test dose may result in little or no food being consumed. After a maximum of 6 h, unconsumed treated diet should be replaced with the sucrose solution alone. The amount of treated diet consumed should be assessed (e.g. measurement of volume/weight of treated diet remaining).

1.6.3.2 *Duration*

The duration of the test is preferably 48 h after the test solution has been replaced with sucrose solution alone. If mortality continues to rise by more than 10 % after the first 24 h, the test duration should be extended to a maximum of 96 h provided that control mortality does not exceed 10 %.

1.6.4 **Observations**

Mortality is recorded at 4 h after starting the test and thereafter at 24 h and 48 h (i.e. after giving dose). If a prolonged observation period is required, further assessments should be made at 24 h intervals, up to a maximum of 96 h, provided that the control mortality does not exceed 10 %.

The amount of diet consumed per group should be estimated. Comparison of the rates of consumption of treated and untreated diet within the given 6 h can provide information about palatability of the treated diet.

All abnormal behavioural effects observed during the testing period should be recorded.

1.6.5 **Limit test**

In some cases (e.g. when a test substance is expected to be of low toxicity) a limit test may be performed, using 100 µg a.s./bee in order to demonstrate that the LD_{50} is greater than this value. The same procedure should be used, including three replicate test groups for the test dose, the relevant controls, the assessment of the amount of treated diet comsumed, and the use of the toxic standard. If mortalities occur, a full study should be conducted. If sublethal effects are observed (see section 1.6.4), these should be recorded.

2. **DATA AND REPORTING**

2.1 DATA

Data should be summarised in tabular form, showing for each treatment group, as well as control and toxic standard groups, the number of bees used, mortality at each observation time and number of bees with adverse behaviour. Analyse the mortality data by appropriate statistical methods (e.g. probit analysis, moving-average, binomial probability) (3)(4). Plot dose-response curves at each recommended observation time and calculate the slopes of the curves and the median lethal doses (LD_{50}) with 95 % confidence limits. Corrections for control mortality could be made using Abbott's correction (4)(5). Where treated diet is not completely consumed, the dose of test substance consumed per group should be determined. LD_{50} should be expressed in µg of test substance per bee.

2.2 TEST REPORT

The test report must include the following information:

2.2.1 **Test substance:**

— physical nature and relevant physical-chemical properties (e.g. stability in water, vapour pressure);

— chemical identification data, including structural formula, purity (i.e. for pesticides, the identity and concentration of active substance(s)).

2.2.2 **Test species:**

— scientific name, race, approximate age (in weeks), collection method, date of collection;

— information on colonies used for collection of test bees including health, any adult disease, any pre-treatment, etc.

2.2.3 **Test conditions:**

— temperature and relative humidity of experimental room;

— housing conditions including type, size and material of cages;

— methods of preparation of stock and test solutions (the solvent and its concentration must be given, when used);

— test design, e.g. number and test concentrations used, number of controls; for each test concentration and control, number of replicate cages and number of bees per cage;

— date of test.

2.2.4 **Results:**

— results of preliminary range-finding study if performed;

— raw data: mortality at each dose tested at each observation time;

— graph of the dose-response curves at the end of the test;

— LD_{50} values with 95 % confidence limits, at each of the recommended observation times, for test substance and toxic standard;

— statistical procedures used for determining the LD_{50};

— mortality in controls;

— other biological effects observed or measured e.g. abnormal behaviour of the bees (including rejection of the test dose), rate of consumption of diet in treated and untreated groups;

— any deviation from the test procedures described here and any other relevant information.

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C.17. HONEYBEES - ACUTE CONTACT TOXICITY TEST

1. METHOD

This acute toxicity test method is a replicate of the OECD TG 214 (1998).

1.1 INTRODUCTION

This toxicity test is a laboratory method, designed to assess the acute contact toxicity of plant protection products and other chemicals to adult worker honeybees.

In the assessment and evaluation of toxic characteristics of substances, determination of acute contact toxicity in honeybees may be required, e.g. when exposure of bees to a given chemical is likely. The acute contact toxicity test is carried out to determine the inherent toxicity of pesticides and other chemicals to bees. The results of this test should be used to define the need for further evaluation. In particular, this method can be used in step-wise programmes for evaluating the hazards of pesticides to bees, based on sequential progression from laboratory toxicity tests to semi-field and field experiments (1). Pesticides can be tested as active substances (a.s.) or as formulated products.

A toxic standard should be used to verify the sensitivity of the bees and the precision of the test procedure.

1.2 DEFINITIONS

Acute contact toxicity: is the adverse effects occurring within a maximum period of 96 h of a topical application of a single dose of a substance.

Dose: is the amount of test substance applied. Dose is expressed as mass (µg) of test substance per test animal (µg/bee).

LD_{50} (Median Lethal Dose) contact: is a statistically derived single dose of a substance that can cause death in 50 % of animals when administered by the contact. The LD_{50} value is given in µg of test substance per bee. For pesticides, the test substance may be either an active substance (a.s.) or a formulated product containing one or more than one active substance.

Mortality: an animal is recorded as dead when it is completely immobile.

1.3 PRINCIPLE OF THE TEST METHOD

Adult worker honeybees (*Apis mellifera*) are exposed to a range of doses of the test substance dissolved in appropriate carrier, by direct application to the thorax (droplets). The test duration is 48 h. If the mortality rate is increasing between 24 h and 48 h whilst control mortality remains at an accepted level, i.e. ≤ 10 %, it is appropriate to extend the duration of the test to a maximum of 96 h. Mortality is recorded daily and compared with control values. The results are analysed in order to calculate the LD_{50} at 24 h and 48 h, and in case the study is prolonged at 72 h and 96 h.

1.4 VALIDITY OF THE TEST

For a test to be valid, the following conditions apply:

— the average mortality for the total numbers of controls must not exceed 10 % at the end of the test;

— the LD_{50} of the toxic standard meets the specified range.

1.5 DESCRIPTION OF THE TEST METHOD

1.5.1 Collection of bees

Young adult worker bees should be used, i.e. bees of the same age, feeding status, race etc. Bees should be obtained from adequately fed, healthy, as far as possible disease-free and queen-right colonies with known history and physiological status. They could be collected in the morning of use or in the evening before test and kept under test conditions to the next day. Bees collected from frames without brood are suitable. Collection in early spring or late autumn should be avoided, as the bees have a changed physiology during the time. If tests have to be conduced in early spring or late autumn, bees can be emerged in an incubator and reared for one week with "bee bread" (pollen collected from the comb) and sucrose solution. Bees treated with chemical substances, such as antibiotics, anti-varroa products, etc., should not be used for toxicity test for four weeks from the time of the end of the last treatment.

1.5.2 Housing and feeding conditions

Easy to clean and well-ventilated cages are used. Any appropriate material can be used, e.g. stainless steel, wire mesh, plastic, disposable wooden cages, etc. The size of test cages should be appropriate to the number of bees, i.e. providing adequate space. Groups of ten bees per cage are preferred.

The bees should be held in the dark in an experimental room at a temperature of 25 ± 2 ^{o}C. The relative humidity, normally around 50-70 %, should be recorded throughout the test. Handling procedures, including treatment and observations may be conducted under (day) light. Sucrose solution in water with a final concentration of 500 g/l (50 % w/v) should be used as food and provided *ad libitum* during the test time, using a bee feeder. This can be a glass tube (ca 50 mm long and 10 mm wide with the open end narrowed to about 2 mm diameter).

1.5.3 Preparation of bees

The collected bees may be anaesthetized with carbon dioxide or nitrogen for application of the test substance. The amount of anaesthetic used and time of exposure should be minimised. Moribund bees should be rejected and replaced by healthy bees before starting the test.

1.5.4 Preparation of doses

The test substance is to be applied as solution in a carrier, i.e. an organic solvent or a water solution with a wetting agent. As organic solvent, acetone is preferred but other organic solvents of low toxicity to bees may be used (e.g. dimethylformamide, dimethylsulfoxide). For water dispersed formulated products and highly polar organic substances not soluble in organic carrier solvents, solutions may be easier to apply if prepared in a weak solution of a commercial wetting agent (e.g. Agral, Cittowett, Lubrol, Triton, Tween).

Appropriate control solutions should be prepared, i.e. where a solvent or a dispersant is used to solubilise the test substance, two separate control groups should be used, one treated with water, and one treated with the solvent/dispersant.

1.6 PROCEDURE

1.6.1 **Test and control groups**

The number of doses and replicates tested should meet the statistical requirements for determination LD_{50} with 95 % confidence limits. Normally five doses in a geometric series, with a factor not exceeding 2.2, and covering the range for LD_{50}, are required for the test. However, the number of doses have to be determined in relation to the slope of the toxicity curve (dose versus mortality) and with consideration taken to the statistical method which is chosen for analysis of the results. A range-finding test enables the choice of the appropriate doses.

A minimum of three replicate test groups, each of ten bees, should be dosed with each test concentration.

A minimum of three control batches, each of ten bees, should be run in addition to the test series. If an organic solvent or a wetting agent is used three additional control batches of each ten bees for the solvent or the wetting agent have to be included.

1.6.2 **Toxic standard**

A toxic standard must be included in the test series. At least three doses should be selected to cover the expected LD_{50} value. A minimum of three replicate cages, each containing ten bees, should be used with each test dose. The preferred toxic standard is dimethoate, for which the reported contact LD_{50}-24 h is in the range 0.10 - 0.30 µg a.s./bee (2). However, other toxic standards would be acceptable where sufficient data can be provided to verify the expected dose response (e.g. parathion).

1.6.3 **Exposure**

1.6.3.1 *Administration of doses*

Anaesthetised bees are individually treated by topical application.The bees are randomly assigned to the different test doses and controls. A volume of 1 µl of solution containing the test substance at the suitable concentration should be applied with a microapplicator to the dorsal side of the thorax of each bee. Other volumes may be used, if justified. After application, the bees are allocated to test cages and supplied with sucrose solutions.

1.6.3.2 *Duration*

The duration of the test is preferably 48 hours. If mortality increases by more than 10 % between 24 h and 48 h, the test duration should be extended up to a maximum of 96 h provided that control mortality does not exceed 10 %.

1.6.4 **Observations**

Mortality is recorded at 4 h after dosing and thereafter at 24 h and 48 h. If a prolonged observation period is required, further assessments should be made, at 24 h intervals, to a maximum of 96 h, provided that the control mortality does not exceeding 10 %.

All abnormal behavioural effects observed during the testing period should be recorded.

1.6.5 **Limit test**

In some cases (e.g. when a test substance is expected to be of low toxicity) limit test may be performed, using 100 µg a.s./bee in order to demonstrate that the LD_{50} is greater than this value. The same procedure should be used, including three replicate test groups for the test dose, the relevant controls, and the use of the toxic standard. If mortalities occur, a full study should be conducted. If sublethal effects are observed (see section 1.6.4) these should be recorded.

2. DATA AND REPORTING

2.1 DATA

Data should be summarised in tabular form, showing for each treatment group, as well as, control and toxic standard groups, the number of bees used, mortality at each observation time and number of bees with adverse behaviour. Analyse the mortality data by appropriate statistical methods (e.g. probit analysis, moving-average, binomial probability) (3)(4). Plot dose-response curves at each recommended observation time (i.e. 24 h, 48 h and, if relevant, 72 h, 96 h) and calculate the slopes of the curves and the median lethal doses (LD_{50}) with 95 % confidence limits. Corrections for control mortality could be made using Abbott's correction (4)(5). LD_{50} should be expressed in µg of test substance per bee.

2.2 TEST REPORT

The test report must include the following information:

2.2.1 **Test substance:**

— physical nature and physical-chemical properties (e.g. stability in water, vapour pressure);

— chemical identification data, including structural formula, purity (i.e. for pesticides, the identity and concentration of active substance(s)).

2.2.2 **Test species:**

— scientific name, race, approximate age (in weeks), collection method, date of collection;

— information on colonies used for collection of test bees including health, any adult disease, any pre-treatment, etc.

2.2.3 **Test conditions:**

— temperature and relative humidity of experimental room;

— housing conditions including type, size and material of cages;

— methods of administration of test substance, e.g. carrier solvent used, volume of test solution applied anaesthetics used;

— test design, e.g. number and test doses used, number of controls; for each test dose and control, number of replicate cages and number of bees per cage;

— date of test.

2.2.4 **Results:**

— results of preliminary range-finding study if performed;

— raw data: mortality at each concentration tested at each observation time;

— graph of the dose-response curves at the end of the test;

— LD_{50} values, with 95 % confidence limits, at each of the recommended observation times, for test substance and toxic standard;

— statistical procedures used for determining the LD_{50};

— mortality in controls;

— other biological effects observed or measured and any abnormal responses of the bees;

— any deviation from the test method procedures described here and any other relevant information.

3. REFERENCES

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(4) Finney, D.J. (1971). Probit Analysis. 3rd ed., Cambridge, London and New-York.

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C.18. ADSORPTION/DESORPTION USING A BATCH EQUILIBRIUM METHOD

1. METHOD

This method is a replicate of the OECD TG 106, for the Determination of Soil Adsorption/Desorption, using a Batch Equilibrium Method (2000).

1.1 INTRODUCTION

The method takes into account a ring test and a workshop for soil selection for the development of an adsorption test (1)(2)(3)(4) and also existing guidelines at national level (5)(6)(7)(8)(9)(10)(11).

Adsorption/desorption studies are useful for generating essential information on the mobility of chemicals and their distribution in the soil, water and air compartments of the biosphere (12)(13)(14)(15)(16)(17)(18)(19)(20)(21). The information can be used in the prediction or estimation, for example, of the availability of a chemical for degradation (22)(23), transformation and uptake by organisms (24); leaching through the soil profile (16)(18)(19)(21)(25)(26)(27)(28); volatility from soil (21)(29)(30); run-off from land surfaces into natural waters (18)(31)(32). Adsorption data can be used for comparative and modelling purposes (19)(33)(34)(35).

The distribution of a chemical between soil and aqueous phases is a complex process depending on a number of different factors: the chemical nature of the substance (12)(36)(37)(38)(39)(40), the characteristics of the soil (4)(12)(13)(14)(41)(42)(43)(44)(45)(46)(47)(48)(49), and climatic factors such as rainfall, temperature, sunlight and wind. Thus, the numerous phenomena and mechanisms involved in the process of adsorption of a chemical by soil cannot be completely defined by a simplified laboratory model such as the present method. However, even if this attempt cannot cover all the environmentally possible cases, it provides valuable information on the environmental relevance of the adsorption of a chemical.

See also General Introduction.

1.2 SCOPE

The method is aimed at estimating the adsorption/desorption behaviour of a substance on soils. The goal is to obtain a sorption value which can be used to predict partitioning under a variety of environmental conditions; to this end, equilibrium adsorption coefficients for a chemical on various soils are determined as a function of soil characteristics (e.g. organic carbon content, clay content and soil texture and pH). Different soil types have to be used in order to cover as widely as possible the interactions of a given substance with naturally occurring soils.

In this method, adsorption represents the process of the binding of a chemical to surfaces of soils; it does not distinguish between different adsorption processes (physical and chemical adsorption) and such processes as surface catalysed degradation, bulk adsorption or chemical reaction. Adsorption that will occur on colloids particles (diameter $< 0.2\ \mu m$) generated by the soils is not taken into account.

The soil parameters that are believed most important for adsorption are: organic carbon content (3)(4)(12)(13)(14)(41)(43)(44)(45)(46)(47)(48); clay content and soil texture (3)(4)(41)(42)(43)(44)(45)(46)(47)(48) and pH for ionizable compounds (3)(4)(42). Other soil parameters which may have an impact on the adsorption/desorption of a particular substance are the effective cation exchange capacity (ECEC), the content of amorphous iron and aluminium oxides, particularly for volcanic and tropical soils (4), as well as the specific surface (49).

The test is designed to evaluate the adsorption of a chemical on different soil types with a varying range of organic carbon content, clay content and soil texture, and pH. It comprises three tiers:

Tier 1: Preliminary study in order to determine:

— the soil/solution ratio;

— the equilibrium time for adsorption and the amount of test substance adsorbed at equilibrium;

— the adsorption of the test substance on the surfaces of the test vessels and the stability of the test substance during the test period.

Tier 2: Screening test: the adsorption is studied in five different soil types by means of adsorption kinetics at a single concentration and determination of distribution coefficient K_d and K_{oc}.

Tier 3: Determination of Freundlich adsorption isotherms to determine the influence of concentration on the extent of adsorption on soils.

Study of desorption by means of desorption kinetics/Freundlich desorption isotherms (Annex 1).

1.3 DEFINITIONS AND UNITS

Symbol	Definition	Units
A_{t_i}	adsorption percentage at the time t_i	%
A_{eq}	adsorption percentage at adsorption equilibrium	%
$m_s^{ads}(t_i)$	mass of the test substance adsorbed on the soil at the time t_i	µg
$m_s^{ads}(\Delta t_i)$	mass of the test substance adsorbed on the soil during the time interval Δt_i	µg
$m_s^{ads}(eq)$	mass of the test substance adsorbed on the soil at adsorption equilibrium	µg
m_0	mass of the test substance in the test tube, at the beginning of the adsorption test	µg
$m_m^{ads}(t_i)$	mass of the test substance measured in an aliquot (v_a^A) at the time point t_i	µg
$m_{aq}^{ads}(eq)$	mass of the substance in the solution at adsorption equilibrium	µg
m_{soil}	quantity of the soil phase, expressed in dry mass of soil	g
C_{st}	mass concentration of the stock solution of the substance	µg cm^{-3}
C_0	initial mass concentration of the test solution in contact with the soil	µg cm^{-3}
$C_{aq}^{ads}(t_i)$	mass concentration of the substance in the aqueous phase at the time t_i that the analysis is performed	µg cm^{-3}

$C_s^{ads}(eq)$	content of the substance adsorbed on soil at adsorption equilibrium an equilibrium	$\mu g\ g^{-1}$
$C_{aq}^{ads}(eq)$	mass concentration of the substance in the aqueous phase at adsorption equilibrium	$\mu g\ cm^{-3}$
V_0	initial volume of the aqueous phase in contact with the soil during the adsorption test	cm^3
v_a^A	volume of the aliquot in which the test substance is measured	cm^3
K_d	distribution coefficient for adsorption	$cm^3\ g^{-1}$
K_{oc}	organic carbon normalised adsorption coefficient	$cm^3\ g^{-1}$
K_{om}	organic matter normalised distribution coefficient	$cm^3\ g^{-1}$
K_F^{ads}	Freundlich adsorption coefficient	$\mu g^{1-1/n} (cm^3)^{1/n} g^{-1}$
1/n	Freundlich exponent	
D_{t_i}	desorption percentage at a point time t_i	%
$D_{\Delta t_i}$	desorption percentage corresponding to a time interval Δt_i	%
K_{des}	apparent desorption coefficient	$cm^3\ g^{-1}$
K_F^{des}	Freundlich desorption coefficient	$\mu g^{1-1/n} (cm^3)^{1/n} g^{-1}$
$m_{aq}^{des}(t_i)$	mass of the test substance desorbed from soil at the time t_i	μg
$m_{aq}^{des}(\Delta t_i)$	mass of the test substance desorbed from soil during the time Δt_i	μg
$m_m^{des}(eq)$	mass of the substance determined analytically in the aqueous phase at desorption equilibrium	μg
$m_{aq}^{des}(eq)$	total mass of the test substance desorbed at desorption equilibrium	μg
$m_s^{des}(\Delta t_i)$	mass of the substance remaining adsorbed on the soil after the time interval Δt_i	μg
m_{aq}^A	mass of the substance left over from the adsorption equilibrium due to incomplete volume replacement	μg
$C_s^{des}(eq)$	content of the test substance remaining adsorbed on the soil at desorption equilibrium	$\mu g\ g^{-1}$
$C_{aq}^{des}(eq)$	mass concentration of the test substance in the aqueous phase at desorption equilibrium	$\mu g\ cm^{-3}$
V_T	total volume of the aqueous phase in contact with the soil during the desorption kinetics experiment performed with the serial method	cm^3
V_R	volume of the supernatant removed from the tube after the attainment of adsorption equilibrium and replaced by the same volume of a 0.01 M $CaCl_2$ solution	cm^3
v_a^D	volume of the aliquot sampled for analytical purpose from the time (i), during the desorption kinetics experiment performed with the serial method	cm^3
V_r^i	volume of the solution taken from the tube (i) for the measurement of the test substance, in desorption kinetics experiment (parallel method)	cm^3

V_r^F	volume of the solution taken from the tube for the measurement of the test substance, at desorption equilibrium	cm^3
MB	mass balance	%
m_E	total mass of the test substance extracted from soil and walls of the test vessel in two steps	µg
V_{rec}	volume of the supernatant recovered after the adsorption equilibrium	cm^3
P_{ow}	octanol/water partition coefficient	
pKa	dissociation constant	
S_w	water solubility	$g\ l^{-1}$

1.4 PRINCIPLE OF THE TEST METHOD

Known volumes of solutions of the test substance, non-labelled or radiolabelled, at known concentrations in 0.01 M $CaCl_2$ are added to soil samples of known dry weight which have been pre-equilibrated in 0.01 M $CaCl_2$. The mixture is agitated for an appropriate time. The soil suspensions are then separated by centrifugation and, if so wished, filtration and the aqueous phase is analysed. The amount of test substance adsorbed on the soil sample is calculated as the difference between the amount of test substance initially present in solution and the amount remaining at the end of the experiment (indirect method).

As an option, the amount of the test substance adsorbed can also be directly determined by analysis of soil (direct method). This procedure which involves stepwise soil extraction with appropriate solvent, is recommended in cases where the difference in the solution concentration of the substance cannot be accurately determined. Examples of such cases are: adsorption of the test substance on surface of the test vessels, instability of the test substance in the time scale of the experiment, weak adsorption giving only small concentration change in the solution; and strong adsorption yielding low concentration which cannot be accurately determined. If radiolabelled substance is used, the soil extraction may be avoided by analysis of the soil phase by combustion and liquid scintillation counting. However, liquid scintillation counting is an unspecific technique which cannot differentiate between parental and transformation products; therefore it should be used only if the test chemical is stable for the duration of the study.

1.5 INFORMATION ON THE TEST SUBSTANCE

Chemical reagents should be of analytical grade. The use of non-labelled test substances with known composition and preferably at least 95% purity or of radiolabelled test substances with known composition and radio-purity, is recommended. In the case of short half-life tracers, decay corrections should be applied.

Before carrying out a test for adsorption-desorption, the following information about the test substance should be available:

a) Water Solubility (A.6);

b) Vapour Pressure (A.4) and/or Henry's Law Constant;

c) Abiotic Degradation: Hydrolysis as a Function of pH (C.7);

d) Partition Coefficient (A.8);

e) Ready Biodegradability (C.4) or Aerobic and Anaerobic Transformation in Soil;

f) pKa of Ionizable Substances**;**

g) Direct Photolysis in Water (i.e. UV-Vis Absorption Spectrum in Water, Quantum Yield) and Photodegradation on Soil.

1.6 APPLICABILITY OF THE TEST

The test is applicable to chemical substances for which an analytical method with sufficient accuracy is available. An important parameter that can influence the reliability of the results, especially when the indirect method is followed, is the stability of the test substance in the time scale of the test. Thus, it is a prerequisite to check the stability in a preliminary study; if a transformation in the time scale of the test is observed, it is recommended that the main study be performed by analysing both soil and aqueous phases.

Difficulties may arise in conducting this test for test substances with low water solubility ($S_w < 10^{-4}$ g l^{-1}), as well as for highly charged substances, due to the fact that the concentration in the aqueous phase cannot be measured analytically with sufficient accuracy. In these cases, additional steps have to be taken. Guidance on how to deal with these problems is given in the relevant sections of this method.

When testing volatile substances, care should be taken to avoid losses during the study.

1.7 DESCRIPTION OF THE METHOD

1.7.1 **Apparatus and chemical reagents**

Standard laboratory equipment, especially the following:

a) Tubes or vessels to conduct the experiments. It is important that these tubes or vessels;
   — fit directly in the centrifuge apparatus in order to minimise handling and transfer errors;
   — be made of an inert material, which minimises adsorption of the test substance on its surface.

b) Agitation device: overhead shaker or equivalent equipment; the agitation device should keep the soil in suspension during shaking.

c) Centrifuge: preferably high-speed, e.g. centrifugation forces > 3000g, temperature controlled, capable of removing particles with a diameter greater than 0.2 µm from aqueous solution. The containers should be capped during agitation and centrifugation to avoid volatility and water losses; to minimise adsorption on them, deactivated caps such as teflon lined screw caps should be used.

d) Optional: filtration device; filters of 0.2 µm porosity, sterile, single use. Special care should be taken in the choice of the filter material, to avoid any losses of the test substance on it; for poorly soluble test substances, organic filter material is not recommended.

e) Analytical instrumentation, suitable for measuring the concentration of the test chemical.

f) Laboratory oven, capable of maintaining a temperature of 103 °C to 110 °C.

1.7.2 **Characterization and selection of soils**

The soils should be characterized by three parameters considered to be largely responsible for the adsorptive capacity: organic carbon, clay content and soil texture, and pH. As already mentioned (see Scope) other physico-chemical properties of the soil may have an impact on the adsorption/desorption of a particular substance and should be considered in such cases.

The methods used for soil characterization are very important and can have a significant influence on the results. Therefore, it is recommended that soil pH should be measured in a solution of 0.01 M $CaCl_2$ (that is the solution used in adsorption/desorption testing) according to the corresponding ISO method (ISO-10390-1). It is also recommended that the other relevant soil properties be determined according to standard methods (for example ISO "Handbook of Soil Analysis"); this permits the analysis of sorption data to be based on globally standardized soil parameters. Some guidance for existing standard methods of soil analysis and characterization is given in references (50-52). For calibration of soil test methods, the use of reference soils is recommended.

Guidance for selection of soils for adsorption/desorption experiments is given in Table 1. The seven selected soils cover soil types encountered in temperate geographical zones. For ionizable test substances, the selected soils should cover a wide range of pH, in order to be able to evaluate the adsorption of the substance in its ionised and unionised forms. Guidance on how many different soils to use at the various stages of the test is given under "Performance of the test" 1.9.

If other soil types are preferred, they should be characterized by the same parameters and should have similar variation in properties to those described in Table 1, even if they do not match the criteria exactly.

Table 1: Guidance for selection of soil samples for adsorption-desorption

Soil Type	pH range (in 0.01 M $CaCl_2$)	Organic carbon content (%)	Clay content (%)	Soil texture*
1	4.5 - 5.5	1.0 - 2.0	65 - 80	clay
2	> 7.5	3.5 - 5.0	20 - 40	clay loam
3	5.5 - 7.0	1.5 - 3.0	15 - 25	silt loam
4	4.0 - 5.5	3.0 - 4.0	15 - 30	loam
5	< 4.0 - 6.0§	< 0.5 - 1.5§‡	< 10 - 15§	loamy sand
6	> 7.0	< 0.5 - 1.0§‡	40 - 65	clay loam/ clay
7	< 4.5	> 10	< 10	sand/ loamy sand

* According to FAO and the US system (85).
§ The respective variables should preferably show values within the range given. If, however, difficulties in finding appropriate soil material occur, values below the indicated minimum are accepted.
‡ Soils with less than 0.3% organic carbon may disturb correlation between organic content and adsorption. Thus, it is recommended the use of soils with a minimum organic carbon content of 0.3%.

1.7.3 **Collection and storage of soil samples**

1.7.3.1 *Collection*

No specific sampling techniques or tools are recommended; the sampling technique depends on the purpose of the study (53)(54)(55)(56)(57)(58).

The following should be considered:

a) detailed information on the history of the field site is necessary; this includes location, vegetation cover, treatments with pesticides and/or fertilisers, biological additions or accidental contamination. Recommendations of the ISO standard on soil sampling (ISO 10381-6) should be followed with respect to the description of the sampling site;

b) the sampling site has to be defined by UTM (Universal Transversal Mercator-Projection/European Horizontal Datum) or geographical co-ordinates; this could allow recollection of a particular soil in the future or could help in defining soil under various classification systems used in different countries. Also, only A horizon up to a maximum depth of 20 cm should be collected. Especially for the soil type n. 7 if a O_h horizon is present as part of the soil, it should be included in the sampling.

The soil samples should be transported using containers and under temperature conditions which guarantee that the initial soil properties are not significantly altered.

1.7.3.2 *Storage*

The use of soils freshly taken from the field is preferred. Only if this is not possible soil can be stored at ambient temperature and should be kept air-dried. No limit on the storage time is recommended, but soils stored for more than three years should be re-analysed prior to the use with respect to their organic carbon content, pH and CEC.

1.7.3.3 *Handling and preparation of soil samples for the test*

The soils are air-dried at ambient temperature (preferably between 20-25 °C). Disaggregation should be performed with minimal force, so that the original texture of the soil will be changed as little as possible. The soils are sieved to a particle size ≤ 2 mm; recommendations of the ISO standard on soil sampling (ISO 10381-6) should be followed with respect to the sieving process. Careful homogenization is recommended, as this enhances the reproducibility of the results. The moisture content of each soil is determined on three aliquots with heating at 105 °C until there is no significant change in weight (approx. 12h). For all calculations the mass of soil refers to oven dry mass, i.e. the weight of soil corrected for moisture content.

1.7.4 **Preparation of the test substance for application to soil**

The test substance is dissolved in a solution of 0.01 M $CaCl_2$ in distilled or de-ionised water; the $CaCl_2$ solution is used as the aqueous solvent phase to improve centrifugation and minimise cation exchange. The concentration of the stock solution should preferably be three orders of magnitude higher than the detection limit of the analytical method used. This threshold safeguards accurate measurements with respect to the methodology followed in this method; additionally, the stock solution concentration should be below water solubility of the test substance.

The stock solution should preferably be prepared just before application to soil samples and should be kept closed in the dark at 4 °C. The storage time depends on the stability of the test substance and its concentration in the solution.

Only for poorly soluble substances ($S_w < 10^{-4}$ g l^{-1}), an appropriate solubilizing agent may be needed when it is difficult to dissolve the test substance. This solubilizing agent: (a) should be miscible with water such as methanol or acetonitrile; (b) its concentration should not exceed 1% of the total volume of the stock solution and should constitute less than that in the solution of the test substance which will come in contact with the soil (preferably less than 0.1%); and (c) should not be a surfactant or undergo solvolytic reactions with the test chemical. The use of a solubilising agent should be stipulated and justified in the reporting of the data.

Another alternative for poorly soluble substances is to add the test substance to the test system by spiking: the test substance is dissolved in an organic solvent, an aliquot of which is added to the system of soil and 0.01 M solution of $CaCl_2$ in distilled or de-ionised water. The content of organic solvent in the aqueous phase should be kept as low as possible, normally not exceeding 0.1%. Spiking from an organic solution may suffer from volume unreproducibility. Thus, an additional error may be introduced as the test substance and co-solvent concentration would not be the same in all tests.

1.8 PREREQUISITES FOR PERFORMING THE ADSORPTION/DESORPTION TEST

1.8.1 **The analytical method**

The key parameters that can influence the accuracy of sorption measurements include the accuracy of the analytical method in analysis of both the solution and adsorbed phases, the stability and purity of the test substance, the attainment of sorption equilibrium, the magnitude of the solution concentration change, the soil/solution ratio and changes in the soil structure during the equilibration process (35)(59-62). Some examples bearing upon the accuracy issues are given in Annex 2.

The reliability of the analytical method used must be checked at the concentration range which is likely to occur during the test. The experimenter should feel free to develop an appropriate method with appropriate accuracy, precision, reproducibility, detection limits and recovery. Guidance on how to perform such a test is given by the experiment below.

An appropriate volume of 0.01 M $CaCl_2$, e.g. 100 cm^3, is agitated during 4 h with a weight of soil, e.g. 20 g, of high adsorbability, i.e. with high organic carbon and clay content; these weights and volumes may vary depending on analytical needs, but a soil/solution ratio of 1:5 is a convenient starting point. The mixture is centrifuged and the aqueous phase may be filtrated. A certain volume of the test substance stock solution is added to the latter to reach a nominal concentration within the concentration range which is likely to occur during the test. This volume should not exceed 10% of the final volume of the aqueous phase, in order to change as little as possible the nature of the pre-equilibration solution. The solution is analysed.

One blank run consisting of the system soil + $CaCl_2$ solution (without test substance) must be included, in order to check for artefacts in the analytical method and for matrix effects caused by the soil.

The analytical methods which can be used for sorption measurements include gas-liquid chromatography (GLC), high-performance liquid chromatography (HPLC), spectrometry (e.g. GC/mass spectrometry, HPLC/mass spectrometry) and liquid scintillation counting (for radiolabelled substances). Independent of the analytical method used, it is considered suitable if the recoveries are between 90% and 110% of the nominal value. In order to allow for detection and evaluation after partitioning has taken place, the detection limits of the analytical method should be at least two orders of magnitude below the nominal concentration.

The characteristics and detection limits of the analytical method available for carrying out adsorption studies play an important role in defining the test conditions and the whole experimental performance of the test. This method follows a general experimental path and provides recommendations and guidance for alternative solutions where the analytical method and laboratory facilities may impose limitations.

1.8.2 **The selection of optimal soil/solution ratios**

Selection of appropriate soil to solution ratios for sorption studies depends on the distribution coefficient K_d and the relative degree of adsorption desired. The change of the substance concentration in the solution determines the statistical accuracy of the measurement based on the form of adsorption equation and the limit of the analytical methodology, in detecting the concentration of the chemical in solution. Therefore, in general practice it is useful to settle on a few fixed ratios, for which the percentage adsorbed is above 20%, and preferably >50% (62), while care should be taken to keep the test substance concentration in the aqueous phase high enough to be measured accurately. This is particularly important in the case of high adsorption percentages.

A convenient approach to selecting the appropriate soil/water ratios, is based on an estimate of the K_d value either by preliminary studies or by established estimation techniques (Annex 3). Selection of an appropriate ratio can then be made based on a plot of soil/solution ratio versus K_d for fixed percentages of adsorption (Fig.1). In this plot it is assumed that the adsorption equation is linear[1]. The applicable relationship is obtained by rearranging equation (4) of the K_d in the form of equation (1):

$$\frac{V_0}{m_{soil}} = \left(\frac{m_0}{m_s^{ads}(eq)} - 1\right) K_d \qquad (1)$$

or in its logarithmic form assuming that $R = m_{soil}/V_0$ and $A_{eq}\%/100 = \frac{m_s^{ads}(eq)}{m_0}$:

$$\log R = - \log K_d + \log\left[\frac{(A_{eq}\%/100)}{(1-A_{eq}\%/100)}\right] \qquad (2)$$

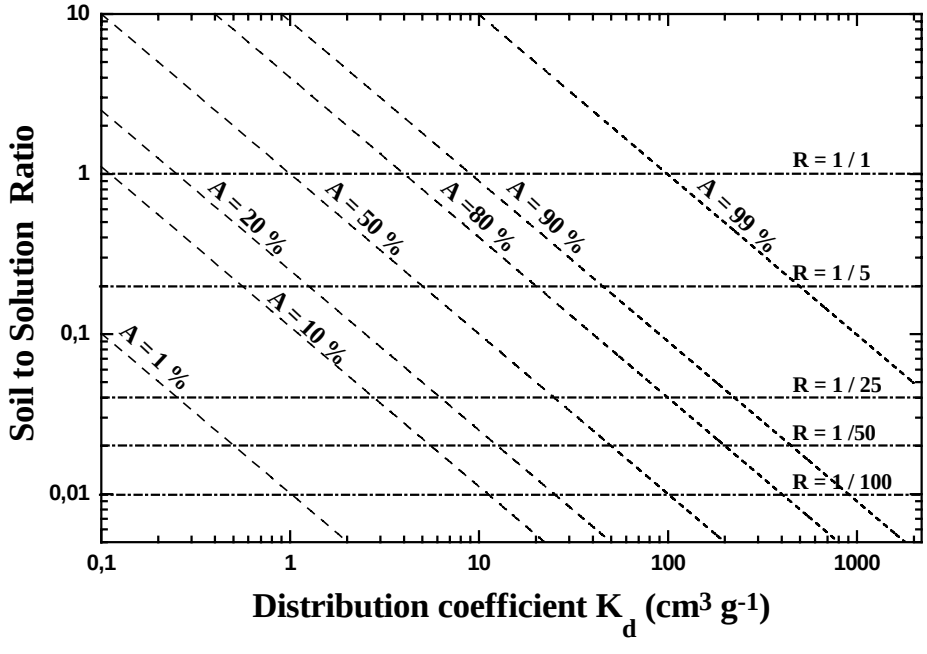

Fig. 1 Relationship between soil to solution ratios and K_d at various percentages of adsorbed test substance

[1] $C_s^{ads}(eq) = K_d \cdot C_{aq}^{ads}(eq)$

Fig. 1 shows soil/solution ratios required as a function of K_d for different levels of adsorption. For example, with a soil/solution ratio of 1:5 and a K_d of 20, approximately 80% adsorption would occur. To obtain 50% adsorption for the same K_d, a 1:25 ratio must be used. This approach to selecting the appropriate soil/solution ratios gives the investigator the flexibility to meet experimental needs.

Areas which are more difficult to deal with are those where the chemical is highly or very slightly adsorbed. Where low adsorption occurs, a 1:1 soil/solution ratio is recommended, although for some very organic soil types smaller ratios may be necessary to obtain a slurry. Care must be taken with the analytical methodology to measure small changes in solution concentration; otherwise the adsorption measurement will be inaccurate. On the other hand, at very high distribution coefficients K_d, one can go up to a 1:100 soil/solution ratio in order to leave a significant amount of chemical in solution. However, care must be taken to ensure good mixing, and adequate time must be allowed for the system to equilibrate. An alternative approach to deal with these extreme cases when adequate analytical methodology is missing, is to predict the K_d value applying estimation techniques based, for example, on P_{ow} values (Annex 3). This could be useful especially for low adsorbed/polar chemicals with $P_{ow} < 20$ and for lipophilic/highly sorptive chemicals with $P_{ow} > 10^4$.

1.9 PERFORMANCE OF THE TEST

1.9.1 **Test conditions**

All experiments are done at ambient temperature and, if possible, at a constant temperature between 20 °C and 25 °C.

Centrifugation conditions should allow the removal of particles larger than 0.2 µm from the solution. This value triggers the smallest sized particle that is considered as a solid particle, and is the limit between solid and colloid particles. Guidance on how to determine the centrifugation conditions is given in Annex 4.

If the centrifugation facilities cannot guarantee the removal of particles larger than 0.2 µm, a combination of centrifugation and filtration with 0.2 µm filters could be used. These filters should be made of a suitable inert material to avoid any losses of the test substance on them. In any case, it should be proven that no losses of the test substance occur during filtration.

1.9.2 **Tier 1- Preliminary study**

The purpose of conducting a preliminary study has already been given in the Scope section. Guidance for setting up such a test is given with the experiment suggested below.

1.9.2.1 *Selection of optimal soil/solution ratios*

Two soil types and three soil/solution ratios (six experiments) are used. One soil type has high organic carbon and low clay content, and the other low organic carbon and high clay content. The following soil to solution ratios are suggested:

- 50 g soil and 50 cm^3 aqueous solution of the test substance (ratio 1/1);
- 10 g soil and 50 cm^3 aqueous solution of the test substance (ratio 1/5);
- 2 g soil and 50 cm^3 aqueous solution of the test substance (ratio 1/25).

The minimum amount of soil on which the experiment can be carried out depends on the laboratory facilities and the performance of analytical methods used. However, it is recommended to use at least 1 g, and preferably 2 g, in order to obtain reliable results from the test.

One control sample with only the test substance in 0.01 M $CaCl_2$ solution (no soil) is subjected to precisely the same steps as the test systems, in order to check the stability of the test substance in $CaCl_2$ solution and its possible adsorption on the surfaces of the test vessels.

A blank run per soil with the same amount of soil and total volume of 50 cm^3 0.01 M $CaCl_2$ solution (without test substance) is subjected to the same test procedure. This serves as a background control during the analysis to detect interfering substances or contaminated soils.

All the experiments, included controls and blanks, should be performed at least in duplicate. The total number of the samples which should be prepared for the study can be calculated with respect to the methodology which will be followed.

Methods for the preliminary study and the main study are generally the same, exceptions are mentioned where relevant.

The air-dried soil samples are equilibrated by shaking with a minimum volume of 45 cm^3 of 0.01 M $CaCl_2$ overnight (12 h) before the day of the experiment. Afterwards, a certain volume of the stock solution of the test substance is added in order to adjust the final volume to 50 cm^3. This volume of the stock solution added: (a) should not exceed 10% of the final 50 cm^3 volume of the aqueous phase in order to change as little as possible the nature of the pre-equilibration solution; and (b) should preferably result in an initial concentration of the test substance being in contact with the soil (C_0) at least two orders of magnitude higher than the detection limit of the analytical method; this threshold safeguards the ability to perform accurate measurements even when strong adsorption occurs (> 90%) and to determine later the adsorption isotherms. It is also recommended, if possible, that the initial substance concentration (C_0) not exceed half of its solubility limit.

An example of how to calculate the concentration of the stock solution (C_{st}) is given below. A detection limit of 0.01 µg cm^{-3} and 90% adsorption are assumed; thus, the initial concentration of the test substance in contact with the soil should preferably be 1 µg cm^{-3} (two orders of magnitude higher than the detection limit). Supposing that the maximum recommended volume of the stock solution is added, i.e. 5 to 45 cm^3 0.01 M $CaCl_2$ equilibration solution (= 10% of the stock solution to 50 cm^3 total volume of aqueous phase), the concentration of the stock solution should be 10 µg cm^{-3}; this is three orders of magnitude higher than the detection limit of the analytical method.

The pH of the aqueous phase should be measured before and after contact with the soil since it plays an important role in the whole adsorption process, especially for ionisable substances.

The mixture is shaken until adsorption equilibrium is reached. The equilibrium time in soils is highly variable, depending on the chemical and the soil; a period of 24 h is generally sufficient (77). In the preliminary study, samples may be collected sequentially over a 48 h period of mixing (for example at 4, 8, 24, 48 h). However, times of analysis should be considered with flexibility with respect to the work schedule of the laboratory.

There are two options for the analysis of the test substance in the aqueous solution: (a) the parallel method and (b) the serial method. It should be stressed that, although the parallel method is experimentally more tedious, the mathematical treatment of the results is simpler (Annex 5). However, the choice of the methodology to be followed, is left to the experimenter who will need to consider the available laboratory facilities and resources.

(a) parallel method: samples with the same soil/solution ratio are prepared, as many as the time intervals at which it is desired to study the adsorption kinetics. After centrifugation and if so wished filtration, the aqueous phase of the first tube is recovered as completely as possible and is measured after, for example, 4 h, that of the second tube after 8 h, that of the third after 24, etc.

(b) serial method: only a duplicate sample is prepared for each soil/solution ratio. At defined time intervals the mixture is centrifuged to separate the phases. A small aliquot of the aqueous phase is immediately analysed for the test substance; then the experiment continues with the original mixture. If filtration is applied after centrifugation, the laboratory should have facilities to handle filtration of small aqueous aliquots. It is recommended that the total volume of the aliquots taken not exceed 1% of the total volume of the solution, in order not to change significantly the soil/solution ratio and to decrease the mass of solute available for adsorption during the test.

The percentage adsorption A_{t_i} is calculated at each time point (t_i) on the basis of the nominal initial concentration and the measured concentration at the sampling time (t_i), corrected for the value of the blank. Plots of the A_{t_i} versus time (Fig. 1 Annex 5) are generated in order to estimate the achievement of equilibrium plateau[2]. The K_d value at equilibrium is also calculated. Based on this K_d value, appropriate soil/solution ratios are selected from Fig.1, so that the percentage adsorption reaches above 20% and preferably >50% (61). All the applicable equations and principles of plotting are given in section on Data and Reporting and in Annex 5.

1.9.2.2 *Determination of adsorption equilibration time and of the amount of test substance adsorbed at equilibrium*

As already mentioned, plots of A_{t_i} or C_{aq}^{ads} versus time permit estimation of the achievement of the adsorption equilibrium and the amount of test substance adsorbed at equilibrium. Figs. 1 and 2 in the Annex 5 show examples of such plots. Equilibration time is the system needs to reach a plateau.

If, with a particular soil, no plateau but a steady increase is found, this may be due to complicating factors such as biodegradation or slow diffusion. Biodegradation can be shown by repeating the experiment with a sterilised sample of the soil. If no plateau is achieved even in this case, the experimenter should search for other phenomena that could be involved in his specific studies; this could be done with appropriate modifications of the experiment conditions (temperature, shaking times, soil/solution ratios). It is left to the experimenter to decide whether to continue the test procedure in spite of a possible failure to achieve an equilibrium.

1.9.2.3 *Adsorption on the surface of the test vessel and stability of the test substance*

Some information on the adsorption of the test substance on the surface of test vessels, as well as its stability, can be derived by analysing the control samples. If a depletion more than the standard error of the analytical method is observed, abiotic degradation and/or adsorption on the surface of the test vessel could be involved. Distinction between these two phenomena could be achieved by thoroughly washing the walls of the vessel with a known volume of an appropriate solvent and subjecting the wash solution to analysis for the test substance. If no adsorption on the surface of the test vessels is observed, the depletion demonstrates abiotic unstability of the test substance. If adsorption is found, changing the material of the test vessels is necessary. However, data on the adsorption on the surface of the test vessels gained from this experiment cannot be directly extrapolated to soil/solution experiment. The presence of soil will affect this adsorption.

Additional information on the stability of the test substance can be derived by determination of the parental mass balance over time. This means that the aqueous phase, extracts of soil and test vessel walls are analysed for the test substance. The difference between the mass of the test chemical added and the sum of the test chemical masses in the aqueous phase, extracts of the soil and test vessel walls is equal to the mass degraded and/or volatilized and/or not extracted. In order to perform a mass balance determination, the adsorbion equilibrium should have been reached within the period of the experiment.

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[2] Plots of the concentration of the test substance in the aqueous phase (C_{aq}^{ads}) versus time could also be used to estimate the achievement of the equilibrium plateau (see Fig. 2 in Annex 5).

The mass balance is performed on both soils and for one soil/solution ratio per soil that gives a depletion above 20% and preferably >50% at equilibrium. When the ratio-finding experiment is completed with the analysis of the last sample of the aqueous phase after 48 h, the phases are separated by centrifugation and, if so wished, filtration. The aqueous phase is recovered as much as possible, and a suitable extraction solvent (extraction coefficient of at least 95%) is added to the soil to extract the test substance. At least two successive extractions are recommended. The amount of test substance in the soil and test vessel extracts is determined and the mass balance is calculated (equation 10, Data and Reporting). If it is less than 90%, the test substance is considered to be unstable in the time scale of the test. However, studies could still be continued, taking into account the unstability of the test substance; in this case it is recommended to analyse both phases in the main study.

1.9.2.4 *Tier 2 - Adsorption kinetics at one concentration of the test substance*

Five soils are used, selected from Table 1. There is an advantage to including some or all of the soils used in the preliminary study, if appropriate, among these five soils. In this case, Tier 2 has not to be repeated for the soils used in preliminary study.

The equilibration time, the soil/solution ratio, the weight of the soil sample, the volume of the aqueous phase in contact with the soil and concentration of the test substance in the solution are chosen based on the preliminary study results. Analysis should preferably be done approximately after 2, 4, 6, 8 (possibly also 10) and 24 h contact time; the agitation time may be extended to a maximum of 48 h in case a chemical requires longer equilibration time with respect to ratio-finding results. However, times of analysis could be considered with flexibility.

Each experiment (one soil and one solution) is done at least in duplicate to allow estimation of the variance of the results. In every experiment one blank is run. It consists of the soil and 0.01 M $CaCl_2$ solution, without test substance, and of weight and volume, respectively, identical to those of the experiment. A control sample with only the test substance in 0.01 M $CaCl_2$ solution (without soil) is subjected to the same test procedure, serving to safeguard against the unexpected.

The percentage adsorption is calculated at each time point A_{t_i} and/or time interval $A_{\Delta t_i}$ (according to the need) and is plotted versus time. The distribution coefficient K_d at equilibrium, as well as the organic carbon normalized adsorption coefficient K_{oc} (for non-polar organic chemicals), are also calculated.

Results of the adsorption kinetics test

The linear K_d value is generally accurate to describe sorptive behaviour in soil (35)(78) and represents an expression of inherent mobility of chemicals in soil. For example, in general chemicals with $K_d \leq 1$ cm^3 g^{-1} are considered to be qualitatively mobile. Similarly, a mobility classification scheme based on K_{oc} values has been developed by MacCall *et al.* (16). Additionally, leaching classification schemes exist based on a relationship between K_{oc} and DT-50[3] (32)(79).

Also, according to error analysis studies (61), K_d values below 0.3 cm^3 g^{-1} cannot be estimated accurately from a decrease in concentration in the aqueous phase, even when the most favourable (from point of view of accuracy) soil/solution ratio is applied, i.e. 1:1. In this case analysis of both phases, soil and solution, is recommended.

[3] DT-50: degradation time for 50% of the test substance.

With respect to the above remarks, it is recommended that the study of the adsorptive behaviour of a chemical in soil and its potential mobility be continued by determining Freundlich adsorption isotherms for these systems, for which an accurate determination of K_d is possible with the experimental protocol followed in this test method. Accurate determination is possible if the value which results by multiplying the K_d with the soil/solution ratio is >0.3, when measurements are based on concentration decrease in the aqueous phase (indirect method), or > 0.1, when both phases are analysed (direct method) (61).

1.9.2.5 *Tier 3 - Adsorption isotherms and desorption kinetics/desorption isotherms*

1.9.2.5.1 Adsorption isotherms

Five test substance concentrations are used, covering preferably two orders of magnitude; in the choice of these concentrations the water solubility and the resulting aqueous equilibrium concentrations should be taken into account. The same soil/solution ratio per soil should be kept along the study. The adsorption test is performed as described above, with the only difference that the aqueous phase is analysed only once at the time necessary to reach equilibrium as determined before in Tier 2. The equilibrium concentrations in the solution are determined and the amount adsorbed is calculated from the depletion of the test substance in the solution or with the direct method. The adsorbed mass per unit mass of soil is plotted as a function of the equilibrium concentration of the test substance (see Data and Reporting).

Results from the adsorption isotherms experiment

Among the mathematical adsorption models proposed so far, the Freundlich isotherm is the one most frequently used to describe adsorption processes. More detailed information on the interpretation and importance of adsorption models is provided in the references (41)(45)(80)(81)(82).

Note: It should be mentioned that a comparison of K_F (Freundlich adsorption coefficient) values for different substances is only possible if these K_F values are expressed in the same units (83).

1.9.2.5.2 Desorption kinetics

The purpose of this experiment is to investigate whether a chemical is reversibly or irreversibly adsorbed on a soil. This information is important, since the desorption process also plays an important role in the behaviour of a chemical in field soil. Moreover, desorption data are useful inputs in the computer modelling of leaching and dissolved run-off simulation. If a desorption study is desired, it is recommended that the study described below be carried out on each system for which an accurate determination of K_d in the preceding adsorption kinetics experiment was possible.

Likewise with the adsorption kinetics study, there are two options to proceed with the desorption kinetics experiment: (a) the parallel method and (b) serial method. The choice of methodology to be followed, is left to the experimenter who will need to consider the available laboratory facilities and resources.

(a) parallel method: for each soil which is chosen to proceed with the desorption study, samples with the same soil/solution ratio are prepared, as many as the time intervals at which it is desired to study the desorption kinetics. Preferably, the same time intervals as in the adsorption kinetics experiment should be used; however, the total time may be extended as appropriate in order the system to reach desorption equilibrium. In every experiment (one soil, one solution) one blank is run. It consists of the soil and 0.01 M $CaCl_2$ solution, without test substance, and of weight and volume, respectively, identical to those of the experiment. As a control sample the test substance in 0.01 M $CaCl_2$ solution (without soil) is subjected to the same test procedure. All the mixtures of the soil with the solution is agitating until to reach adsorption equilibrium (as determined before in Tier 2). Then, the phases are separated by centrifugation and the aqueous phases are removed as much as possible. The volume of solution removed is replaced by an equal volume of 0.01 M $CaCl_2$ without test substance and the new mixtures are agitated again. The aqueous phase of the first tube is recovered as completely as possible and is measured after, for example, 2 h, that of the second tube after 4 h, that of the third after 6 h, etc. until the desorption equilibrium is reached.

(b) serial method: after the adsorption kinetics experiment, the mixture is centrifuged and the aqueous phase is removed as much as possible. The volume of solution removed is replaced by an equal volume of 0.01 M $CaCl_2$ without test substance. The new mixture is agitated until the desorption equilibrium is reached. During this time period, at defined time intervals, the mixture is centrifuged to separate the phases. A small aliquot of the aqueous phase is immediately analysed for the test substance; then, the experiment continues with the original mixture. The volume of each individual aliquot should be less than 1% of the total volume. The same quantity of fresh 0.01 M $CaCl_2$ solution is added to the mixture to maintain the soil to solution ratio, and the agitation continues until the next time interval.

The percentage desorption is calculated at each time point (D_{t_i}) and/or time interval ($D_{\Delta t_i}$) (according to the needs of the study) and is plotted versus time. The desorption coefficient of K_{des} at equilibrium is also calculated. All applicable equations are given in Data and Reporting and Annex 5.

Results from desorption kinetics experiment

Common plots of the percentage desorption D_{t_i} and adsorption A_{t_i} versus time, allow estimation of the reversibility of the adsorption process. If the desorption equilibrium is attained even within twice the time of the adsorption equilibrium, and the total desorption is more than 75% of the amount adsorbed, the adsorption is considered to be reversible.

1.9.2.5.3 Desorption isotherms

Freundlich desorption isotherms are determined on the soils used in the adsorption isotherms experiment. The desorption test is performed as described in the section “Desorption kinetics”, with the only difference that the aqueous phase is analysed only once, at desorption equilibrium. The amount of the test substance desorbed is calculated. The content of test substance remaining adsorbed on soil at desorption equilibrium is plotted as a function of the equilibrium concentration of the test substance in solution (see Data and Reporting and Annex 5).

2. DATA AND REPORTING

The analytical data are presented in tabular form (see Annex 6). Individual measurements and averages calculated are given. Graphical representations of adsorption isotherms are provided. The calculations are made as described below.

For the purpose of the test, it is considered that the weight of 1 cm^3 of aqueous solution is 1g. The soil/solution ratio may be expressed in units of w/w or w/vol with the same figure.

2.1 ADSORPTION

The adsorption (A_{t_i}) is defined as the percentage of substance adsorbed on the soil related to the quantity present at the beginning of the test, under the test conditions. If the test substance is stable and does not adsorb significantly to the container wall, A_{t_i} is calculated at each time point t_i, according to the equation:

$$A_{t_i} = \frac{m_s^{ads}(t_i) \cdot 100}{m_0} \quad (\%) \qquad (3)$$

where:

A_{t_i} = adsorption percentage at the time point t_i (%);

$m_s^{ads}(t_i)$ = mass of the test substance adsorbed on the soil at the time t_i (µg);

m_0 = mass of the test substance in the test tube, at the beginning of the test (µg).

Detailed information on how to calculate the percentage of adsorption A_{t_i} for the parallel and serial methods is given in Annex 5.

The distribution coefficient K_d is the ratio between the content of the substance in the soil phase and the mass concentration of the substance in the aqueous solution, under the test conditions, when adsorption equilibrium is reached.

$$K_d = \frac{C_s^{ads}(eq)}{C_{aq}^{ads}(eq)} = \frac{m_s^{ads}(eq)}{m_{aq}^{ads}(eq)} \frac{V_0}{m_{soil}} \quad (cm^3 g^{-1}) \qquad (4)$$

where:

$C_s^{ads}(eq)$ = content of the substance adsorbed on the soil at adsorption equilibrium (µg g^{-1});

$C_{aq}^{ads}(eq)$ = mass concentration of the substance in the aqueous phase at adsorption equilibrium (µg cm^{-3}). This concentration is analytically determined taking into account the values given by the blanks;

$m_s^{ads}(eq)$ = mass of the substance adsorbed on the soil at adsorption equilibrium (µg);

$m_{aq}^{ads}(eq)$ = mass of the substance in the solution at adsorption equilibrium (µg);

m_{soil} = quantity of the soil phase, expressed in dry mass of soil (g);

V_0 = initial volume of the aqueous phase in contact with the soil (cm^3).

The relation between A_{eq} and K_d is given by:

$$K_d = \frac{A_{eq}}{100 - A_{eq}} \cdot \frac{V_0}{m_{soil}} \quad (cm^3 g^{-1}) \qquad (5)$$

where:

A_{eq} = percentage of adsorption at adsorption equilibrium, %.

The organic carbon normalised adsorption coefficient K_{oc} relates the distribution coefficient K_d to the content of organic carbon of the soil sample:

$$K_{OC} = K_d \cdot \frac{100}{\%OC} \quad (cm^3\,g^{-1}) \qquad (6)$$

where:

%OC = percentage of organic carbon in the soil sample ($g\,g^{-1}$).

K_{oc} coefficient represents a single value which characterises the partitioning mainly of non-polar organic chemicals between organic carbon in the soil or sediment and water. The adsorption of these chemicals is correlated with the organic content of the sorbing solid (7); thus, K_{oc} values depend on the specific characteristics of the humic fractions which differ considerably in sorption capacity, due to differences in origin, genesis, etc.

2.1.1 **Adsorption isotherms**

The Freundlich adsorption isotherms equation relates the amount of the test substance adsorbed to the concentration of the test substance in solution at equilibrium (equation 8).

The data are treated as under "Adsorption" and, for each test tube, the content of the test substance adsorbed on the soil after the adsorption test ($C_s^{ads}(eq)$, elsewhere denoted as x/m) is calculated. It is assumed that equilibrium has been attained and that $C_s^{ads}(eq)$ represents the equilibrium value:

$$C_s^{ads}(eq) = \frac{m_s^{ads}(eq)}{m_{soil}} = \frac{\left[C_0 - C_{aq}^{ads}(eq)\right] \cdot V_0}{m_{soil}} \ (\mu g\,g^{-1}) \qquad (7)$$

The Freundlich adsorption equation is shown in (8):

$$C_s^{ads}(eq) = K_F^{ads} \cdot C_{aq}^{ads}(eq)^{1/n} \quad (\mu g\,g^{-1}) \qquad (8)$$

or in the linear form:

$$\log C_s^{ads}(eq) = \log K_F^{ads} + 1/n \cdot \log C_{aq}^{ads}(eq) \qquad (9)$$

where:

K_F^{ads} = Freundlich adsorption coefficient; its dimension is $cm^3\ g^{-1}$ only if 1/n = 1; in all other cases, the slope 1/n is introduced in the dimension of K_F^{ads} $(\mu g^{1-1/n}\ (cm^3)^{1/n}\ g^{-1})$;

n = regression constant; 1/n generally ranges between 0.7 - 1.0, indicating that sorption data is frequently slightly non-linear.

Equations (8) and (9) are plotted and the values of K_F^{ads} and 1/n are calculated by regression analysis using the equation 9. The correlation coefficient r^2 of the log equation is also calculated. An example of such plots is given in Fig.2.

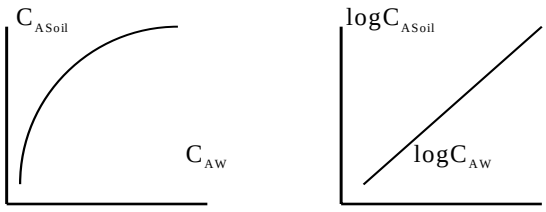

Fig. 2. Freundlich Adsorption Plot, normal and linearized

2.1.2 **Mass balance**

The mass balance (MB) is defined as the percentage of substance which can be analytically recovered after an adsorption test versus the nominal amount of substance at the beginning of the test.

The treatment of data will differ if the solvent is completely miscible with water. In the case of water-miscible solvent, the treatment of data described under "Desorption" may be applied to determine the amount of substance recovered by solvent extraction. If the solvent is less miscible with water, the determination of the amount recovered has to be made.

The mass balance MB for the adsorption is calculated as follows; it is assumed that the term (m_E) corresponds to the sum of the test chemical masses extracted from the soil and surface of the test vessel with an organic solvent:

$$MB = \frac{(V_{rec} \cdot C_{aq}^{ads}(eq) + m_E) \cdot 100}{V_0 \cdot C_0} \quad (\%) \qquad (10)$$

where:

MB = mass balance (%);

m_E = total mass of test substance extracted from the soil and walls of the test vessel in two steps (µg);

C_0 = initial mass concentration of the test solution in contact with the soil ($\mu g\ cm^{-3}$);

V_{rec} = volume of the supernatant recovered after the adsorption equilibrium (cm^{-3}).

2.2 DESORPTION

The desorption (D) is defined as the percentage of the test substance which is desorbed, related to the quantity of substance previously adsorbed, under the test conditions:

$$D_{t_i} = \frac{m_{aq}^{des}(t_i)}{m_s^{ads}(eq)} \cdot 100 \quad (\%) \qquad (11)$$

where:

D_{t_i} = desorption percentage at a time point t_i (%);

$m_{aq}^{des}(t_i)$ = mass of the test substance desorbed from soil at a time point t_i (µg);

$m_s^{ads}(eq)$ = mass of the test substance adsorbed on soil at adsorption equilibrium (µg).

Detailed information on how to calculate the percentage of desorption D_{t_i} for the parallel and serial methods is given in Annex 5.

The apparent desorption coefficient (K_{des}) is, under the test conditions, the ratio between the content of the substance remaining in the soil phase and the mass concentration of the desorbed substance in the aqueous solution, when desorption equilibrium is reached:

$$K_{des} = \frac{m_s^{ads}(eq) - m_{aq}^{des}(eq)}{m_{aq}^{des}(eq)} \frac{V_T}{m_{soil}} \quad (cm^3\ g^{-1}) \qquad (12)$$

where:

K_{des} = desorption coefficient ($cm^3\ g^{-1}$);

$m_{aq}^{des}(eq)$ = total mass of the test substance desorbed from soil at desorption equilibrium (µg);

V_T = total volume of the aqueous phase in contact with the soil during the desorption kinetics test (cm^3).

Guidance for calculating the $m_{aq}^{des}(eq)$ is given in Annex 5 under the heading "Desorption".

Remark

If the adsorption test which was preceded, was performed with the parallel method the volume V_T in the equation (12) is considered to be equal to V_0.

2.2.1 **Desorption isotherms**

The Freundlich desorption isotherms equation relates the content of the test substance remaining adsorbed on the soil to the concentration of the test substance in solution at desorption equilibrium (equation 16).

For each test tube, the content of the substance remaining adsorbed on soil at desorption equilibrium is calculated as follows:

$$C_s^{des}(eq) = \frac{m_s^{ads}(eq) - m_{aq}^{des}(eq)}{m_{soil}} \quad (\mu g\ g^{-1}) \qquad (13)$$

$m_{aq}^{des}(eq)$ is defined as :

$$m_{aq}^{des}(eq) = m_{m}^{des}(eq) \cdot \frac{V_0}{V_r^F} - m_{aq}^{A} \quad (\mu g) \qquad (14)$$

where:

$C_s^{des}(eq)$ = content of the test substance remaining adsorbed on the soil at desorption equilibrium ($\mu g\ g^{-1}$);

$m_{m}^{des}(eq)$ = mass of substance determined analytically in the aqueous phase at desorption equilibrium (μg);

m_{aq}^{A} = mass of the test substance left over from the adsorption equilibrium due to incomplete volume replacement (μg);

$m_{aq}^{des}(eq)$ = mass of the substance in the solution at adsorption equilibrium (μg);

$$m_{aq}^{A} = m_{aq}^{ads}(eq) \cdot \left(\frac{V_0 - V_R}{V_0} \right) \qquad (15)$$

V_r^F = volume of the solution taken from the tube for the measurement of the test substance, at desorption equilibrium (cm^3);

V_R = volume of the supernatant removed from the tube after the attainment of adsorption equilibrium and replaced by the same volume of a 0.01 M $CaCl_2$ solution (cm^3);

The Freundlich desorption equation is shown in (16):

$$C_s^{des}(eq) = K_F^{des} \cdot C_{aq}^{des}(eq)^{1/n} \quad (\mu g\ g^{-1}) \qquad (16)$$

or in the linear form:

$$\log C_s^{des}(eq) = \log K_F^{des} + 1/n \cdot \log C_{aq}^{des}(eq) \qquad (17)$$

where:

K_F^{des} = Freundlich desorption coefficient;

n = regression constant;

$C_{aq}^{des}(eq)$ = mass concentration of the substance in the aqueous phase at desorption equilibrium ($\mu g\ cm^{-3}$).

The equations (16) and (17) can be plotted and the value of K_F^{des} and 1/n are calculated by regression analysis using the equation 17.

Remark:

If the Freundlich adsorption or desorption exponent 1/n is equal to 1, the Freundlich adsorption or desorption binding constant (K_F^{ads} and K_F^{des}) will be equal to the adsorption or desorption equilibrium constants (K_d and K_{des}) respectively, and plots of C_s vs C_{aq} will be linear. If the exponents are not equal to 1, plots of C_s vs C_{aq} will be non-linear and the adsorption and desorption constants will vary along the isotherms.

2.2.2 **TEST REPORT**

The test report should include the following information:

— Complete identification of the soil samples used including:

- geographical reference of the site (latitude, longitude);
- date of sampling;
- use pattern (e.g. agricultural soil, forest, etc.);
- depth of sampling;
- sand/silt/clay content;
- pH values (in 0.01 M $CaCl_2$);
- organic carbon content;
- organic matter content;
- nitrogen content;
- C/N ratio;
- Cation Exchange Capacity (mmol/kg);
- all information relating to the collection and storage of soil samples;
- where appropriate, all relevant information for the interpretation of the adsorption - desorption of the test substance;
- reference of the methods used for the determination of each parameter.

— information on the test substance as appropriate;

— temperature of the experiments;

— centrifugation conditions;

— analytical procedure used to analyse the test substance;

— justification for any use of solubilising agent for the preparation of the stock solution of the test substance;

— explanations of corrections made in the calculations, if relevant;

— data according to the form sheet (Annex 6) and graphical presentations;

— all information and observations helpful for the interpretation of the test results.

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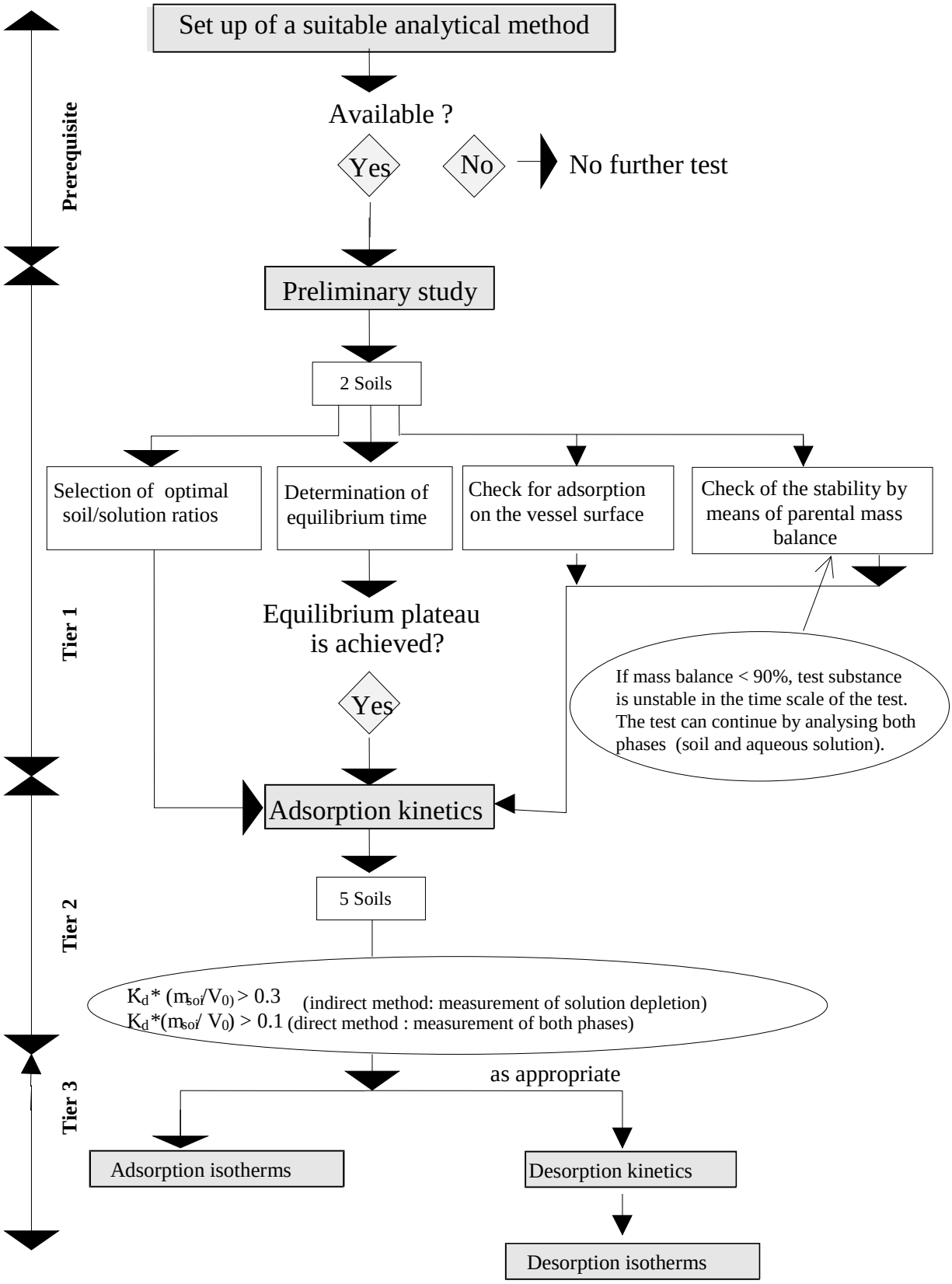
ANNEX 1

Testing Scheme



ANNEX 2

INFLUENCE OF ACCURACY OF ANALYTICAL METHOD AND CONCENTRATION CHANGE ON ACCURACY OF ADSORPTION RESULTS

From the following table (84) it becomes obvious that when the difference between the initial mass (m_0=110 µg) and equilibrium mass ($m_{aq}^{ads}(eq)$ =100 µg) of the test substance in the solution is very small, an error of 5% in the measurement of equilibrium concentration results in an error of 50% in the calculation of the mass of the substance adsorbed in soil ($m_s^{ads}(eq)$) and of 52.4% in the calculation of the K_d.

Amount of soil m_{soil} = 10 g
Volume of solution V_0 = 100 cm^3

	$m_{aq}^{ads}(eq)$ (µg)	$C_{aq}^{ads}(eq)$ (µg cm^{-3})	R	$m_s^{ads}(eq)$ * (µg)	$C_s^{ads}(eq)$ * (µg g^{-1})	R‡	K_d*	R‡
	FOR A = 9%							
m_0 = 110 µg or C_0=1.100 µg / cm^3	100	1.000	true value	10	1.00	true value	1	
	101	1.010	1%	9	0.90	10%	0.891	10.9%
	105	1.050	5%	5	0.50	50%	0.476	52.4%
	109	1.090	9%	1	0.10	90%	0.092	90.8%
	FOR A = 55%							
m_0= 110 µg or C_0=1.100µg/cm^3	50.0	0.500	true value	60.0	6.00	true value	12.00	
	50.5	0.505	1%	59.5	5.95	0.8%	11.78	1.8%
	52.5	0.525	5%	57.5	5.75	4.0%	10.95	8.8%
	55.0	0.550	10%	55.0	5.50	8.3%	10.00	16.7%
	FOR A = 99%							
m_0 = 110 µg or C_0=1.100 µg / cm^3	1.100	0.011	true value	108.9	10.89	true value	990	
	1.111	0.01111	1%	108.889	10.8889	0.01%	980	1.0%
	1.155	0.01155	5%	108.845	10.8845	0.05%	942	4.8%
	1.21	0.0121	10%	108.790	10.8790	0.10%	899	9.2%

$$* m_s^{ads}(eq) = m_0 - m_{aq}^{ads}(eq), \quad C_s^{ads}(eq) = \frac{\left[C_0 - C_{aq}^{ads}(eq)\right] V_0}{m_{soil}}, \quad Kd = \frac{m_s^{ads}(eq)}{m_{aq}^{ads}(eq)} \frac{V_0}{m_{soil}}$$

$m_s^{ads}(eq)$ = mass of the test substance in the soil phase at equilibrium, µg;

$m_{aq}^{ads}(eq)$ = mass of the test substance in the aqueous phase at equilibrium, µg;

$C_s^{ads}(eq)$ = content of the test substance in the soil phase at equilibrium, µg g^{-1};

$C_{aq}^{ads}(eq)$ = mass concentration of the test substance in the aqueous phase at equilibrium, µg cm^{-3};

R = analytical error in the determination of the $m_{aq}^{ads}(eq)$;

$R^{\ddagger}$ = calculated error due to the analytical error R.

ANNEX 3

ESTIMATION TECHNIQUES FOR K_d

1. Estimation techniques permit prediction of K_d based on correlations with, for example, P_{ow} values (12)(39)(63-68), water solubility data (12)(19)(21)(39)(68-73), or polarity data derived by application of HPLC on reversed phase (74-76). As shown in Tables 1 and 2, is the K_{oc} or K_{om} that are calculated from these equations and then, indirectly, the K_d from the equations:

$$Koc = Kd \cdot \frac{100}{\%oc} \quad (cm^3\ g^{-1}) \qquad Kom = \frac{Kd}{1.724} \cdot \frac{100}{\%oc} \quad (cm^3\ g^{-1})$$

2. The concept of these correlations is based on two assumptions: (1) It is the organic matter of the soil that mainly influences the adsorption of a substance; and (2) The interactions involved are mainly non-polar. As a result, these correlations: (1) are not, or are only to some extent, applicable to polar substances, and (2) are not applicable in cases where the organic matter content of the soil is very small (12). In addition, although satisfactory correlations have been found between P_{ow} and adsorption (19), the same cannot be said for the relationship between water solubility and extent of adsorption (19)(21); so far the studies are very contradictory.

3. Some examples of correlations between the adsorption coefficient and the octanol-water partition coefficient, as well as water solubility are given in Tables 1 and 2, respectively.

Table 1. Examples of correlations between the adsorption distribution coefficient and the octanol-water partition coefficient; for further examples (12) (68).

Substances	**Correlations**	**Authors**
Substituted ureas	$\log K_{om} = 0.69 + 0.52 \log P_{ow}$	Briggs (1981) (39)
Aromatic chlorinated	$\log K_{oc} = - 0.779 + 0.904 \log P_{ow}$	Chiou *et al*. (1983) (65)
Various pesticides	$\log K_{om} = 4.4 + 0.72 \log P_{ow}$	Gerstl and Mingelgrin (1984) (66)
Aromatic hydrocarbons	$\log K_{oc} = -2.53 + 1.15 \log P_{ow}$	Vowles and Mantoura (1987) (67)

Table 2. Examples of correlations between the adsorption distribution coefficient and water solubility; for further examples see (68) (69).

Compounds	**Correlations**	**Authors**
Various pesticides	$\log K_{om} = 3.8 - 0.561 \log S_w$	Gerstl and Mingelgrin (1984) (66)
Aliphatic, aromatic chlorinated substances	$\log K_{om} = (4.040 +/- 0.038) - (0.557 +/- 0.012) \log S_w$	Chiou *et al*. (1979) (70)
α-naphtol	$\log K_{oc} = 4.273 - 0.686 \log S_w$	Hasset et al. (1981) (71)
Cyclic, aliphatic aromatic substances	$\log K_{oc} = - 1.405 - 0.921 \log S_w - 0.00953$ (mp-25)	Karickhoff (1981) (72)
Various compounds	$\log K_{om} = 2.75 - 0.45 \log S_w$	Moreale van Blade (1982) (73)

ANNEX 4

CALCULATIONS FOR DEFINING THE CENTRIFUGATION CONDITIONS

1. The centrifugation time is given by the following formula, assuming spherical particles:

$$t = \frac{9}{2}\left[\frac{\eta}{\omega^2 rp^2(\rho_s - \rho_{aq})}\right]\ln\left(Rb/Rt\right) \qquad (1)$$

For simplification purposes, all parameters are described in non-SI units (g, cm).

where:

ω	=	rotational speed (=2 π rpm/60), rad s^{-1};
rpm	=	revolutions per minute;
η	=	viscosity of solution, g s^{-1} cm^{-1};
r_p	=	particle radius, cm;
ρ_s	=	soil density, g cm^{-3};
ρ_{aq}	=	solution density, g cm^{-3};
R_t	=	distance from the centre of centrifuge rotor to top of solution in centrifuge tube, cm;
R_b	=	distance from the centre of centrifuge rotor to bottom in centrifuge tube, cm;
R_b-R_t	=	length of the soil/solution mixture in the centrifuge tube, cm.

In general practice, double the calculated times is used to ensure complete separation.

2. The equation (1) can be simplified further if we consider the viscosity (η) and the density (ρ_{aq}) of the solution as equal to the viscosity and density of water at 25 °C; thus, η = 8.95 x 10^{-3}g $s^{-1}cm^{-1}$ and ρ_{aq} = 1.0 g.cm^{-3}.

Then, the centrifugation time is given by the equation (2):

$$t = \frac{3.7}{(rpm)^2 \cdot rp^2\,(\rho s - 1)} \ln\frac{Rb}{Rt} \qquad (2)$$

3. From the equation (2) it becomes apparent that two parameters are important in defining the centrifugation condition, i.e. time (t) and speed (rpm), in order to achieve separation of particles with a specific size (in our case 0.1 µm radius): (1) the density of the soil and (2) the length of the mixture in the centrifuge tube (R_b-R_t), i.e. the distance which a soil particle covers from the top of the solution to the bottom of the tube; obviously, for a fixed volume the length of the mixture in the tube will depend on the square of the radius of the tube.

4. Fig. 1 presents variations in the centrifugation time (t) versus centrifugation speed (rpm) for different soil densities (ρ_s) (Fig.1a) and different lengths of the mixture in the centrifuge tubes (Fig.2a). From Fig.1a the influence of the soil density appears obvious; for example, for a classical centrifugation of 3000 rpm the centrifugation time is approx. 240 min for 1.2 g cm^3 soil density, while it is only 50 min for 2.0 g cm^3. Similarly, from Fig 1b, for a classical centrifugation of 3000 rpm the centrifugation time is approx. 50 min for a length of the mixture of 10 cm and only 7 min for a length of 1 cm. However, it is important to find an optimal relation between centrifugation which requires the less length possible and easy handling for the experimenter in separating the phases after centrifugation.

5. Moreover, when defining the experimental conditions for the separation of soil/solution phases, it is important to consider the possible existence of a third "pseudo-phase", the colloids. These particles, with a size less than 0.2 μm, can have an important impact on the whole adsorption mechanism of a substance in a soil suspension. When centrifugation is performed as described above, colloids remain in the aqueous phase and are subjected to analysis together with the aqueous phase. Thus, the information about their impact is lost.

If the conducting laboratory has ultracentrifugation or ultrafiltration facilities, the adsorption/desorption of a substance in soil could be studied more in depth, including information on the adsorption of the substance on the colloids. In this case, an ultracentrifugation at 60,000 rpm/min or an ultrafiltration with filter porosity of 100,000 Daltons should be applied in order to separate the three phases soil, colloids, solution. The test protocol should also be modified accordingly, in order all three phases to be subjected to substance analysis.

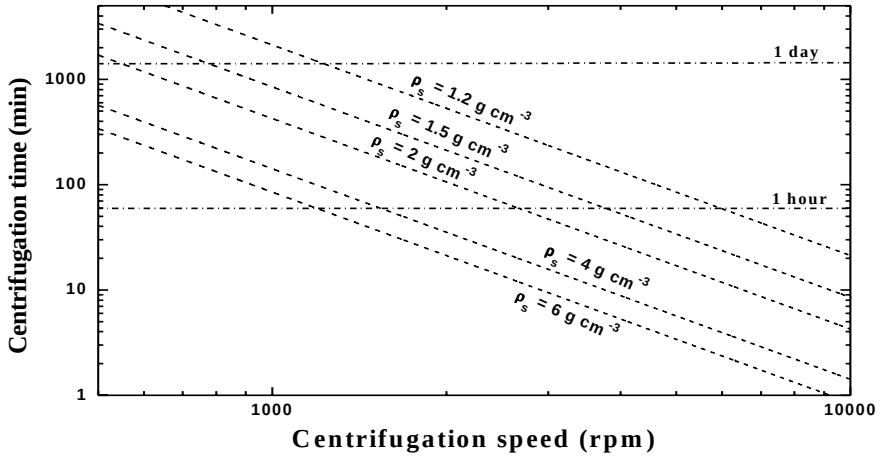


Fig. 1a. Variations of centrifugation time (t) versus centrifugation speed (rpm) for different soil densities (ρ_s). R_t = 10 cm, R_b -R_t = 10 cm, η = 8.95 x 10^{-3}g s^{-1}cm^{-1} and ρ_{aq} = 1.0 g.cm^{-3} at 25 °C.

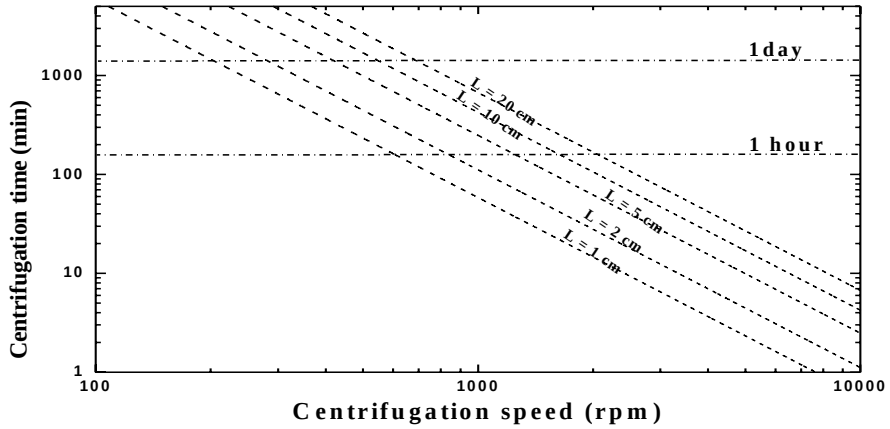
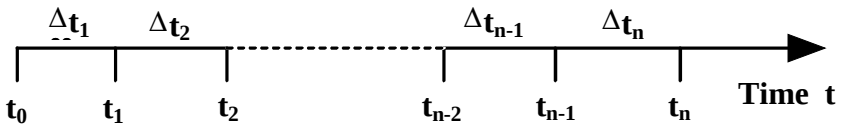


Fig. 1b. Variations of centrifugation time (t) versus centrifugation speed (rpm) for different lengths of the mixture in the centrifuge tube (Rb -Rt) = L; R_t = 10 cm, η = 8.95 x 10^{-3}g s^{-1}cm^{-1}, ρ_{aq} = 1.0 g.cm^{-3} at 25 °C and ρ_s = 2.0 g cm^{-3}.

ANNEX 5

CALCULATION OF ADSORPTION A (%) AND DESORPTION D (%)

The time scheme of the procedure is:



For all the calculations it is assumed that the test substance is stable and does not adsorb significantly to the container walls.

ADSORPTION A (A%)

a) Parallel method

The percentage adsorption is calculated for each test tube (i) at each time point (t_i), according to the equation:

$$A_{t_i} = \frac{m_s^{ads}(t_i) \cdot 100}{m_0} \quad (\%) \qquad (1)^4$$

The terms of this equation may be calculated as follows:

$$m_0 = C_0 \cdot V_0 \ (\mu g) \qquad (2)$$

$$m_s^{ads}(t_i) = m_0 - C_{aq}^{ads}(t_i) \cdot V_0 \ (\mu g) \qquad (3)$$

where:

A_{t_i} = adsorption percentage (%) at the time point t_i;

$m_s^{ads}(t_i)$ = mass of the test substance on soil at the time t_i that the analysis is performed (µg);

m_0 = mass of test substance in the test tube, at the beginning of the test (µg);

C_0 = initial mass concentration of the test solution in contact with the soil (µg cm^{-3});

$C_{aq}^{ads}(t_i)$ = mass concentration of the substance in the aqueous phase at the time t_i that the analysis is performed (µg cm^{-3}); this concentration is analytically determined taking into account the values given by the blanks.

V_0 = initial volume of the test solution in contact with the soil (cm^3).

The values of the adsorption percentage A_{t_i} or $C_{aq}^{ads}(t_i)$ are plotted versus time and the time after which the sorption equilibrium is attained is determined. Examples of such plots are given in Fig.1 and Fig.2 respectively.

[4] Equation applicable to both direct and indirect methods. All the other equations are applicable only to indirect method.

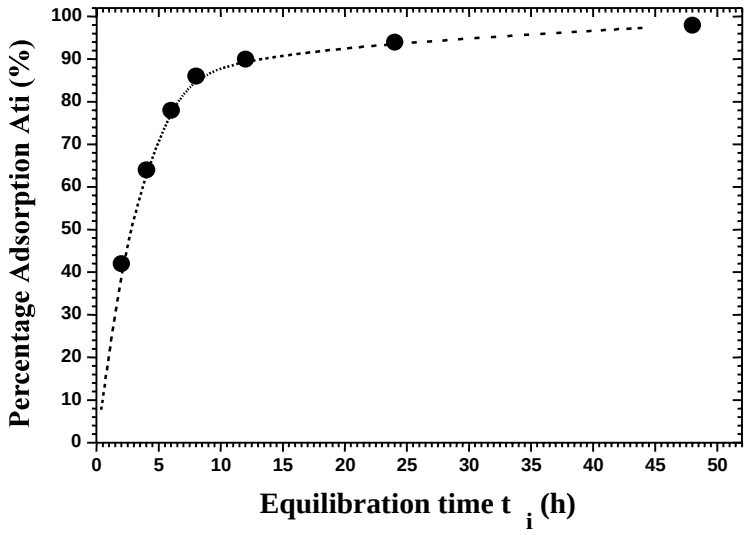


Fig. 1. Adsorption Equilibrium Plot

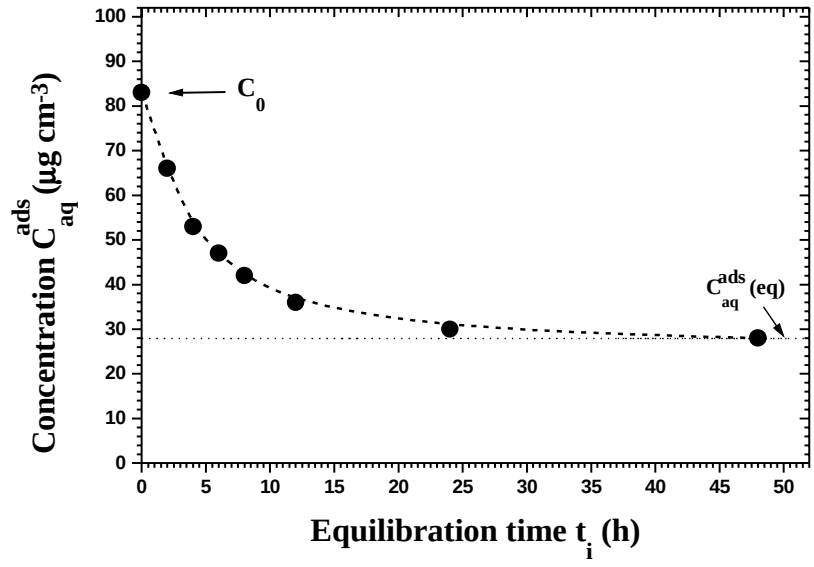


Fig.2. Mass concentration of the test substance in the aqueous phase (C_{aq}) versus time

b) Serial method

The following equations take into account that the adsorption procedure is carried out by measurements of the test substance in small aliquots of the aqueous phase at specific time intervals.

- During each time interval the amount of the substance adsorbed on the soil is calculated as follows:

- for the first time interval $\Delta t_1 = t_1 - t_0$

$$m_s^{ads}(\Delta t_1) = m0 - m_m^{ads}(t_1) \cdot \left(\frac{V_0}{v_a^A} \right) \quad (4)$$

- for the second time interval $\Delta t_2 = t_2 - t_1$

$$m_s^{ads}(\Delta t_2) = m_m^{ads}(t_1) \cdot \left(\frac{V_0}{v_a^A} \right) - m_m^{ads}(t_2) \cdot \left(\frac{V_0 - v_a^A}{v_a^A} \right) \quad (5)$$

- for the third time interval $\Delta t_3 = t_3 - t_2$

$$m_s^{ads}(\Delta t_3) = m_m^{ads}\ (t_2) \cdot \left(\frac{V_0 - v_a^A}{v_a^A} \right) - m_m^{ads}(t_3) \cdot \left(\frac{V_0 - 2 \cdot v_a^A}{v_a^A} \right) \quad (6)$$

- for the n^{th} time interval $\Delta t_n = t_n - t_{n-1}$

$$m_s^{ads}(\Delta t_n) = m_m^{ads}\ (t_{n-1}) \cdot \left(\frac{V_0 - (n-2) \cdot v_a^A}{v_a^A} \right) - m_m^{ads}(t_n) \cdot \left(\frac{\left(V_0 - (n-1) \cdot v_a^A \right)}{v_a^A} \right) \quad (7)$$

- The percentage of adsorption at each time interval, $A_{\Delta t_i}$, is calculated using the following equation:

$$A_{\Delta t_i} = \frac{m_s^{ads}(\Delta t_i)}{m_0} \cdot 100 \quad (\%) \quad (8)^5$$

while the percentage of adsorption (A_{t_i}) at a time point t_i is given by the equation:

$$A_{t_i} = \frac{\sum_{j=\Delta t_1}^{\Delta t_i} m_s^{ads}(j)}{m_0} \cdot 100 \quad (\%) \quad (9)^5$$

[5] Equations applicable to both direct and indirect methods. All the other equations are applicable only to indirect method.

The values of the adsorption A_{t_i} or $A_{\Delta t_i}$ (with respect to the needs of the study) are plotted versus time and the time after which the sorption equilibrium is attained is determined.

- At the equilibration time t_{eq}:

- the mass of the test substance adsorbed on the soil is:

$$m_s^{ads}(eq) = \sum_{\Delta t_i=1}^{n} m_s^{ads}(\Delta t_i) \qquad (10)^5$$

- the mass of the test substance in the solution is:

$$m_{aq}^{ads}(eq) = m_0 - \sum_{\Delta t_i=1}^{n} m_s^{ads}(\Delta t_i) \qquad (11)^5$$

- and the percentage of adsorption at equilibrium is:

$$A_{eq} = \frac{m_s^{ads}(eq)}{m_0} \cdot 100 \quad (\%) \qquad (12)^5$$

The parameters used above are defined as:

$m_s^{ads}(\Delta t_1),\ m_s^{ads}(\Delta t_2),\ldots,m_s^{ads}(\Delta t_n)$	= mass of the substance adsorbed on the soil during the time intervals Δ_{t1}, Δ_{t2},..., Δ_{tn} respectively (μg);
$m_m^{ads}(t_1),\ m_m^{ads}(t_2),\ldots,m_n^{ads}(t_n)$	= mass of the substance measured in an aliquot v_a^A at the time points t_1, t_2,...t_n respectively (μg);
$m_s^{ads}(eq)$	= mass of the substance adsorbed on the soil at adsorption equilibrium (μg);
$m_{aq}^{ads}(eq)$	= mass of the substance in the solution at adsorption equilibrium (μg);
v_a^A	= volume of the aliquot in which the test substance is measured (cm^3);
$A_{\Delta t_i}$	= percentage of adsorption corresponding at a time interval Δt_i (%);
A_{eq}	= percentage of adsorption at adsorption equilibrium (%).

DESORPTION D (%)

The time t_0 that the desorption kinetics experiment begins, is considered as the moment that the maximal recovered volume of the test substance solution (after that the adsorption equilibrium is attained) is replaced by an equal volume of 0.01 M $CaCl_2$ solution.

a) Parallel method

At a time point t_i, the mass of the test substance is measured in the aqueous phase taken from the tube i (V_r^i), and the mass desorbed is calculated according to the equation:

$$m_{aq}^{des}(t_i) = m_m^{des}(t_i) \cdot \left(\frac{V_0}{V_r^i} \right) - m_{aq}^A \qquad (13)$$

At desorption equilibrium $t_i = t_{eq}$ and therefore $m_{aq}^{des}(t_i) = m_{aq}^{des}(eq)$.

The mass of the test substance desorbed during a time interval (Δt_i) is given by the equation:

$$m_{aq}^{des}(\Delta t_i) = m_{aq}^{des}(t_i) - \sum_{j=1}^{i-1} m_{aq}^{des}(j) \qquad (14)$$

The percentage of desorption is calculated:

- at a time point t_i from the equation:

$$D_{t_i} = \frac{m_{aq}^{des}(t_i)}{m_s^{ads}(eq)} \cdot 100 \quad (\%) \qquad (15)$$

- and during a time interval (Δt_i) from the equation:

$$D_{\Delta t_i} = \frac{m_{aq}^{des}(\Delta t_i)}{m_s^{ads}(eq)} \cdot 100 \quad (\%) \qquad (16)$$

where:

D_{t_i} = desorption percentage at a time point t_i (%);

$D_{\Delta t_i}$ = desorption percentage corresponding to a time interval Δt_i (%);

$m_{aq}^{des}(t_i)$ = mass of the test substance desorbed at a time point t_i, (µg);

$m_{aq}^{des}(\Delta t_i)$ = mass of the test substance desorbed during a time interval Δt_i (µg);

$m_m^{des}(t_i)$ = mass of the test substance analytically measured at a time t_i in a solution volume V_r^i ,which is taken for the analysis (µg);

m_{aq}^A = mass of the test substance left over from the adsorption equilibrium due to incomplete volume replacement (µg);

$$m_{aq}^{A} = m_{aq}^{ads}(eq) \cdot \left(\frac{V_0 - V_R}{V_0} \right) \quad (17)$$

$m_{aq}^{ads}(eq)$ = mass of the test substance in the solution at adsorption equilibrium (μg);

V_R = volume of the supernatant removed from the tube after the attainment of adsorption equilibrium and replaced by the same volume of a 0.01 M $CaCl_2$ solution (cm^3);

V_r^i = volume of the solution taken from the tube (i) for the measurement of the test substance, in desorption kinetics experiment (cm^3).

The values of desorption D_{t_i} or $D_{\Delta t_i}$ (according to the needs of the study) are plotted versus time and the time after which the desorption equilibrium is attained is determined.

b) Serial method

The following equations take into account that the adsorption procedure, which was preceded, was carried out by measurement of test substance in small aliquots (v_a^A) of the aqueous phase (serial method in "Performance of the test" 1.9). It is assumed that: a) the volume of the supernatant removed from the tube after the adsorption kinetics experiment was replaced by the same volume of 0.01 M $CaCl_2$ solution (V_R) and b) and the total volume of the aqueous phase in contact with the soil (V_T) during the desorption kinetics experiment remains constant and is given by the equation:

$$V_T = V_o - \sum_{i=1}^{n} v_a^A(i) \quad (18)$$

<u>At a time point t_i:</u>

- The mass of the test substance is measured in a small aliquot (v_a^D) and the mass desorbed is calculated, according to the equation:

$$m_{aq}^{des}(t_i) = m_m^{des}(t_i) \cdot \left(\frac{V_T}{v_a^D} \right) - m_{aq}^{A} \cdot \left(\frac{\left(V_T - (i-1) \cdot v_a^D \right)}{V_T} \right) \quad (19)$$

- At desorption equilibrium $t_i = t_{eq}$ and therefore $m_{aq}^{des}(t_i) = m_{aq}^{des}(eq)$.

- The percentage of desorption D_{t_i} is calculated, from the following equation:

$$D_{t_i} = \frac{m_{aq}^{des}(t_i)}{m_s^{ads}(eq)} \cdot 100 \quad (\%) \quad (20)$$

At a time interval (Δt_i):

- During each time interval the amount of the substance desorbed is calculated as follows:

— for the first time interval $\Delta t_1 = t_1 - t_0$

$$m_{aq}^{des}(\Delta t_1) = m_m^{des}(t_1) \cdot \left(\frac{V_T}{v_a^D} \right) - m_{aq}^A \quad \text{and} \quad m_s^{des}(t_1) = m_s^{aq}(eq) - m_{aq}^{des}(\Delta t_1) \quad (21)$$

— for the second time interval $\Delta t_2 = t_2 - t_1$

$$m_{aq}^{des}(\Delta t_2) = m_m^{des}(t_2) \cdot \left(\frac{V_T}{v_a^D} \right) - m_{aq}^{des}(\Delta t_1) \cdot \left(\frac{\left(V_T - v_a^D \right)}{V_T} \right) - m_{aq}^A \cdot \left(\frac{\left(V_T - v_a^D \right)}{V_T} \right) \quad \text{and}$$

$$m_s^{des}(t_2) = m_s^{ads}(eq) - \left[m_{aq}^{des}(\Delta t_1) + m_{aq}^{des}(\Delta t_2) \right] \quad (22)$$

— for the n^{th} interval $\Delta t_n = t_n - t_{n-1}$

$$m_{aq}^{des}(\Delta t_n) = \left[m_m^{des}(t_n) \cdot \left(\frac{V_T}{v_a^D} \right) - m_{aq}^A \cdot \left(\frac{\left(V_T - \left(n-1 \right) \cdot v_a^D \right)}{V_T} \right) - \sum_{i=1, n \neq 1}^{n-1} \left(\frac{\left(V_T - \left(n-i \right) \cdot v_a^D \right)}{V_T} \cdot m_{aq}^{des}(\Delta t_i) \right) \right]$$

and $$m_s^{des}(t_n) = m_s^{ads}(eq) - \sum_{i=1, n \neq 1}^{n} m_{aq}^{des}(\Delta t_i) \quad (23)$$

Finally, the percentage of desorption at each time interval, $D_{\Delta t_i}$, is calculated using the following equation:

$$D_{\Delta t_i} = \frac{m_{aq}^{des}(\Delta t_i)}{m_s^{ads}(eq)} \cdot 100 \quad (\%) \quad (24)$$

while the percentage of desorption D_{t_i} at a time point t_i is given by the equation:

$$D_{t_i} = \frac{\sum_{j=\Delta t_1}^{\Delta t_i} m_{aq}^{des}(j)}{m_s^{ads}(eq)} \cdot 100 = \frac{m_{aq}^{des}(t_i)}{m_s^{ads}(eq)} \cdot 100 \, (\%) \quad (25)$$

where the above used parameters are defined as:

$m_s^{des}(\Delta t_1), m_s^{des}(\Delta t_2), \ldots, m_s^{des}(\Delta t_n)$ = mass of the substance remaining adsorbed on the soil after the time intervals Δt_1, Δt_2,..., Δt_n respectively (μg);

$m_{aq}^{des}(\Delta t_1), m_{aq}^{des}(\Delta t_2), \ldots, m_{aq}^{des}(\Delta t_n)$ = mass of the test substance desorbed during the time intervals Δt_1, Δt_2,..., Δt_n respectively (μg);

$m_m^{des}(t_1)$, $m_m^{des}(t_2)$,... , $m_m^{des}(t_n)$ = mass of the substance measured in an aliquot (v_a^D) at time points t_1,t_2,..., t_n, respectively (μg);

V_T = total volume of the aqueous phase in contact with the soil during the desorption kinetics experiment performed with the serial method (cm^3);

m_{aq}^A = mass of the test substance left over from the adsorption equilibrium due to incomplete volume replacement (μg);

$$m_{aq}^A = \left(\frac{\left(V_0 - \sum_{i=1}^{n} v_a^A(i) \right) - V_R}{\left(V_0 - \sum_{i=1}^{n} v_a^A(i) \right)} \right) \cdot m_{aq}^{ads}(eq) \qquad (26)$$

V_R = volume of the supernatant removed from the tube after the attainment of adsorption equilibrium and replaced by the same volume of a 0.01 M $CaCl_2$ solution (cm^3);

v_a^D = volume of the aliquot sampled for analytical purpose from the tube (i), during the desorption kinetics experiment performed with the serialmethod (cm^3);

$$v_a^D \leq 0.02 \cdot V_T \qquad (27)$$

ANNEX 6

ADSORPTION-DESORPTION IN SOILS: DATA REPORTING SHEETS

Substance tested:

Soil tested:

Dry mass content of the soil (105 °C, 12 h):……..%

Temperature:.....°C

Suitability of the analytical method

Weighed soil	g	
Soil: dry mass	g	
Volume $CaCl_2$ sol.	cm^3	
Nominal conc. final sol.	$\mu g\ cm^{-3}$	
Analytical conc. final sol.	$\mu g\ cm^{-3}$	

Principle of the analytical method used:

Calibration of the analytical method:

Substance tested:

Soil tested

Dry mass content of the soil (105 °C, 12 h):……%

Temperature:.....°C

Analytical methodology followed: Indirect ☐ Parallel ☐ Serial ☐

Direct ☐

Adsorption test: test samples

	Symbol	Units	Equilibration Time		Equilibration Time		Equilibration Time		Equilibration Time	
Tube No										
Weighed soil	-	g								
Soil: dry mass	m_{soil}	g								
Water volume in weighed soil (calculated)	V_{WS}	cm^3								
Volume 0.01 M $CaCl_2$ sol. to equilibrate the soil		cm^3								
Volume of stock solution		cm^3								
Total volume of aq. phase in contact with soil	V_0	cm^3								
Initial concentration Test solution	C_0	$\mu g\ cm^{-3}$								
Mass test subst. at the begining of the test	m_0	µg								
After agitation and centrifugation										
Indirect Method										
Parallel method										
Concentration test subst. aq. phase Blank correction included	$C_{aq}^{ads}(t_i)$	$\mu g\ cm^{-3}$								
Serial method										
Measured mass test subst. in the aliquot V_a^A	$m_m^{ads}(t_i)$	µg								
Direct method										
Mass test substance adsorbed on soil	$m_s^{ads}(t_i)$	µg								
Calculation of adsorption										
Adsorption	A_{t_i}	%								
	$A_{\Delta t_i}$	%								
Means										
Adsorption coefficient	K_d	$cm^3\ g^{-1}$								
Means										
Adsorption coefficient	K_{oc}	$cm^3\ g^{-1}$								
Means										

Substance tested:

Soil tested:

Dry mass content of the soil (105 °C, 12 h):……%

Temperature:……°C

Adsorption test: blanks and control

	Symbol	**Units**	**Blank**		**Blank**		**Control**	
Tube N^{o}								
Weighed soils		g					0	0
Water amount in weighed soil (calculated)		cm^3					-	-
Volume of 0.01 M $CaCl_2$ solution added		cm^3						
Volume of the stock solution of the test substance added		cm^3	0	0				
Total volume of aq. phase (calculated)		cm^3					-	-
Initial concentration of the test substance in aqueous phase		$\mu g\ cm^{-3}$						
After agitation and centrifugation								
Concentration in aqueous phase		$\mu g\ cm^{-3}$						

Remark: Add columns if necessary

Substance tested:

Soil tested:

Dry mass content of the soil (105 °C 12 h):……%

Temperature:.....…….°C

Mass balance

	Symbol	Units				
Tube No.						
Weighed soil	-	g				
Soil: dry mass	ms_{oil}	g				
Water volume in weighed soil (calculated)	V_{WS}	ml				
Volume 0.01 M $CaCl_2$ sol. to equilibrate the soil		ml				
Volume of stock solution		cm^3				
Total volume of aq. phase in contact with soil	V_0	cm^3				
Initial concentration test solution	C_0	$\mu g\ cm^{-3}$				
Equilibration time	-	h				
After agitation and centrifugation						
Concentr. test subst. aq. phase at adsorption equilibrium blank correction included	$C_{aq}^{ads}(eq)$	$\mu g\ cm^{-3}$				
Equalibration time	t_{eq}	h				
1st dilution with solvent						
Removed volume aq. phase	V_{rec}	cm^3				
Added volume of solvent	ΔV	cm^3				
1st extraction with solvent						
Signal analyzed in solvent	S_{E1}	var.				
Conc. test subst. in solvent	C_{E1}	$\mu g\ cm^{-3}$				
Mass of substance extracted from soil and vessel walls	m_{E1}	µg				
2nd dilution with solvent						
Removed volume of solvent	ΔV_s	cm^3				
Added volume of solvent	$\Delta V'$	cm^3				
2nd extraction with solvent						
Signal analyzed in solvent phase	S_{E2}	var.				
Conc. test subst. in solvent	C_{E2}	$\mu g\ cm^{-3}$				
Mass of substance extracted from soil and vessel walls	m_{E2}	µg				
Total mass test subst. extracted in two steps	m_E	µg				
Mass balance	MB	%				

Substance tested:

Soil tested:

Dry mass content of the soil (105 °C, 12 h):……%

Temperature:……..°C

Adsorption isotherms

	Symbol	**Units**								
Tube No.										
Weighed soil	-	g								
Soil: dry mass	E	g								
Water volume in weighed soil (calculated)	V_{WS}	cm^3								
Volume 0.01 M $CaCl_2$ sol. to equilibrate the soil		cm^3								
Volume of stock solution added		cm^3								
Total volume of aq. phase in contact with soil (calculated)	V_0	cm^3								
Concentration solution	C_0	$\mu g\ cm^{-3}$								
Equilibration time	-	h								
After agitation and centrifugation										
Concentration subst. aq. phase, blank correction included	$C_{aq}^{ads}(eq)$	$\mu g\ cm^{-3}$								
Temperature		°C								
Adsorb. mass per unit soil	$C_s^{ads}(eq)$	$\mu g\ g^{-1}$								

Regression analysis:

value of K_F^{ads} :

value of l/n:

regression coefficient r^2:

Substance tested:

Soil tested:

Dry mass content of the soil (105 °C, 12 h):……..%

Temperature:..... .....……..°C

Analytical methodology followed: Indirect ☐ Parallel ☐ Serial ☐

Desorption test

		Symbol	Units	Time interval	Time interval	Time interval	Time interval
Tube No. coming from adsorption step							
Mass of substance adsorbed on soil at adsorption equilibrium		$m_s^{ads}(eq)$	µg				
Removed volume aq. phase, replaced by 0.01 M $CaCl_2$		V_R	cm^3				
Total volume of aq. phase in contact with soil	PM	V_0	cm^3				
	SM	V_T	cm^3				
Mass test subst. left over the adsorption equilibrium due to incomplete volume replacement		m_{aq}^A	µg				
Desorption kinetics							
Measured mass of substance desorbed from soil at time t_i		$m_m^{des}(t_i)$	µg				
Volume of the solution taken from the tube (i) for the measurement of the test substance	PM	v_r^i	cm^3				
	SM	v_a^D	cm^3				
Mass of substance desorbed from soil at time t_i (calculated)		$m_{aq}^{des}(t_i)$	µg				
Mass of substance desorbed from soil during time interval Δt_i (calculated)		$m_{aq}^{des}(\Delta t_i)$	µg				
Desorption percentage							
Desorption at time t_i		D_{t_i}	%				
Desorption at time interval Δt_i		$D_{\Delta t_i}$	%				
Apparent desorption coefficient		K_{des}					

PM: Parallel method
SM: Serial method

C.19. ESTIMATION OF THE ADSORPTION COEFFICIENT (K_{OC}) ON SOIL AND ON SEWAGE SLUDGE USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

1. METHOD

This method is a replicate of OECD TG121 (2000).

1.1 INTRODUCTION

The sorption behavior of substances in soils or sewage sludges can be described through parameters experimentally determined by means of the Test Method C.18. An important parameter is the adsorption coefficient which is defined as the ratio between the concentration of the substance in the soil/sludge and the concentration of the substance in the aqueous phase at adsorption equilibrium. The adsorption coefficient normalized to the organic carbon content of the soil K_{oc} is a useful indicator of the binding capacity of a chemical on organic matter of soil and sewage sludge and allows comparisons to be made between different chemicals. This parameter can be estimated through correlations with the water solubility and the n-octanol/water partition coefficient (1)(2)(3)(4)(5)(6)(7).

The experimental method described in this test uses HPLC for the estimation of the adsorption coefficient K_{oc} in soil and in sewage sludge (8). The estimates are of higher reliability than those from QSAR calculations (9). As an estimation method it cannot fully replace batch equilibrium experiments used in the Test Method C18. However, the estimated K_{oc} may be useful for choosing appropriate test parameters for adsorption/desorption studies according to the Test Method C.18 by calculating K_d (distribution coefficient) or K_f (Freundlich adsorption coefficient) according to the equation 3 (see section 1.2).

1.2 DEFINITIONS

K_d : Distribution coefficient is defined as the ratio of equilibrium concentrations C of a dissolved test substance in a two phase system consisting of a sorbent (soil or sewage sludge) and an aqueous phase; it is a dimensionless value when concentrations in both phases are expressed on a weight/weight base. In case the concentration in the aqueous phase is given on a weight/volume base then the units are $ml \cdot g^{-1}$. K_d can vary with sorbent properties and can be concentration dependent.

$$K_d = \frac{C_{soil}}{C_{aq}} \text{ or } \frac{C_{sludge}}{C_{aq}} \qquad (1)$$

where:

C_{soil} = concentration of test substance in soil at equilibrium ($\mu g \cdot g^{-1}$)
C_{sludge} = concentration of test substance in sludge at equilibrium ($\mu g \cdot g^{-1}$)
C_{aq} = concentration of test substance in aqueous phase at equilibrium ($\mu g \cdot g^{-1}$, $\mu g \cdot ml^{-1}$).

K_f : Freundlich adsorption coefficient is defined as the concentration of the test substance in soil or sewage sludge (x/m) when the equilibrium concentration C_{aq} in the aqueous phase is equal to one; units are $\mu g.g^{-1}$ sorbent. The value can vary with sorbent properties.

$$\log \frac{x}{m} = \log K_f + \frac{1}{n} \cdot \log C_{aq} \qquad (2)$$

where:

x/m = amount of test substance x (µg) adsorbed on amount of sorbent m (g) at equilibrium
1/n = slope of Freundlich adsorption isotherm
C_{aq} = concentration of test substance in aqueous phase at equilibrium ($\mu g \cdot ml^{-1}$)

At $C_{aq} = 1$; $\log K_f = \log \frac{x}{m}$

K_{oc}: Distribution coefficient (K_d) or Freundlich adsorption coefficient (K_f) normalized to the organic carbon content (f_{oc}) of a sorbent; particularly for non-ionized chemicals, it is an approximate indicator for the extent of adsorption between a substance and the sorbent and allows comparisons to be made between different chemicals. Depending on the dimensions of K_d and K_f, K_{oc} can be dimensionless or have the units $ml \cdot g^{-1}$ or $\mu g \cdot g^{-1}$ organic matter.

$$Koc = \frac{Kd}{foc} (\text{dimensionless or } ml \cdot g^{-1}) \text{ or } \frac{Kf}{foc} (\mu g \cdot g^{-1}) \qquad (3)$$

The relationship between K_{oc} and K_d is not always linear and thus K_{oc} values can vary from soil to soil but their variability is greatly reduced compared to K_d or K_f values.
The adsorption coefficient (K_{oc}) is deduced from the capacity factor (k′) using a calibration plot of log k′ versus log K_{oc} of the selected reference compounds.

$$k' = \frac{t_R - t_0}{t_0} \qquad (4)$$

where:

t_R : HPLC retention time of test and reference substance (minutes)
t_0 : HPLC dead time (minutes) (see section 1.8.2).

P_{ow}: The octanol-water partition coefficient is defined as the ratio of the concentrations of dissolved substance in n-octanol and water; it is a dimensionless value.

$$Pow = \frac{Coc\ tan\ ol}{Caq} (= Kow) \qquad (5)$$

1.3 REFERENCE SUBSTANCES

The structural formula, the purity and the dissociation constant (if appropriate) should be known before using the method. Information on solubility in water and organic solvents, on octanol-water partition coefficient and on hydrolysis characteristics is useful.

To correlate the measured HPLC-retention data of a test substance with its adsorption coefficient K_{oc}, a calibration graph of log K_{oc} versus log k' has to be established. A minimum of six reference points, at least one above and one below the expected value of the test substance should be used. The accuracy of the method will be significantly improved if reference substances that are structurally related to the test substance are used. If such data are not available, it is up to the user to select the appropriate calibration substances. A more general set of structurally heterogeneous substances should be chosen in this case. Substances and K_{oc}-values which may be used are listed in the Annex in Table 1 for sewage sludge and in Table 3 for soil. The selection of other calibration substances should be justified.

1.4 PRINCIPLE OF THE TEST METHOD

HPLC is performed on analytical columns packed with a commercially available cyanopropyl solid phase containing lipophilic and polar moieties. A moderately polar stationary phase based on a silica matrix is used:

– O – Si	– CH_2 – CH_2 – CH_2	– CN
silica	non-polar spacer	polar moiety

The principle of the test method is similar to Testing Method A.8 (Partition Coefficient, HPLC Method). While passing through the column along with the mobile phase the test substance interacts with the stationary phase. As a result of partitioning between mobile and stationary phases the test substance is retarded. The dual composition of the stationary phase having polar and non-polar sites allows for interaction of polar and non-polar groups of a molecule in a similar way as is the case for organic matter in soil or sewage sludge matrices**.** This enables the relationship between the retention time on the column and the adsorption coefficient on organic matter to be established.

pH has a significant influence on sorption behavior in particular for polar substances. For agricultural soils or tanks of sewage treatment plants pH normally varies between pH 5.5 and 7.5. For ionisable substances, two tests should be performed with both ionized and non-ionized forms in appropriate buffer solutions but only in cases where at least 10 % of the test compound will be dissociated within pH 5.5 to 7.5.

Since only the relationship between the retention on the HPLC column and the adsorption coefficient is employed for the evaluation, no quantitative analytical method is required and only the determination of the retention time is necessary. If a suitable set of reference substances is available and standard experimental conditions can be used, the method provides a fast and efficient way to estimate the adsorption coefficient K_{oc}.

1.5 APPLICABILITY OF THE TEST

The HPLC method is applicable to chemical substances (unlabelled or labelled) for which an appropriate detection system (e.g. spectrophotometer, radioactivity detector) is available and which are sufficiently stable during the duration of the experiment. It may be particularly useful for chemicals which are difficult to study in other experimental systems (i.e. volatile substances; substances which are not soluble in water at a concentration which can be measured analytically; substances with a high affinity to the surface of incubation systems). The method can be used for mixtures which give unresolved elution bands. In such a case, upper and lower limits of the log K_{oc} values of the compounds of the test mixture should be stated.

Impurities may sometimes cause problems for interpretation of HPLC results, but they are of minor importance as long as the test substance can analytically be clearly identified and separated from the impurities.

The method is validated for the substances listed in Table 1 in the Annex and was also applied to a variety of other chemicals belonging to the following chemical classes:

- aromatic amines (e.g. trifluralin, 4-chloroaniline, 3,5-dinitroaniline, 4-methylaniline, N-methylaniline, 1-naphthylamine);
- aromatic carboxilic acid esters (e.g. benzoic acid methylester, 3,5-dinitrobenzoic acid ethylester);
- aromatic hydrocarbons (e.g. toluene, xylene, ethylbenzene, nitrobenzene);
- aryloxyphenoxypropionic acid esters (e.g. diclofop-methyl, fenoxaprop-ethyl, fenoxaprop-P-ethyl);
- benzimidazole and imidazole fungicides (e.g. carbendazim, fuberidazole, triazoxide);
- carboxilic acid amides (e.g. 2-chlorobenzamide, N,N-dimethylbenzamide, 3,5-dinitrobenzamide, N-methylbenzamide, 2-nitrobenzamide, 3-nitrobenzamide);
- chlorinated hydrocarbons (e.g. endosulfan, DDT, hexachlorobenzene, quintozene, 1,2,3-trichlorobenzene);
- organophosphorus insecticides (e.g. azinphos-methyl, disulfoton, fenamiphos, isofenphos, pyrazophos, sulprofos, triazophos);
- phenols (e.g. phenol, 2-nitrophenol, 4-nitrophenol, pentachlorophenol, 2,4,6-trichlorophenol, 1-naphthol);
- phenylurea derivatives (e.g. isoproturon, monolinuron, pencycuron);
- pigment dyestuffs (e.g. Acid Yellow 219, Basic Blue 41, Direct Red 81);
- polyaromatic hydrocarbons (e.g. acenaphthene, naphthalene);
- 1,3,5-triazine herbicides (e.g. prometryn, propazine, simazine, terbutryn);
- triazole derivatives (e.g. tebuconazole, triadimefon, tradimenol, triapenthenol).

The method is not applicable for substances which react either with the eluent or the stationary phase. It is also not applicable for substances that interact in a specific way with inorganic components (e.g. formation of cluster complexes with clay minerals). The method may not work for surface active substances, inorganic compounds and moderate or strong organic acids and bases. Log K_{oc} values ranging from 1.5 to 5.0 can be determined. Ionisable substances must be measured using a buffered mobile phase, but care has to be taken to avoid precipitation of buffer components or test substance.

1.6 QUALITY CRITERIA

1.6.1 **Accuracy**

Normally, the adsorption coefficient of a test substance can be estimated to within +/– 0.5 log unit of the value determined by the batch equilibrium method (see Table 1 in the Annex). Higher accuracy may be achieved if the reference substances used are structurally related to the test substance.

1.6.2 **Repeatability**

Determinations should be run at least in duplicate. The values of log K_{oc} derived from individual measurements should be within a range of 0.25 log unit.

1.6.3 **Reproducibility**

Experience gained so far in the application of the method is supportive of its validity. An investigation of the HPLC method, using 48 substances (mostly pesticides) for which reliable data on K_{oc} on soils were available gave a correlation coefficient of R = 0.95 (10) (11).

An inter-laboratory comparison test with 11 participating laboratories was performed to improve and validate the method (12). Results are given in Table 2 of the Annex**.**

1.7 DESCRIPTION OF THE TEST METHOD

1.7.1 **Preliminary Estimation of the Adsorption Coefficient**

The octanol-water partition coefficient P_{ow} (= K_{ow}) and, to some extent, the water solubility can be used as indicators for the extent of adsorption, particularly for non-ionized substances, and thus may be used for preliminary range finding. A variety of useful correlations have been published for several groups of chemicals (1)(2)(3)(4)(5)(6)(7).

1.7.2 **Apparatus**

A liquid chromatograph, fitted with a pulse-free pump and a suitable detection device is required. The use of an injection valve with an injection loop is recommended. Commercial cyanopropyl chemically bound resins on a silica base shall be used (e.g. Hypersil and Zorbax CN). A guard column of the same material may be positioned between the injection system and the analytical column. Columns from different suppliers may vary considerably in their separation efficiency. As a guidance, the following capacity factors k' should be reached: log k'> 0.0 for log K_{oc} = 3.0 and log k' > – 0.4 for log K_{oc} = 2.0 when using methanol/water 55/45 % as mobile phase.

1.7.3 **Mobile phases**

Several mobile phases have been tested and the following two are recommended:

- methanol/water (55/45% v/v)
- methanol/0.01M citrate-buffer pH 6.0 (55/45% v/v)

HPLC grade methanol and distilled water or citrate-buffer are used to prepare the eluting solvent. The mixture is degassed before use. Isocratic elution should be employed. If methanol/water mixtures are not appropriate, other organic solvent/water mixtures may be tried, e.g. ethanol/water or acetonitrile/water mixtures. For ionisable compounds the use of buffer solution is recommended to stabilize pH. Care must be taken to avoid salt precipitation and column deterioration, which may occur with some organic phase/buffer mixtures.

No additives such as ion pair reagents may be used because they can affect the sorption properties of the stationary phase. Such changes of the stationary phase may be irreversible. For this reason, it is mandatory that experiments using additives are carried out on separate columns.

1.7.4 **Solutes**

Test and reference substances should be dissolved in the mobile phase.

1.8 PERFORMANCE OF THE TEST

1.8.1 **Test condition**

The temperature during the measurements should be recorded. The use of a temperature controlled column compartment is highly recommended to guarantee constant conditions during calibration and estimation runs and measurement of the test substance.

1.8.2 **Determination of dead time t_o**

For the determination of the dead time t_o two different methods may be used (see also section 1.2).

1.8.2.1 *Determination of the dead time t_o by means of a homologous series*

This procedure has proven to yield reliable and standardized t_o values. For details see Testing Method A.8: Partition Coefficient (n-octanol/water), HPLC Method.

1.8.2.2 *Determination of the dead time t_o by inert substances which are not retained by the column*

This technique is based on the injection of solutions of formamide, urea or sodium nitrate. Measurements should be performed at least in duplicate.

1.8.3 **Determination of the retention times t_R**

Reference substances should be selected as described in section 1.3. They may be injected as a mixed standard to determine their retention times, provided it has been confirmed that the retention time of each reference standard is unaffected by the presence of the other reference standards. The calibration should be performed at regular intervals at least twice daily in order to account for unexpected changes in column performance. For best practice the calibration injections should be carried out before and after injections of the test substance to confirm retention times have not drifted. The test substances are injected separately in quantities as small as possible (to avoid column overload) and their retention times are determined.

In order to increase the confidence in the measurement, at least duplicate determinations should be made. The values of log K_{oc} derived from individual measurements should fall within a range of 0.25 log unit.

1.8.4 **Evaluation**

The capacity factors k′ are calculated from the dead time t_o and retention times t_R of the selected reference substances according to equation 4 (see section 1.2). The log k′ data of the reference substances are then plotted against their log K_{oc} values from batch equilibrium experiments given in Tables 1 and 3 of the Annex. Using this plot, the log k′ value of a test substance is then used to determine its log K_{oc} value. If the actual results show that the log K_{oc} of the test substance is outside the calibration range the test should be repeated using different, more appropriate reference substances.

2. **DATA AND REPORTING**

The report must include the following information:

- identity of test and reference substances and their purity, and pK_a values if relevant;
- description of equipment and operating conditions, e.g. type and dimension of analytical (and guard) column, means of detection, mobile phase (ratio of components and pH), temperature range during measurements;
- dead time and the method used for its determination;
- quantities of test and reference substances introduced in the column;
- retention times of reference compounds used for calibration;
- details of fitted regression line (log k' vs log K_{oc}) and a graph of the regression line;
- average retention data and estimated d log K_{oc} value for the test compound;
- chromatograms.

3. REFERENCES

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(12) W. Kördel, G. Kotthoff, J. Müller (1995). HPLC-screening method for the determination of the adsorption coefficient on soil-results of a ring test. Chemosphere, 30(7), 1373-1384.

ANNEX

Table 1

Comparison of K_{oc} values for soils and sewage sludges, and calculated values by the HPLC screening method [1,2]

substance	CAS-No.	log K_{oc} sewage sludges	log K_{oc} HPLC	Δ	log K_{oc} soils	log K_{oc} HPLC	Δ
Atrazine	1912-24-9	1.66	2.14	0.48	1.81	2.20	0.39
Linuron	330-55-2	2.43	2.96	0.53	2.59	2.89	0.30
Fenthion	55-38-9	3.75	3.58	0.17	3.31	3.40	0.09
Monuron	150-68-5	1.46	2.21	0.75	1.99	2.26	0.27
Phenanthrene	85-01-8	4.35	3.72	0.63	4.09	3.52	0.57
Benzoic acid phenylester	93-99-2	3.26	3.03	0.23	2.87	2.94	0.07
Benzamide	55-21-0	1.60	1.00	0.60	1.26	1.25	0.01
4-Nitrobenzamide	619-80-7	1.52	1.49	0.03	1.93	1.66	0.27
Acetanilide	103-84-4	1.52	1.53	0.01	1.26	1.69	0.08
Aniline	62-53-3	1.74	1.47	0.27	2.07	1.64	0.43
2,5-Dichloroaniline	95-82-9	2.45	2.59	0.14	2.55	2.58	0.03

[1] W. Kördel, D. Hennecke, M. Herrmann (1997). Application of the HPLC-screening method for the determination of the adsorption coefficient on sewage sludges. Chemosphere, 35(1/2), 121 - 128.

[2] W. Kördel, D. Hennecke, C. Franke (1997). Determination of the adsorption-coefficients of organic substances on sewage sludges. Chemosphere, 35 (1/2), 107 – 119.

Table 2

Results of a laboratory inter-comparison test (11 participating laboratories) performed to improve and validate the HPLC-method[1]

substance	CAS-No.	log K_{oc} [OECD 106]	K_{oc} [HPLC-method]	log K_{oc} [HPLC-method]
Atrazine	1912-24-9	1.81	78 ± 16	1.89
Monuron	150-68-5	1.99	100 ± 8	2.00
Triapenthenol	77608-88-3	2.37	292 ± 58	2.47
Linuron	330-55-2	2.59	465 ± 62	2.67
Fenthion	55-38-9	3.31	2062 ± 648	3.31

[1] W. Kördel, G. Kotthoff, J. Müller (1995). HPLC-screening method for the determination of the adsorption coefficient on soil-results of a ring test. Chemosphere, 30(7), 1373-1384.

Table 3

Recommended reference substances for the HPLC screening method based on soil adsorption data.

Reference substance	CAS-No.	log K_{oc} mean values from batch equilibrium	number of K_{oc} data	log S.D.	source
Acetanilide	103-84-4	1.25	4	0.48	a
Phenol	108-95-2	1.32	4	0.70	a
2-Nitrobenzamide	610-15-1	1.45	3	0.90	b
N.N-dimethylbenzamide	611-74-5	1.52	2	0.45	a
4-Methylbenzamide	619-55-6	1.78	3	1.76	a
Methylbenzoate	93-58-3	1.80	4	1.08	a
Atrazine	1912-24-9	1.81	3	1.08	c
Isoproturon	34123-59-6	1.86	5	1.53	c
3-Nitrobenzamide	645-09-0	1.95	3	1.31	b
Aniline	62-53-3	2.07	4	1.73	a
3,5-Dinitrobenzamide	121-81-3	2.31	3	1.27	b
Carbendazim	10605-21-7	2.35	3	1.37	c
Triadimenol	55219-65-3	2.40	3	1.85	c
Triazoxide	72459-58-6	2.44	3	1.66	c
Triazophos	24017-47-8	2.55	3	1.78	c
Linuron	330-55-2	2.59	3	1.97	c
Naphthalene	91-20-3	2.75	4	2.20	a
Endosulfan-diol	2157-19-9	3.02	5	2.29	c
Methiocarb	2032-65-7	3.10	4	2.39	c
Acid Yellow 219	63405-85-6	3.16	4	2.83	a
1,2,3-Trichlorobenzene	87-61-6	3.16	4	1.40	a
γ-HCH	58-89-9	3.23	5	2.94	a
Fenthion	55-38-9	3.31	3	2.49	c
Direct Red 81	2610-11-9	3.43	4	2.68	a
Pyrazophos	13457-18-6	3.65	3	2.70	c
α-Endosulfan	959-98-8	4.09	5	3.74	c
Diclofop-methyl	51338-27-3	4.20	3	3.77	c
Phenanthrene	85-01-8	4.09	4	3.83	a
Basic Blue 41 (mix)	26850-47-5 12270-13-2	4.89	4	4.46	a
DDT	50-29-3	5.63	1	–	b

/a/ W. Kördel, J. Müller (1994). Bestimmung des Adsorptionskoeffizienten organischer Chemikalien mit der HPLC. UBA R & D Report No. 106 01 044 (1994).

/b/ B.V. Oepen, W. Kördel, W. Klein. (1991). Chemosphere, 22, 285-304.

/c/ Data provided by industry.

C.20 *DAPHNIA MAGNA* REPRODUCTION TEST

1. METHOD

This Reproduction toxicity test method is a replicate of the OECD TG 211 (1998).

1.1 INTRODUCTION

The primary objective of the test is to assess the effect of chemicals on the reproductive output of *Daphnia magna*.

1.2 DEFINITIONS AND UNITS

Parent Animals: are those female *Daphnia* present at the start of the test and of which the reproductive output is the object of the study.

Offspring: are the young *Daphnia* produced by the parent animals in the course of the test.

Lowest Observed Effect Concentration (LOEC): is the lowest tested concentration at which the substance is observed to have a statistically significant effect on reproduction and parent mortality (at $p < 0.05$) when compared with the control, within a stated exposure period. 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, which when compared with the control, has no statistically significant effect ($p < 0.05$), within a stated exposure period.

EC_x: is the concentration of the test substance dissolved in water that results in a x per cent reduction in reproduction of *Daphnia magna* within a stated exposure period.

Intrinsic rate of increase: is a measure of population growth which integrates reproductive output and age-specific mortality (20) (21) (22). In steady state populations it will be zero. For growing populations it will be positive and for shrinking populations it will be negative. Clearly, the latter is not sustainable and ultimately will lead to extinction.

Limit of Detection: is the lowest concentration that can be detected but not quantified.

Limit of Determination: is the lowest concentration that can be measured quantitatively.

Mortality: an animal is recorded as dead when it is immobile, i.e. when it is not able to swim, or if there is no observed movement of appendages or postabdomen, within 15 seconds after gentle agitation of the test container. (If another definition is used, this must be reported together with its reference).

1.3 PRINCIPLE OF THE TEST METHOD

Young female *Daphnia* (the parent animals), aged less than 24 hours at the start of the test, are exposed to the test substance added to water at a range of concentrations. The test duration is 21 days. At the end of the test, the total number of living offspring produced per parent animal alive at the end of the test is assessed. This means that juveniles produced by adults that die during the test are excluded from the calculations. Reproductive output of parent animals can be expressed in other ways (e.g. number of living offspring produced per animal per day from the first day offspring were observed) but these should be reported in addition to the total number of juveniles produced per parent alive at the end of the test. The reproductive output of the animals exposed to the test substance is compared to that of the control(s) in order to determine the lowest observed effect concentration (LOEC) and hence the no observed effect concentration (NOEC). In addition, and as far as possible, the data are analysed using a regression model in order to estimate the concentration that would cause a x % reduction in reproductive output (i.e. the EC_{50}, EC_{20}, or EC_{10}).

The survival of the parent animals and time to production of first brood must also be reported. Other substance-related effects on parameters such as growth (e.g. length) and possibly intrinsic rate of increase, may also be examined.

1.4 INFORMATION ON THE TEST SUBSTANCE

Results of an acute toxicity test (see Method C.2, Part I) performed with *Daphnia magna* should be available. The result may be useful in selecting an appropriate range of test concentrations in the reproduction tests. The water solubility and the vapour pressure of the test substance should be known and a reliable analytical method for the quantification of the substance in the test solutions with reported recovery efficiency and limit of determination should be available.

Information on the test substance which may be useful in establishing the test conditions includes the structural formula, purity of the substance, stability in light, stability under the conditions of the test, pKa, P_{ow} and results of the test for ready biodegradability (see Method C.4).

1.5 VALIDITY OF THE TEST

For a test to be valid, the following performance criteria should be met in the control(s):

— the mortality of the parent animals (female *Daphnia*) does not exceed 20 % at the end of the test;

—- the mean number of live offspring produced per parent animal surviving at the end of the test is ≥ 60.

1.6 DESCRIPTION OF THE TEST METHOD

1.6.1 **Apparatus**

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 test vessels will normally be glass beakers.

In addition, some or all of the following equipment will be required:

— oxygen meter (with microelectrode or other suitable equipment for measuring dissolved oxygen in low volume samples);

— adequate apparatus for temperature control;

— pH meter;

— equipment for the determination of the hardness of water;

— equipment for the determination of the total organic carbon concentration (TOC) of water or equipment for the determination of the chemical oxygen demand (COD);

— adequate apparatus for the control of the lighting regime and the measurement of light intensity.

1.6.2 **Test Organism**

The species to be used in the test is *Daphnia magna* Straus. Other *Daphnia* species may be used providing they meet the validity criteria as appropriate (the validity criterion relating to the reproductive output in the controls should be relevant for the *Daphnia* species). If other species of *Daphnia* are used they must be clearly identified and their use justified.

Preferably, the clone should have been identified by genotyping. Research (1) has shown that the reproductive performance of Clone A (which originated from IRCHA in France) (3) consistently meets the validity criterion of a mean of ≥ 60 offspring per parent animal surviving when cultured under the conditions described in this method. However, other clones are acceptable provided that the *Daphnia* culture is shown to meet the validity criteria for a test.

At the start of the test, the animals should be less than 24 hours old and must not be first brood progeny. They should be derived from a healthy stock (i.e. showing no signs of stress such as high mortality, presence of males and ephippia, delay in the production of the first brood, discoloured animals etc.). The stock animals must be maintained in culture conditions (light, temperature, medium, feeding and animals per unit volume) similar to those to be used in the test. If the *Daphnia* culture medium to be used in the test is different from that used for routine *Daphnia* culture, it is good practice to include a pre-test acclimation period of normally about 3 weeks (i.e. one generation) to avoid stressing the parent animals.

1.6.3 **Test Medium**

It is recommended that a fully defined medium be used in this test. This can avoid the use of additives (e.g. seaweed, soil extract etc.), which are difficult to characterise, and therefore improves the opportunities for standardisation between laboratories. Elendt M4 (4) and M7 media (see Annex 1) have been found to be suitable for this purpose. However, other media (e.g. (5) (6)) are acceptable providing the performance of the *Daphnia* culture is shown to meet the validity criteria for the test.

If media are used which include undefined additives, these additives should be specified clearly and information should be provided in the test report on composition, particularly with regard to carbon content as this may contribute to the diet provided. It is recommended that the total organic carbon (TOC) and/or chemical oxygen demand (COD) of the stock preparation of the organic additive is determined and an estimate of the resulting contribution to the TOC/COD in the test medium made. It is recommended that TOC levels in the medium
(i.e. before addition of the algae) be below 2 mg/l (7).

When testing substances containing metals, it is important to recognise that the properties of the test medium (e.g. hardness, chelating capacity) may have a bearing on the toxicity of the test substance. For this reason, a fully defined medium is desirable. However, at present, the only fully defined media which are known to be suitable for long-term culture of *Daphnia magna* are Elendt M4 and M7. Both media contain the chelating agent EDTA. Work has shown (2) that the 'apparent toxicity' of cadmium is generally lower when the reproduction test is performed in M4 and M7 media than in media containing no EDTA. M4 and M7 are not, therefore, recommended for testing substances containing metals, and other media containing known chelating agents should also be avoided. For metal-containing substances it may be advisable to use an alternative medium such as, for example, ASTM reconstituted hard fresh water (7), which contains no EDTA, with added seaweed extract (8). This combination of ASTM reconstituted hard fresh water and seaweed extract is also suitable for long-term culture and testing of *Daphnia magna* (2), although it still exerts a mild chelating action due to the organic component in the added seaweed extract.

At the beginning and during the test, the dissolved oxygen concentration should be above 3 mg/l. The pH should be within the range 6-9, and normally it should not vary by more than 1.5 units in any one test. Hardness above 140 mg/l (as $CaCO_3$) is recommended. Tests at this level and above have demonstrated reproductive performance in compliance with the validity criteria (9) (10).

1.6.4 **Test Solutions**

Test solutions of the chosen concentrations are usually prepared by dilution of a stock solution. Stock solutions should preferably be prepared by dissolving the substance in test medium.

The use of organic solvents or dispersants may be required in some cases in order to produce a suitably concentrated stock solution, but every effort should be made to avoid the use of such materials. Examples of suitable solvents are acetone, ethanol, methanol, dimethylformamide and triethylene glycol. Examples of suitable dispersants are Cremophor RH40, methylcellulose 0.01 % and HCO-40. In any case, the test substance in the test solutions should not exceed the limit of solubility in the test medium.

Solvents are used to produce a stock solution which can be dosed accurately into water. At the recommended solvent concentration in the final test medium (i.e. ≤ 0.1 ml/l), the solvents listed above will not be toxic and will not increase the water solubility of a substance.

Dispersants may assist in accurate dosing and dispersion. At the recommended concentration in the final test medium (≤ 0.1 ml/l), the dispersants listed above will not be toxic and will not increase the water solubility of a substance.

1.7 TEST DESIGN

Treatments should be allocated to the test vessels and all subsequent handling of the test vessels should be done in a random fashion. Failure to do this may result in bias that could be construed as being a concentration effect. In particular, if experimental units are handled in treatment or concentration order, then some time-related effect, such as operator fatigue or other error, could lead to greater effects at the higher concentrations. Furthermore, if the test results are likely to be affected by an initial or environmental condition of the test, such as position in the laboratory, then consideration should be given to blocking the test.

1.8 PROCEDURE

1.8.1 **Conditions of exposure**

1.8.1.1 *Duration*

The test duration is 21 days.

1.8.1.2 *Loading*

Parent animals are maintained individually, one per test vessel, with 50-100 ml of medium in each vessel.

Larger volumes may sometimes be necessary to meet requirements of the analytical procedure used for determination of the test substance concentration, although pooling of replicates for chemical analysis is also allowable. If volumes greater than 100 ml are used, the ration given to the *Daphnia* may need to be increased to ensure adequate food availability and compliance with the validity criteria. For flow-through tests, alternative designs may, for technical reasons, be considered (e.g. four groups of 10 animals in a larger test volume), but any changes to the test design should be reported.

1.8.1.3 *Number of animals*

For semi-static tests, at least 10 animals individually held at each test concentration and at least 10 animals individually held in the control series.

For flow-through tests, 40 animals divided into four groups of 10 animals at each test concentration has been shown to be suitable (1). A smaller number of test organisms may be used and a minimum of 20 animals per concentration divided into two or more replicates with an equal number of animals (e.g. four replicates each with five daphnids) is recommended. Note that for tests where animals are held in groups, it will not be possible to express the reproductive output as the total number of living offspring produced per parent animal alive at the end of the test, if parent animals die. In these cases reproductive output should be expressed as 'total number of living offspring produced per parent present at the beginning of the test'.

1.8.1.4 *Feeding*

For semi-static tests, feeding should preferably be done daily, but at least three times per week (i.e. corresponding to media changes). Deviations from this (e.g. for flow-through tests) should be reported.

During the test the diet of the parent animals should preferably be living algal cells of one or more of the following: *Chlorella* sp, *Selenastrum capricornutum* (now *Pseudokirchneriella subcapitata* (11)) and *Scenedesmus subspicatus*. The supplied diet should be based on the amount of organic carbon (C) provided to each parent animal. Research (12) has shown that, for *Daphnia magna*, ration levels of between 0.1 and 0.2 mg C/*Daphnia*/day are sufficient for achieving the required number of offspring to meet the test validity criteria. The ration can be supplied either at a consistent rate throughout the period of the test, or, if desired, a lower rate can be used at the beginning and then increased during the test to take account of growth of the parent animals. In this case, the ration should still remain within the recommended range of 0.1 - 0.2 mg C/*Daphnia*/day at all times.

If surrogate measures, such as algal cell number or light absorbance, are to be used to feed the required ration level (i.e. for convenience since measurement of carbon content is time consuming), each laboratory must produce its own nomograph relating the surrogate measure to carbon content of the algal culture (see Annex 2 for advice on nomograph production). Nomographs should be checked at least annually and more frequently if algal culture conditions have changed. Light absorbance has been found to be a better surrogate for carbon content than cell number (13).

A concentrated algal suspension should be fed to the *Daphnia* to minimise the volume of algal culture medium transferred to the test vessels. Concentration of the algae can be achieved by centrifugation followed by resuspension in distilled water, deionised water or *Daphnia* culture medium.

1.8.1.5 *Light*

16 hours light at an intensity not exceeding 15-20 $\mu E \cdot m^{-2} \cdot s^{-1}$.

1.8.1.6 *Temperature*

The temperature of the test media should be within the range 18-22°C. However, for any one test, the temperature should not, if possible, vary by more than 2°C within these limits (e.g. 18-20, 19-21 or 20-22°C). It may be appropriate to use an additional test vessel for the purposes of temperature monitoring.

1.8.1.7 *Aeration*

The test vessels must not be aerated during the test.

1.8.2 **Test concentration**

Normally, there should be at least five test concentrations arranged in a geometric series with a separation factor preferably not exceeding 3.2, and the appropriate number of replicates for each test concentration should be used (see section 1.8.1.3). Justification should be provided if fewer than five concentrations are used. Substances should not be tested above their solubility limit in the test medium.

In setting the range of concentrations, the following should be borne in mind:

i. If the aim is to obtain the LOEC/NOEC, the lowest test concentration must be low enough so that the fecundity at that concentration is not significantly lower than that in the control. If this is not the case, the test will have to be repeated with a reduced lowest concentration.

ii. If the aim is to obtain the LOEC/NOEC, the highest test concentration must be high enough so that the fecundity at that concentration is significantly lower than that in the control. If this is not the case, the test will have to be repeated with an increased highest concentration.

iii. If the EC_X for effects on reproduction is estimated, it is advisable that sufficient concentrations are used to define the EC_X with an appropriate level of confidence. If the EC_{50} for effects on reproduction is estimated, it is advisable that the highest test concentration is greater than this EC_{50}. Otherwise, although it will still be possible to estimate the EC_{50}, the confidence interval for the EC_{50} will be very wide and it may not be possible to satisfactorily assess the adequacy of the fitted model.

iv. The range of test concentration should preferably not include any concentrations that have a statistically significant effect on adult survival since this would change the nature of the test from simply a reproduction test to a combined reproduction and mortality test requiring much more complex statistical analysis.

Prior knowledge of the toxicity of the test substance (e.g. from an acute test and/or from range-finding studies) should help in selecting appropriate test concentrations.

Where a solvent or dispersant is used to aid preparation of test solutions (see section 1.6.4), its final concentration in the test vessels should not be greater than 0.1 ml/l and should be the same in all test vessels.

1.8.3 **Controls**

One test-medium control series and also, if relevant, one control series containing the solvent or dispersant should be run in addition to the test series. When used, the solvent or dispersant concentration should be the same as that used in the vessels containing the test substance. The appropriate number of replicates should be used (see section 1.8.1.3).

Generally, in a well-run test, the coefficient of variation around the mean number of living offspring produced per parent animal in the control(s) should be $\leq$ 25 %, and this should be reported for test designs using individually held animals.

1.8.4 **Test medium renewal**

The frequency of medium renewal will depend on the stability of the test substance, but should be at least three times per week. If, from preliminary stability tests (see section 1.4) the test substance concentration is not stable (i.e. outside the range 80 -120 % of nominal or falling below 80 % of the measured initial concentration) over the maximum renewal period (i.e. 3 days), consideration should be given to more frequent medium renewal, or to the use of a flow-through test.

When the medium is renewed in semi-static tests, a second series of test vessels are prepared and the parent animals transferred to them by, for example, a glass pipette of suitable diameter. The volume of medium transferred with the *Daphnia* should be minimised.

1.8.5 **Observations**

The results of the observations made during the test should be recorded on data sheets (see examples in Annexes 3 and 4). If other measurements are required (see 1.3 and 1.8.8) additional observations may be required.

1.8.6 **Offspring**

The offspring produced by each parent animal should preferably be removed and counted daily from the appearance of the first brood, to prevent them consuming food intended for the adult. For the purpose of this method it is only the number of living offspring that needs to be counted, but the presence of aborted eggs or dead offspring should be recorded.

1.8.7 **Mortality**

Mortality among the parent animals should be recorded preferably daily, at least at the same times as offspring are counted.

1.8.8 **Other parameters**

Although this method is designed principally to assess effects on reproduction, it is possible that other effects may also be sufficiently quantified to allow statistical analysis. Growth measurements are highly desirable since they provide information on possible sublethal effects, which may be more useful than reproduction measurement alone; the measurement of the length of the parent animals (i.e. body length excluding the anal spine) at the end of the test is recommended. Other parameters that can be measured or calculated include time to production of first brood (and subsequent broods), number and size of broods per animal, number of aborted broods, presence of males or ephippia and the intrinsic rate of population increase.

1.8.9 **Frequency of analytical determinations and measurements**

Oxygen concentration, temperature, hardness and pH values should be measured at least once a week, in fresh and old media, in the control(s) and in the highest test substance concentration.

During the test, the concentrations of test substance are determined at regular intervals.

In semi-static tests where the concentration of the test substance is expected to remain within ± 20 % of the nominal (i.e. within the range 80-120 % - see 1.4 and 1.8.4), it is recommended that, as a minimum, the highest and lowest test concentrations be analysed when freshly prepared and at the time of renewal on one occasion during the first week of the test (i.e. analyses should be made on a sample from the same solution - when freshly prepared and at renewal). These determinations should be repeated at least at weekly intervals thereafter.

For tests where the concentration of the test substance is not expected to remain within ± 20 % of the nominal, it is necessary to analyse all test concentrations, when freshly prepared and at renewal. However, for those tests where the measured initial concentration of the test substance 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 initial concentrations), chemical determinations could be reduced in weeks 2 and 3 of the test to the highest and lowest test concentrations. In all cases, determination of test substance concentrations prior to renewal need only be performed on one replicate vessel at each test concentration.

If a flow-through test is used, a similar sampling regime to that described for semi-static tests is appropriate (but measurement of 'old' solutions is not applicable in this case). However, it may be advisable to increase the number of sampling occasions during the first week (e.g. three sets of measurements) to ensure that the test concentrations are remaining stable. In these types of test, the flow-rate of diluent and test substance should be checked daily.

If there is evidence that the concentration of the substance being tested has been satisfactorily maintained within ± 20 % of the nominal or measured initial concentration throughout the test, then results can be based on nominal or measured initial values. If the deviation from the nominal or measured initial concentration is greater than ± 20 %, results should be expressed in terms of the time-weighed mean (see Annex 5).

2. DATA AND REPORTING

2.1 TREATMENT OF RESULTS

The purpose of this test is to determine the effect of the test substance on the total number of living offspring produced per parent animal alive at the end of the test. The total number of offspring per parent animal should be calculated for each test vessel (i.e. replicate). If, in any replicate the parent animal dies during the test or turns out to be male, then the replicate is excluded from the analysis. The analysis will then be based on a reduced number of replicates.

For the estimation of the LOEC, and hence the NOEC, for effects of the chemical on reproductive output, it is necessary to calculate the mean reproductive output across replicates for each concentration and the pooled residual standard deviation, and this can be done using analysis of variance (ANOVA). The mean for each concentration must then be compared with the control mean using an appropriate multiple comparison method. Dunnett's or Williams' tests may be useful (14)(15)(16)(17). It is necessary to check whether the ANOVA assumption of homogeneity of variance holds. It is recommended that this be done graphically rather than via a formal significance test (18); a suitable alternative is to run a Bartlett's test. If this assumption does not hold, then consideration should be given to transforming the data to homogenise variances prior to performing the ANOVA, or to carrying out a weighted ANOVA. The size of the effect detectable using ANOVA (i.e. the least significant difference) should be calculated and reported.

For the estimation of the concentration which would cause a 50 % reduction in reproductive output (i.e. the EC_{50}), a suitable curve, such as the logistic curve, should be fitted to the data using a statistical method such as least squares. The curve could be parameterized so that the EC_{50} and its standard error can be estimated directly. This would greatly ease the calculation of the confidence limits about the EC_{50}. Unless there are good reasons to prefer different confidence levels, two-sided 95 % confidence limits should be quoted. The fitting procedure should preferably provide a means for assessing the significance of the lack of fit. This can be done graphically or by dividing the residual sum of squares into 'lack of fit' and 'pure error components' and performing a significance test for lack of fit. Since treatments giving high fecundity are likely to have greater variance in the number of juveniles produced than treatments giving low fecundity, consideration to weighting the observed values to reflect the different variances in the different treatment groups should be given (see for background information ref. 18).

In the analysis of the data from the final ring test (2), a logistic curve was fitted using the following model, although other suitable models can be used:

$$Y = \frac{c}{1 + \left(\frac{x}{x_0}\right)^b}$$

where:

Y: the total number of juveniles per parent animal alive at the end of the test (calculated for each vessel)

x: the substance concentration

c : the expected number of juveniles when x = 0

x_0: the EC_{50} in the population

b : the slope parameter

This model is likely to be adequate in a large number of situations, but there will be tests for which it is not appropriate. A check should be made on the validity of the model as suggested above. In some cases, a hormesis model in which low concentrations give enhanced effects may be appropriate (19).

Other Effect Concentrations, such as the EC_{10} or EC_{20} can also be estimated, although it may be preferable to use a different parameterisation of the model from that used to estimate the EC_{50}.

2.2 TEST REPORT

The test report must include the following:

2.2.1 **Test substance:**

— physical nature and relevant physicochemical properties;

— chemical identification data, including purity.

2.2.2 **Test species:**

— the clone (whether it has been genetically typed), supplier or source (if known) and the culture conditions used. If a different species to *Daphnia magna* is used, this should be reported and justified.

2.2.3 **Test conditions:**

— test procedure used (e.g. semi-static or flow-through, volume, loading in number of *Daphnia* per litre);

— photoperiod and light intensity;

— test design (e.g. number of replicates, number of parents per replicate);

— details of culture medium used;

— if used, additions of organic material including the composition, source, method of preparation, TOC/COD of stock preparations, estimation of resulting TOC/COD in test medium;

— detailed information on feeding, including amount (in mg C/*Daphnia*/day) and schedule (e.g. type of food(s), including for algae the specific name(species) and, if known, the strain, the culture conditions);

— method of preparation of stock solutions and frequency of renewal (the solvent or dispersant and its concentration must be given, when used).

2.2.4 **Results:**

— results from any preliminary studies on the stability of the test substance;

— the nominal test concentrations and the results of all analyses to determine the concentration of the test substance in the test vessels (see example data sheets in Annex 4); the recovery efficiency of the method and the limit of determination should also be reported;

— water quality within the test vessels (i.e. pH, temperature and dissolved oxygen concentration, and TOC and/or COD and hardness where applicable) (see example data sheet in Annex 3);

— the full record of living offspring by each parent animal (see example data sheet in Annex 3);

— the number of deaths among the parent animals and the day on which they occurred (see example data sheet in Annex 3);

— the coefficient of variation for control fecundity (based on total number of living offspring per parent animal alive at the end of the test);

— plot of total number of living offspring per parent animal (for each replicate) alive at the end of the test vs concentration of the test substance;

— the Lowest Observed Effect Concentration (LOEC) for reproduction, including a description of the statistical procedures used and an indication of what size of effect could be detected and the No Observed Effect Concentration (NOEC) for reproduction; where appropriate, the LOEC/NOEC for mortality of the parent animals should also be reported;

— where appropriate, the EC_x for reproduction and confidence intervals and a graph of the fitted model used for its calculation, the slope of the dose-response curve and its standard error;

— other observed biological effects or measurements: report any other biological effects which were observed or measured (e.g. growth of parent animals) including any appropriate justification;

— an explanation for any deviation from the Test Method.

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ANNEX 1

PREPARATION OF FULLY DEFINED ELENDT M7 AND M4 MEDIA

Acclimation to Elendt M7 and M4 media

Some laboratories have experienced difficulty in directly transferring *Daphnia* to M4 (1) and M7 media. However, some success has been achieved with gradual acclimation, i.e. moving from own medium to 30 % Elendt, then to 60 % Elendt and then to 100 % Elendt. The acclimation periods may need to be as long as one month.

PREPARATION

Trace elements

Separate stock solutions (I) of individual trace elements are first prepared in water of suitable purity, e.g. deionised, distilled or reverse osmosis. From these different stock solutions (I) a second single stock solution (II) is prepared, which contains all trace elements (combined solution), i.e.:

Stock solutions I (single substance)	**Amount added to water mg/l**	**Concentration (in relation to medium M4) fold**	**To prepare the combined stock-solution II add the following amount of stock solution I to water ml/l**	
			M 4	**M 7**
H_3BO_3	57 190	20 000	1.0	0.25
$MnCl_2$ * 4 H_2O	7 210	20 000	1.0	0.25
LiCl	6 120	20 000	1.0	0.25
RbCl	1 420	20 000	1.0	0.25
$SrCl_2$ * 6 H_2O	3 040	20 000	1.0	0.25
NaBr	320	20 000	1.0	0.25
Na_2MoO_4 * 2 H_2O	1 260	20 000	1.0	0.25
$CuCl_2$ * 2 H_2O	335	20 000	1.0	0.25
$ZnCl_2$	260	20 000	1.0	1.0
$CoCl_2$ * 6 H_2O	200	20 000	1.0	1.0
KI	65	20 000	1.0	1.0
Na_2SeO_3	43.8	20 000	1.0	1.0
NH_4VO_3	11.5	20 000	1.0	1.0
Na_2EDTA * 2 H_2O	5 000	2 000	–	–
$FeSO_4$ * 7 H_2O	1 991	2 000	–	–
Both Na_2EDTA and $FeSO_4$ solutions are prepared singly, poured together and autoclaved immediately. This gives:				
21 Fe-EDTA solution		1 000-fold	20.0	5.0

M4 and M7 media

M4 and M7 media are prepared using stock solution II, the macro-nutrients and vitamins as follows:

	Amount added to water mg/l	Concentration (related to medium M4) fold	Amount of stock solution added to prepare medium ml/l M 4	M 7
Stock solution II combined trace elements		20	50	50
Macro-nutrient stock solutions (single substance)				
$CaCl_2$ * 2 H_2O	293 800	1 000	1.0	1.0
$MgSO_4$ * 7 H_2O	246 600	2 000	0.5	0.5
KCl	58 000	10 000	0.1	0.1
$NaHCO_3$	64 800	1 000	1.0	1.0
Na_2SiO_3 * 9 H_2O	50 000	5 000	0.2	0.2
$NaNO_3$	2 740	10 000	0.1	0.1
KH_2PO_4	1 430	10 000	0.1	0.1
K_2HPO_4	1 840	10 000	0.1	0.1
Combined Vitamin stock	-	10 000	0.1	0.1
The combined vitamin stock solution is prepared by adding the 3 vitamins to 1 litre water as show below:				
Thiamine hydrochloride	750	10 000	–	–
Cyanocobalamine (B_{12})	10	10 000	–	–
Biotine	7.5	10 000	–	–

The combined vitamin stock is stored frozen in small aliquots. Vitamins are added to the media shortly before use.

N.B. To avoid precipitation of salts when preparing the complete media, add the aliquots of stock solutions to about 500-800 ml deionized water and then fill up to 1 litre.

N.N.B. The first publication of the M4 medium can be found in Elendt, B.P. (1990). Selenium deficiency in crustacea; an ultrastructural approach to antennal damage in *Daphnia magna* Straus. Protoplasma, 154, 25-33.

ANNEX 2

TOTAL ORGANIC CARBON (TOC) ANALYSIS AND

PRODUCTION OF A NOMOGRAPH FOR TOC CONTENT OF ALGAL FEED

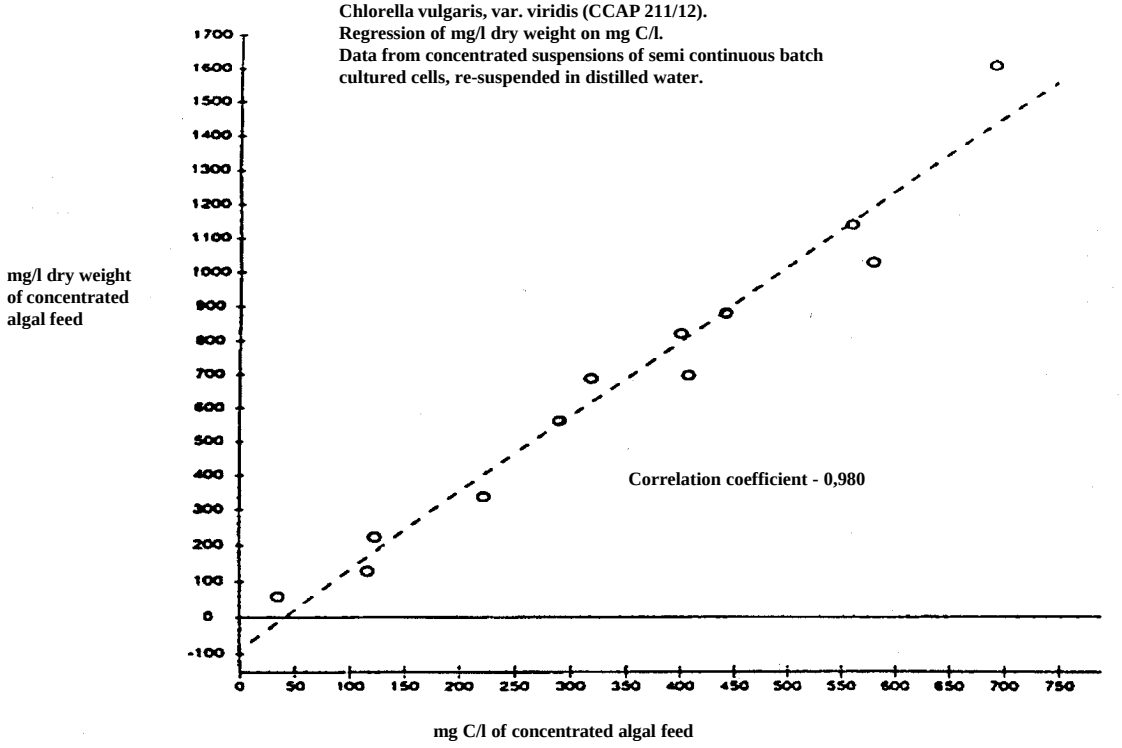
It is recognised that the carbon content of the algal feed will not normally be measured directly but from correlations (i.e. nomographs) with surrogate measures such as algal cell number or light absorbance).

TOC should be measured by high temperature oxidation rather than by UV or persulphate methods. (See: The Instrumental Determination of Total Organic Carbon, Total Oxygen Demand and Related Determinands 1979, HMSO 1980; 49 High Holborn, London WC1V 6HB).

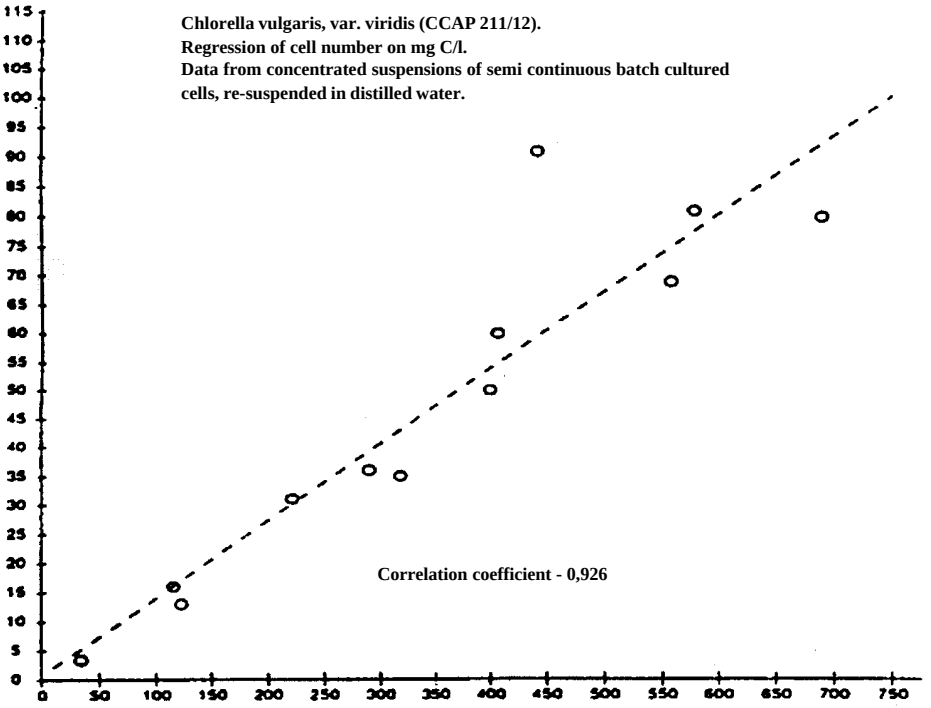
For nomograph production, algae should be separated from the growth medium by centrifugation followed by resuspension in distilled water. Measure the surrogate parameter and TOC concentration in each sample in triplicate. Distilled water blanks should be analysed and the TOC concentration deducted from that of the algal sample TOC concentration.

Nomograph should be linear over the required range of carbon concentrations. Examples are shown below.

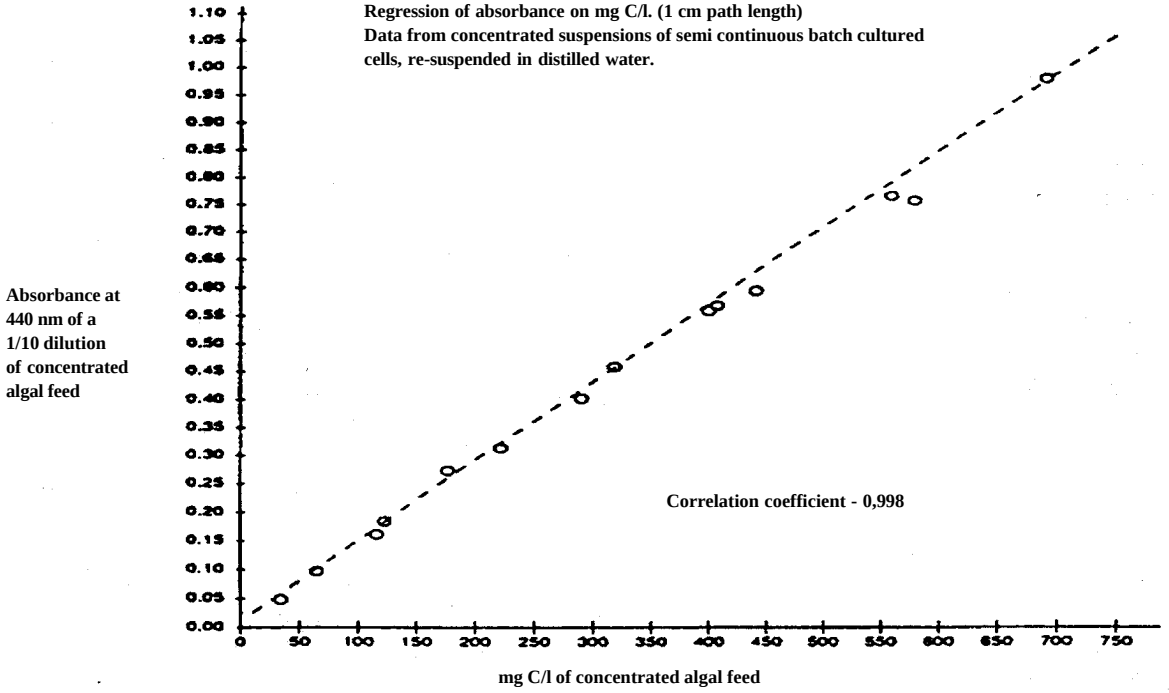
N.B. These should not be used for conversions; it is essential that laboratories prepare their own nomographs.



Chlorella vulgaris, var. viridis (CCAP 211/12).
Regression of cell number on mg C/l.
Data from concentrated suspensions of semi continuous batch cultured cells, re-suspended in distilled water.



Chlorella vulgaris, var. viridis (CCAP 112/12).
Regression of absorbance on mg C/l. (1 cm path length)
Data from concentrated suspensions of semi continuous batch cultured cells, re-suspended in distilled water.



ANNEX 3

EXAMPLE DATA SHEET RECORDING MEDIUM RENEWAL, PHISICAL/CHEMICAL MONITORING DATA, FEEDING,

DAPHNIA REPRODUCTION AND ADULT MORTALITY

Experiment N^0: Data starded : Clone : Medium : Type of food : Test Substance : Nominal conc.:

Day	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21		
Medium renewal (tick)																								
PH *																							new	
																							old	
O_2 mg/l *																							new	
																							old	
Temp (^oC) *																							new	
																							old	
Food provided (tick)																								
N^o live offspring †																								Total
Vessel 1																								
2																								
3																								
4																								
5																								
6																								
7																								
8																								
9																								
10																								
																							Total	
Cumulative adult mortality ‡																								

*Indicate which vessel was used for the experiment

‡Record mortality of any adult animals as ‘M’ in relevant box

†Record aborted broods as ‘AB’ in relevant box

ANNEX 4

EXAMPLE DATA SHEET FOR RECORDING RESULTS OF CHEMICAL ANALYSIS

(A) Measured concentrations

Nominal conc.	Week 1 sample		Week 2 sample		Week 3 sample	
	Fresh	Old	Fresh	Old	Fresh	Old

(b) Measured concentrations as a percentage of nominal

Nominal conc.	Week 1 sample		Week 2 sample		Week 3 sample	
	Fresh	Old	Fresh	Old	Fresh	Old

ANNEX 5

CALCULATION OF A TIME-WEIGHTED MEAN

Time-weighted mean

Given that the concentration of the test substance can decline over the period between medium renewals, it is necessary to consider what concentration should be chosen as representative of the range of concentrations experienced by the parent *Daphnia*. The selection should be based on biological considerations as well as statistical ones. For example, if reproduction is thought to be affected mostly by the peak concentration experienced, then the maximum concentration should be used. However, if the accumulated or longer term effect of the toxic substance is considered to be more important, then an average concentration is more relevant. In this case, an appropriate average to use is the time-weighted mean concentration, since this takes account of the variation in instantaneous concentration over time.

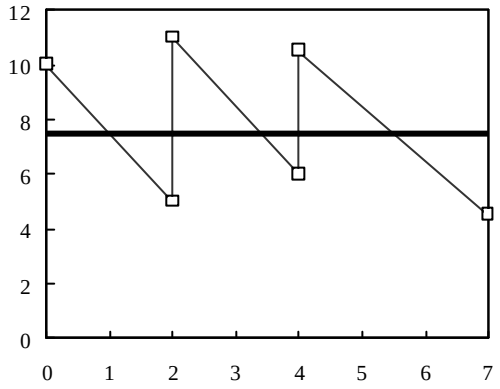


Figure 1 : Example of time-weighted mean

Figure 1 shows an example of a (simplified) test lasting 7 days with medium renewal at Days 0, 2 and 4.

- The thin zig-zag line represents the concentration at any point in time. The fall in concentration is assumed to follow an exponential decay process.
- The 6 plotted points represent the observed concentrations measured at the start and end of each renewal period.
- The thick solid line indicates the position of the time-weighted mean.

The time-weighted mean is calculated so that the area under the time-weighted mean is equal to the area under the concentration curve. The calculation for the above example is illustrated in Table 1.

Table 1: Calculation of Time-weighted mean

Renewal N°	Days	Conc0	Conc1	Ln(Conc0)	Ln(Conc1)	Area
1	2	10.000	4.493	2.303	1.503	13.767
2	2	11.000	6.037	2.398	1.798	16.544
3	3	10.000	4.066	2.303	1.403	19.781
Total Days :	7				Total Area	50.091
					TW Mean	7.156

Days is the number of days in the renewal period
Conc0 is the measured concentration at the start of each renewal period
Conc1 is the measured concentration at the end of each renewal period
Ln(Conc0) is the natural logarithm of Conc0
Ln(Conc1) is the natural logarithm of Conc1
Area is the area under the exponential curve for each renewal period. It is calculated by :

$$Area = \frac{Conc0 - Conc1}{Ln(Conc0) - Ln(Conc1)} \text{ x } Days$$

The time-weighted mean (TW *Mean*) is the *Total Area* divided by the *Total Days*.

Of course, for the *Daphnia* reproduction test the table would have to be extended to cover 21 days.

It is clear that when observation are taken only at the start and end of each renewal period, it is not possible to confirm that the decay process is, in fact, exponential. A different curve would result in a different calculation for *Area*. However, an exponential decay process is not implausible and is probably the best curve to use in the absence of other information.

However, a work of caution is required if the chemical analysis fails to find any substance at the end of the renewal period. Unless it is possible to estimate how quickly the substance disappeared from the solution, it is impossible to obtain a realistic area under the curve, and hence it is impossible to obtain a reasonable time-weighted mean.