COMMISSION OF THE EUROPEAN COMMUNITIES

Brussels, 29 October 2003
COM (2003) 644

2003/zzz (COD)
VOLUME V

Proposal for a

REGULATION OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL

concerning the Registration, Evaluation, Authorisation and Restrictions of Chemicals (REACH)

ORIGINAL: EN

(presented by the Commission)

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C.1. ACUTE TOXICITY FOR FISH

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1.1.   INTRODUCTION

The purpose of this test is to determine the acute lethal toxicity of a substance to fish in fresh water. It is desirable to have, as far as possible, information on the water solubility, vapour pressure, chemical stability, dissociation constants and biodegradability of the substance to help in the selection of the most appropriate test method (static, semi-static or flow-through) for ensuring satisfactorily constant concentrations of the test substance over the period of the test.

Additional information (for instance structural formula, degree of purity, nature and percentage of significant impurities, presence and amounts of additives, and n-octanol/water partition coefficient) should be taken into consideration in both the planning of the test and interpretation of the results.

1.2.   DEFINITIONS AND UNITS

Acute toxicity is the discernible adverse effect induced in an organism within a short time (days) of exposure to a substance. In the present test, acute toxicity is expressed as the median lethal concentration (LC_{50}), that is the concentration in water which kills 50% of a test batch of fish within a continuous period of exposure which must be stated.

All concentrations of the test substance are given in weight by volume (milligrams per litre). They may also be expressed as weight by weight ($mg.kg^{-1}$).

1.3.   REFERENCE SUBSTANCES

A reference substance may be tested as a means of demonstrating that under the laboratory test conditions the response of tested species have not changed significantly.

No reference substances are specified for this test.

1.4.   PRINCIPLE OF THE TEST METHOD

A limit test may be performed at 100 mg per litre in order to demonstrate that the LC_{50} is greater than this concentration.

The fish are exposed to the test substance added to water at a range of concentrations for a period of 96 hours. Mortalities are recorded at least at 24-hour intervals, and the concentrations killing 50% of the fish (LCso) at each observation time are calculated where possible.

1.5.   QUALITY CRITERIA

The quality criteria shall apply to the limit test as well as the full test method.

The mortality in the controls must not exceed 10% (or one fish if less than ten are used) by the end of the test.

The dissolved oxygen concentration must have been more than 60% of the air-saturation value throughout.

The concentrations of the test substance shall be maintained to within 80% of the initial concentrations throughout the duration of the test.

For substances which dissolve easily in the test medium, yielding stable solutions i.e. those which will not to any significant extent volatilize, degrade, hydrolyze or adsorb, the initial concentration can be taken as being equivalent to the nominal concentration. Evidence shall be presented that the concentrations have been maintained throughout the test and that the quality criteria have been satisfied.

For substances that are:

(i)   poorly soluble in the test medium, or

(ii)   capable of forming stable emulsions or dispersions, or

(iii)   not stable in aqueous solutions,

the initial concentration shall be taken as the concentration measured in solution (or, if technically not possible, measured in the water column) at the start of the test. The concentration shall be determined after a period of equilibration but before the introduction of the test fish.

In any of these cases, further measurements must be made during the test to confirm the actual exposure concentrations or that the quality criteria have been met.

The pH should not vary by more than 1 unit.

1.6.   DESCRIPTION OF THE TEST METHOD

Three types of procedure can be used:

Static test:

Toxicity test in which no flow of test solution occurs. (Solutions remain unchanged throughout the duration of the test.)

Semi-static test:

Test without flow of test solution, but with regular batchwise renewal of test solutions after prolonged periods (e.g. 24 hours).

Flow-through test:

Toxicity test in which the water is renewed constantly in the test chambers, the chemical under test being transported with the water used to renew the test medium.

1.6.1. Reagents

1.6.1.1. Solutions of test substances

Stock solutions of the required strength are prepared by dissolving the substance in deionized water or water according to 1.6.1.2.

The chosen test concentrations are prepared by dilution of the stock solution. If high concentrations are tested, the substance may be dissolved in the dilution water directly.

The substances should normally only be tested up to the limit of solubility. For some substances (e.g. substances having low solubility in water, or high P_{ow}, or those forming stable dispersion rather than true solution in water), it is acceptable to run a test concentration above the solubility limit of the substance to ensure that the maximum soluble/stable concentration has been obtained. It is important, however, that this concentration will not otherwise disturb the test system (e.g. film of the substance on the water surface preventing the oxygenation of the water, etc.).

Ultrasonic dispersion, organic solvents, emulsifiers or dispersants may be used as an aid to prepare stock solutions of substances with low aqueous solubility or to help to disperse these substances in the test medium. When such auxiliary substances are used, all test concentrations should contain the same amount of auxiliary substance, and additional control fish should be exposed to the same concentration of the auxiliary substance as that used in the test series. The concentration of such auxiliaries should be minimized, but in no case should exceed 100 mg per litre in the test medium.

The test should be carried out without adjustment of the pH. If there is evidence of marked change in the pH, it is advised that the test should be repeated with pH adjustment and the results reported. In that case, the pH value of the stock solution should be adjusted to the pH value of the dilution water unless there are specific reasons not to do so. HCl and NaOH are preferred for this purpose. This pH adjustment should be made in such a way that the concentration of test substance in the stock solution is not changed to any significant extent. Should any chemical reaction or physical precipitation of the test compound be caused by the adjustment, this should be reported.

1.6.1.2. Holding and dilution water

Orinking-water supply (uncontaminated by potentially harmful concentrations of chlorine, heavy metals or other substances), good-quality natural water or reconstituted water (See Appendix I) may be used. Waters with a total hardness of between 10 and 250 mg per litre (as $CaCO_3$) and with a pH from 6,0 to 8,5 are preferred.

1.6.2. Apparatus

All apparatus must be made of chemically inert material.

– automatic dilution system (for flow-through test),

– oxygen meter,

– equipment for determination of hardness of water,

– adequate apparatus for temperature control,

–   pH meter.

1.6.3. Test fish

The fish should be in good health and free from any apparent malformation.

The species used should be selected on the basis of practical criteria, such as their ready availability throughout the year, ease of maintenance, convenience for testing, relative sensitivity to chemicals, and any economic, biological or ecological factors which have any bearing. The need for comparability of the data obtained and existing international harmonization (reference 1) should also be borne in mind when selecting the fish species.

A list of fish species which are recommended for the performance of this test is given in Appendix 2; Zebra fish and rainbow trout are the preferred species.

1.6.3.1. Holding

Test fish should preferably come from a single stock of similar length and age. The fish must be held for at least 12 days, in the following conditions:

loading:

appropriate to the system (recirculation or flow-through) and the fish species,

water:

see 1.6.1.2,

light:

12 to 16 hours illumination daily,

dissolved oxygen concentration:

at least 80 % of air-saturation value,

feeding:

three times per week or daily, ceasing 24 hours before the start of the test.

1.6.3.2. Mortality

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

–   greater than 10 % of population in seven days:

       rejection of entire batch,

–   between 5 and 10% of population:

       holding period continued for seven additional days. If no further mortalities occur, the batch is acceptable, otherwise it must be rejected,

–   less than 5 % of population:

acceptance of the batch.

1.6.4. Adaptation

All fish must be exposed to water of the quality and the temperature to be used in the test for at least seven days before they are used.

1.6.5. Test Procedure

A range-finding test can precede a definitive test, in order to obtain information about the range of concentrations to be used in the main test.

One control without the test substance is run and, if relevant, one control containing the auxiliary substance is also run, in addition to the test series.

Depending on the physical and chemical properties of the test compound, a static, semi-static, or a flow-through test should be selected as appropriate, to fulfil the quality criteria.

Fish are exposed to the substance as described below:

–   duration: 96 hours

–   number of animals: at least 7 per concentration,

–   tanks: of suitable capacity in relation to the recommended loading,

–   loading: maximum loading of 1 g per litre for static and semi-static tests is recommended; for flow-through systems, higher loading is acceptable,

–   test concentration: At least five concentrations differing by a constant factor not exceeding 2,2 and as far as possible spanning the range of 0 to 100 % mortality,

–   water: see 1.6.1.2,

–   light: 12 to 16 hours illumination daily,

–   temperature: appropriate to the species (Appendix 2) but within ± 1 °C within any particular test,

–   dissolved oxygen concentration: not less than 60 % of the air-saturation value at the selected temperature,

–   feeding: none.

The fish are inspected after the first 2 to 4 hours and at least at 24-hour intervals. Fish are considered dead if touching of the caudal peduncle produces no reaction, and no breathing movements are visible. Dead fish are removed when observed and mortalities are recorded. Records are kept of visible abnormalities (e.g. loss of equilibrium, changes in swimming behaviour, respiratory function, pigmentation, etc.).

Measurements of pH, dissolved oxygen and temperature must be carried out daily.

Limit test

Using the procedures described in this test method, a limit test may be performed at 100 mg per litre in order to demonstrate that the LC_{50} is greater than this concentration.

If the nature of the substance is such that a concentration of 100 mg per litre in the test water cannot be attained, the limit test should be performed at a concentration equal to the solubility of the substance (or the maximum concentration forming a stable dispersion) in the medium used (see also point 1.6.1.1).

The limit test should be performed using 7 to 10 fish, with the same number in the control(s). (Binomial theory dictates that when 10 fish are used with zero mortality, there is a 99,9% confidence that the LC_{50} is greater than the concentration used in the limit test. With 7, 8 or 9 fish, the absence of mortality provides at least 99 % confidence that the LC_{50} is greater than the concentration used.)

If mortalities occur, a full study must be carried out. If sublethal effects are observed, these should be recorded.

2.   DATA AND EVALUATION

For each period where observations were recorded (24, 48, 72 and 96 hours), plot percentage mortality for each recommended exposure period against concentration on logarithmic-probability paper.

When possible and for each observation time, the LC_{50} and the confidence limits (p = 0,05) should be estimated using standard procedures; these values should be rounded off to one, or at most two significant figures (examples of rounding off to two figures: 170 for 173,5; 0,13 for 0,127; 1,2 for 1,21).

In those cases where the slope of the concentration/percentage response 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 2,2 give only 0 and 100% mortality, these two values are sufficient to indicate the range within which the LC_{50} falls.

If it is observed that the stability or homogeneity of the test substance cannot be maintained, this should be reported and care should be taken in the interpretation of the results.

3.   REPORTING

The test report shall, if possible, include the following information:

–   information about test fish (scientific name, strain, supplier, any pretreatment, size and number used in each test concentration);

–   dilution-water source and major chemical characteristics (pH, hardness, temperature);

–   in the case of a substance of low aqueous solubility, the method of preparation of stock and test solutions;

–   concentration of any auxiliary substances;

– list of the concentrations used and any available information on the stability at the concentrations of the tested chemical in the test solution;

– if chemical analyses are performed, methods used and results obtained;

– results of the limit test if conducted;

– reasons for the choice and details of the test procedure used (e.g. static, semi-static, dosing rate, flow-through rate, whether aerated, fish loading, etc.);

– description of test equipment;

– lighting regime;

– dissolved oxygen concentrations, pH values and temperatures of the test solutions every 24 hours;

– evidence that the quality criteria have been fulfilled;

– a table showing the cumulative mortality at each concentration and the control (and control with the auxiliary substance if required) at each of the recommended observation times;

– graph of the concentration/ percentage response curve at the end of the test;

– if possible, the LC_{50} values at each of the recommended observation times (with 95 % confidence limits);

– statistical procedures used for determining the LC_{50} values;

– if a reference substance is used, the results obtained,

– highest test concentration causing no mortality within the period of the test;

– lowest test concentration causing 100% mortality within the period of the test.

4.   REFERENCES

1) OECD, Paris, 1981, Test Guideline 203, Decision of the Council C(81) 30 final and updates.

2) AFNOR -Determination of the acute toxicity of a substance to *Brachydanio rerio* - Static and Flow Through methods -NFT 90-303 June 1985.

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18) Sprague, J.B. Measurement of pollutant toxicity to fish. II Utilising and applying bioassay results. Water Res. 1970, vol. 4, 3-32.

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Example of a suitable dilution water

All chemicals must be of analytical grade.

The water should be good-quality distilled water, or deionized water with a conductivity less than 5 μScm^{-1}.

Apparatus for distillation of water must not contain any parts made of copper.

Stock solutions

CaCI$_2$. 2H2O (calcium chloride dihydrate):	11,76 g
Dissolve in, and make up to 1 litre with water .	
MgSO$_4$. 7H2O (magnesium sulphate heptahydrate):	4,93 g
Dissolve in, and make up to 1litre with water.	
NaHCO$_3$ (sodium hydrogen carbonate):	2,59 g
Dissolve in, and make up to 1 litre with water.	
KCI (potassium chloride):	0,23 g
Dissolve in, and make up to 11itre with water.	

Reconstituted dilution water

Mix 25 ml of each of the four stock solutions and make up to 1 litre with water.

Aerate until the dissolved oxygen concentration equals the air-saturation value.

The pH should be 7,8 ± 0,2.

If necessary adjust the pH with NaOH (sodium hydroxide) or HCI (hydrochloric acid).

The dilution water so prepared is set aside for about 12 hours and must not be further aerated.

The sum of the Ca and Mg ions in this solution is 2,5 mmol per litre. The ratio of Ca:Mg ions is 4:1 and of Na:K ions is 10:1. The total alkalinity of this solution is 0,8 mmol per litre.

Any deviation in the preparation of the dilution water must not change the composition or properties of the water.

APPENDIX 2: FISH SPECIES RECOMMENDED FOR TESTING

Recommended species	Recommended range of test temperature (°C)	Recommended total length of test animal (cm)
Brachydanio rerio (Teleostei, Cyprinidae) (Hamilton-Buchanan) Zebrab-fish	20 to 24	3,0 ± 0,5
Pimephales promelas (Teleostei, Cyprinidae) (Rafinesque) Fathead minnow	20 to 24	5,0 ± 2,5
Cyprinus carpio (Teleostei, Cyprinidae) (Linneaus 1758) Common carp	20 to 24	6,0 ± 2,0
Oryzias latipes (Teleostei, Poeciliidae) Cyprinodontidae (Tomminck and Schlege 1850) Red killifish	20 to 24	3,0 ± 1,0
Poecilia reticulata (Teleostei, Poeciliidae) (Peters 1859) Guppy	20 to 24	3,0 ± 1,0
Lepomis macrochirus (Teleostei, Centrarchidae) (Rafinesque Linneaus 1758) Bluegill	20 to 24	5,0 ± 2,0
Onchorhynchus mykiss (Teleostei, Salmonidae) (Walbaum 1988) Rainbow trout	12 to 17	6,0 ± 2,0
Leuciscus idus (Teleostei, Cyprinidae) (Linneaus 1758) Golden Orfe	20 to 24	6,0 ± 2,0

Collection

The fish listed above are easy to rear and/or are widely available throughout the year. 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.

APPENDIX 3: EXAMPLE OF CONCENTRATION: PERCENTAGE MORTALITY

Appendix 3

Example of concentration: percentage mortality

Example of determination of LC_{50} using log-probit paper
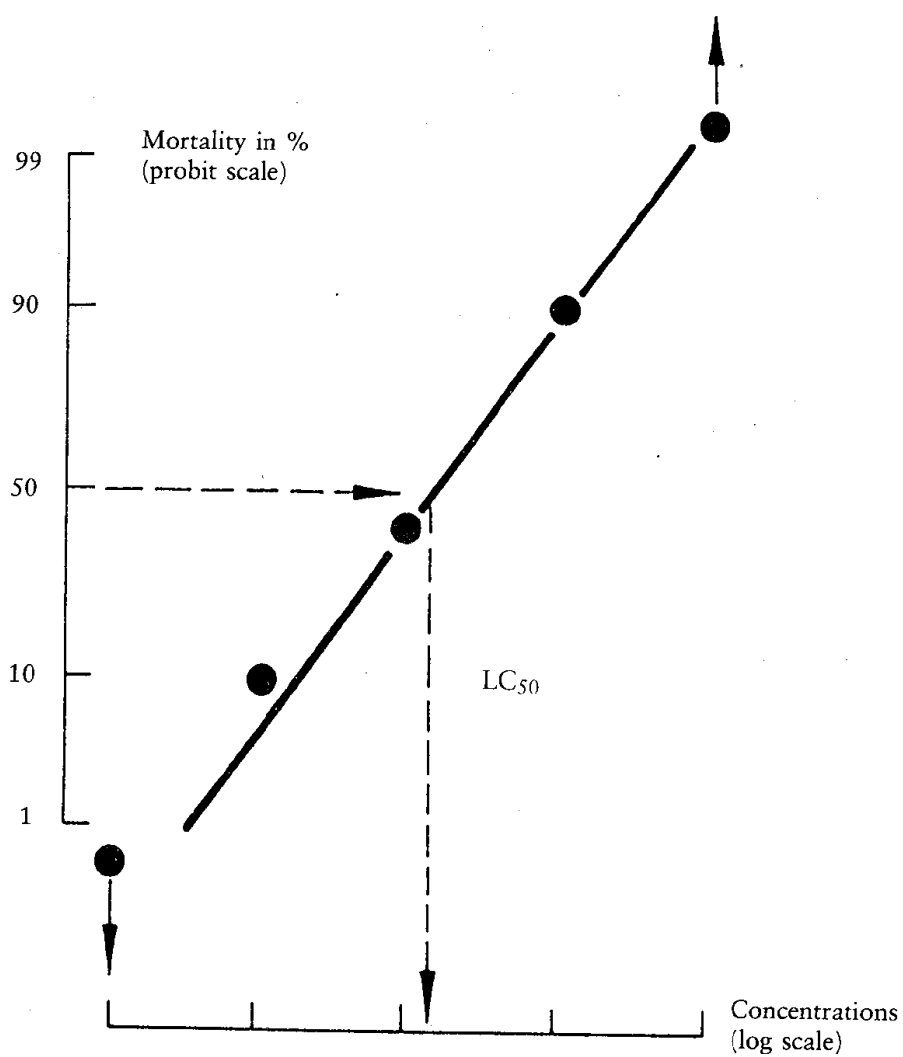
C.2. ACUTE TOXICITY FOR DAPHNIA

1. METHOD

1.1. INTRODUCTION

The purpose of this test is to determine the median effective concentration for immobilization (EC_{50}) of a substance to *Daphnia* in fresh water. It is desirable to have, as far as possible, information on the water solubility, vapour pressure, chemical stability, dissociation constants and biodegradability of the substance before starting the test.

Additional information (for instance structural formula, degree of purity, nature and percentage of significant impurities, presence and amount of additives, and n-octanol/water partition coefficient) should be taken into consideration in both the planning of the test and interpretation of the results.

1.2. DEFINITIONS AND UNITS

The Directive requirement for the LC_{50} for *Daphnia* is considered to be fulfilled by the determination of the EC_{50} as described in this test method.

Acute toxicity is expressed in this test as the median effective concentration (EC_{50}) for immobilization. This is the concentration, in terms of initial values, which immobilizes 50% of the *Daphnia* in a test batch within a continuous period of exposure which must be stated.

Immobilization:

Those animals which are not able to swim within 15 seconds after gentle agitation of the test container are considered to be immobile.

All concentrations of the test substance are given in weight by volume (milligrams per litre). They may also be expressed as weight by weight ($mg.kg^{-1}$).

1.3. REFERENCE SUBSTANCES

A reference substance may be tested as a means of demonstrating that under the laboratory test conditions the sensitivity of the test species has not changed significantly.

The summary of the results of an EEC ring-test, using four different substances, is given in Appendix 2.

1.4. PRINCIPLE OF THE TEST METHOD

A limit test may be performed at 100 mg per litre in order to demonstrate that the EC_{50} is greater than this concentration.

The *Daphnia* are exposed to the test substance added to water at a range of concentrations for 48 hours. If a shorter test is used, justification should be given in the test report.

Under otherwise identical test conditions, and an adequate range of test substance concentrations, different concentrations of a test substance exert different average degrees of effect on the swimming ability of *Daphnia*. Different concentrations result in different percentages of *Daphnia* being no longer capable of swimming at the end of the test. The concentrations causing zero or 100 % immobilization are derived directly from the test observations whereas the 48-hour EC_{50} is determined by calculation if possible.

A static system is used for this method, hence test solutions are not renewed during the exposure period.

1.5. QUALITY CRITERIA

The quality criteria shall apply to the limit test as well as the full test method.

Immobilization in the controls must not exceed 10% at the end of the test.

Test *Daphnia* in the control groups must not have been trapped at the surface of the water.

It is desirable that concentration of dissolved oxygen in the test vessels should remain above 3 mg l^{-1} throughout the course of the test. However, in no circumstances should the dissolved oxygen concentration fall below 2 mg l^{-1}.

The concentration of the test substance shall be maintained to within 80% of the initial concentration throughout the duration of the test.

For substances which dissolve easily in the test medium, yielding stable solutions i.e. those which will not to any significant extent volatilize, degrade, hydrolyze or adsorb, the initial concentration can be taken as being equivalent to the nominal concentration. Evidence shall be presented that the concentrations have been maintained throughout the test and that the quality criteria have been satisfied.

For substances that are:

(i)   poorly soluble in the test medium, or

(ii)   capable of forming stable emulsions or dispersions, or

(iii)   not stable in aqueous solutions,

the initial concentration shall be taken as the concentration measured in solution (or, if technically not possible, measured in the water column) at the start of the test. The concentration shall be determined after a period of equilibration but before the introduction of the test organisms.

In any of these cases, further measurements must be made during the test to confirm the actual exposure concentrations or that the quality criteria have been met.

The pH should not vary by more than 1 unit.

1.6. DESCRIPTION OF TEST METHOD

1.6.1. Reagents

1.6.1.1. Solutions of test substances

Stock solutions of the required strength are prepared by dissolving the substance in deionized water or water according to 1.6.1.2.

The chosen test concentrations are prepared by dilution of the stock solution. If high concentrations are tested, the substance may be dissolved in the dilution water directly.

The substances should normally only be tested up to the limit of solubility. For some substances (e. g. substances having low solubility in water, or high P_{ow}, or those forming stable dispersion rather than true solution in water), it is acceptable to run a test concentration above the solubility limit of the substance to ensure that the maximum soluble/stable concentration has been obtained. It is important, however, that this concentration will not otherwise disturb the test system (e.g. film of the substance on the water surface preventing the oxygenation of the water, etc.).

Ultrasonic dispersion, organic solvents, emulsifiers or dispersants may be used as an aid to prepare stock solutions of substances with low aqueous solubility or to help to disperse these substances in the test medium. When such auxiliary substances are used, all test concentrations should contain the same amount of auxiliary substances, and additional control *Daphnia* should be exposed to the same concentration of the auxiliary substance as that used in the test series. The concentration of such auxiliaries should be minimized, but in no case should exceed 100 mg per litre in the test medium.

The test should be carried out without adjustment of the pH. If there is evidence of marked change in the pH, it is advised that the test should be repeated with pH adjustment and the results reported. In that case, the pH value of the stock solution should be adjusted to the pH value of the solution water unless there are specific reasons not to do so. HCl and NaOH are preferred for this purpose. This pH adjustment should be made in such a way that the concentration of test substance in the stock solution is not changed to any significant extent. Should any chemical reaction or physical precipitation of the test compound be caused by the adjustment, this should be reported.

1.6.1.2. Test water

Reconstituted water is used in this test (see Appendix 1 and reference (2) : ISO 6341). To avoid the necessity for acclimation prior to the test, it is recommended that the culture water should be of similar quality (pH, hardness) as the water used for the test.

1.6.2. Apparatus

Normal laboratory apparatus and equipment should be used. Equipment which will come into contact with the test solutions should preferably be made entirely of glass:

– 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 hardness of water.

1.6.3. Test organism

Daphnia magna is the preferred test species although *Daphnia pulex* is also permitted. The test animals shall be less than 24 hours old, at the beginning of the test, laboratory bred, free from overt disease and with a known history (e.g. breeding -any pretreatments, etc.).

1.6.4. Test procedure

A range-finding test can precede the definitive test, in order to obtain information about the range of concentrations to be used in the main test.

One control without the test substance is run and, if relevant, one control containing the auxiliary substance is also run in addition to the test series.

Daphnia are exposed to the substance as described below:

– duration: preferably 48 hours,

– number of animals: at least 20 animals at each test concentration preferably divided into four batches of five animals each or two batches of 10,

– loading: at least 2 ml of test solutions should be provided for each animal,

– test concentration: the test solution should be prepared immediately before introduction of the *Daphnia*, preferably without using any solvent other than water. The concentrations are made up in a geometric series, at a concentration ratio not exceeding 2.2. Concentrations sufficient to give 0 and 100% immobilization after 48 hours and a range of intermediate degrees of immobilizations permitting calculation of the 48 hour EC_{50} should be tested together with controls,

– water: see 1.6.1.2,

– light: a light-dark cycle is optional,

– temperature: the test temperature should be between 18 and 22 °C, but for each single test it should be constant within ± 1 °C,

– aeration: the test solutions must not be bubble-aerated,

– feeding: none.

The pH and the oxygen concentration of the controls and of all the test concentrations should be measured at the end of the test; the pH of the test solutions should not be modified.

Volatile compounds should be tested in completely filled closed containers, large enough to prevent lack of oxygen.

Daphnia are inspected at least after 24 hours exposure and again after 48 hours.

Limit test

Using the procedures described in this method, a limit test may be performed at 100 mg per litre in order to demonstrate that the EC_{50} is greater than this concentration.

If the nature of the substance is such that a concentration of 100 mg per litre in the test water cannot be attained, the limit test should be performed at a concentration equal to the solubility of the substance (or the maximum concentration forming a stable dispersion) in the medium used (see also point 1.6.1.1).

The limit test should be performed using 20 *Daphnia,* divided in two or four batches, with the same number in the control(s). If immobilisations occur, a full study must be carried out.

2.   DATA AND EVALUATION

For each period where observations were recorded (24 and 48 h), the percentage mortality is plotted against concentration on logarithmic-probability paper.

When possible and for each observation time, the EC_{50} and the confidence limits (p = 0,05) should be estimated using standard procedures; these values should be rounded off to one, or at most two significant figures (examples of rounding off to two figures: 170 for 173,5; 0,13 for 0,127; 1,2 for 1,21).

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

When two immediately consecutive concentrations at a ratio of 2,2 give only 0 and 100% immobilization these two values are sufficient to indicate the range within which the EC_{50} falls.

If it is observed that the stability or homogeneity of the test substance cannot be maintained, this should be reported and care taken in the interpretation of the results.

3.   REPORTING

The test report shall, if possible, include the following information:

–   information about the test organism (scientific name, strain, supplier or source, any pretreatment, breeding method -including source, kind and amount of food, feeding frequency);

–   dilution water source and major chemical characteristics (i. e. pH, temperature, hardness);

–   in the case of substance of low aqueous solubility, the method of preparation of stock and test solution;

–   concentration of any auxiliary substances;

–   list of the concentrations used and any available information on the stability at the concentrations of the tested chemical in the test solutions;

–   if chemical analyses are performed, methods used and results obtained;

– results of the limit test, if conducted;

– description of test equipment;

– lighting regime;

– dissolved oxygen concentrations, pH values and temperatures of the test solutions;

– evidence that the quality criteria have been fulfilled;

– a table showing the cumulative immobilisation at each concentration and the control (and control with the auxiliary substance if required) at each of the recommended observation times (24 and 48 h);

– graph of the concentration/percentage response curve at the end of the test;

– if possible, the EC_{50} values at each of the recommended observation times (with 95% confidence limits);

– statistical procedures used for determining the EC_{50} values;

– if a reference substance is used, the results obtained;

– highest tested concentration causing no immobilization within the period of the test;

– lowest tested concentration causing 100% immobilization within the period of the test.

4. REFERENCES

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APPENDIX 1: RECONSTITUTED WATER

Example of a suitable dilution water (according to ISO 6341)

All chemicals must be of analytical grade.

The water should be good-quality distilled water, or deionized water with a conductivity less than 5 μScm^{-1}.

The apparatus for distillation of water must not contain any parts made of copper.

Stock solutions

$CaCl_2.2 H_2O$ (calcium chloride dihydrate):   11,76 g
dissolve in, and make up to 1 litre with water

$MgSO_4.7H_2O$ (magnesium sulphate heptahydrate):   4,93 g
dissolve in, and make up to 1 litre with water

$NaHCO_3$ (sodium hydrogen carbonate):   2,59 g
dissolve in, and make up to 1 litre with water

KCI (potassium chloride):   0,23 g
dissolve in, and make up to 1 litre with water

Reconstituted dilution water

Mix 25 ml of each of the four stock solutions and make up to 1litre with water.

Aerate until the dissolved oxygen concentrarion equals the air-saturation value.

The pH should be 7,8 ± 0,2.

If necessary adjust the pH with NaOH (sodium hydroxide) or HCI (hydrochloric acid).

The dilution water so prepared is set aside for about 12 hours and need not be further aerated.

The sum of the Ca and Mg ions in this solution is 2,5 mmol per litre. The ratio of Ca:Mg ions is 4:1 and of Na:K ions is 10:1. The total alkalinity of this solution is 0,8 mmol per litre.

Any deviation in the preparation of the dilution water must not change the composition or properties of the water.

APPENDIX 2

Summary of the results of an EEC ring-test performed in 1978 (also cited in reference 2)

Caution: the purpose of this ring-test was the determination of the EC_{50} 24 hours.

Substances used:

1)   Potassium dichromate

2)   Tetrapropylbenzenesulphonic acid

3)   Tetrapropylbenzenesulphonic acid, sodium salt

4)   Trichloro-2,4,5-phenoxyacetic acid, potassium salt

Substance	Number of participating laboratories	Number of results of calculation	EC_{50}-24 h mg/l mean
1	46	129	1,5
2	36	108	27
3	31	84	27
4	32	72	770

APPENDIX 3: EXAMPLE OF CONCENTRATION: PERCENTAGE IMMOBILISATION

Appendix 3

Example of concentration: percentage immobilisation

Example of determination of EC_{50} using log-probit paper
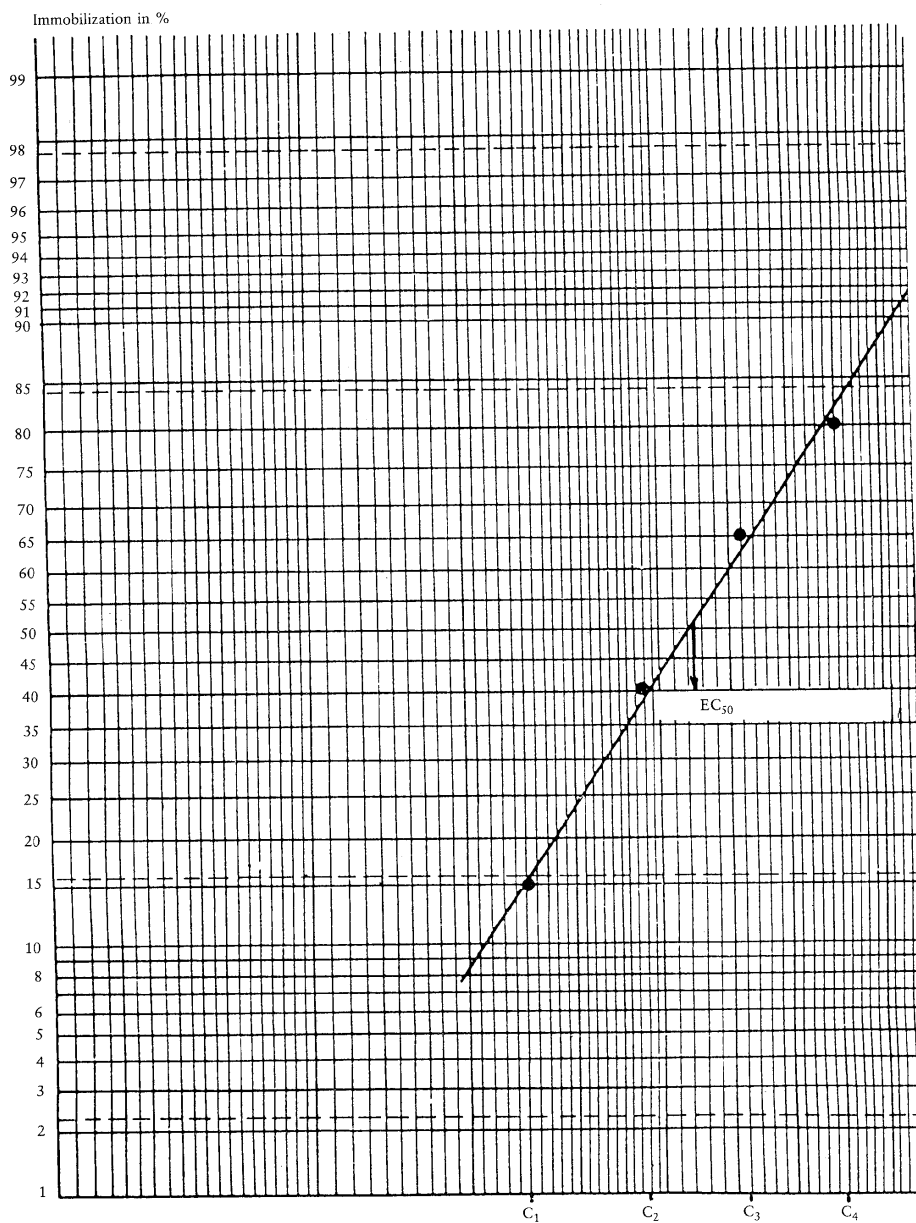
C.3. ALGAL INHIBITION TEST

1. METHOD

1.1. INTRODUCTION

The purpose of this test is to determine the effects of a substance on the growth of a unicellular green algal species. Relatively brief (72 hours) tests can assess effects over several generations. This method can be adapted for use with several unicellular algal species, in which case a description of the method used must be provided with the test report.

This method is most easily applied to water-soluble substances which, under the conditions of the test, are likely to remain in the water.

The method can be used for substances that do not interfere directly with the measurement of algal growth.

It is desirable to have, as far as possible, information on the water solubility, vapour pressure, chemical stability, dissociation constants and biodegradability of the substance before starting the test.

Additional information (for instance structural formula, degree of purity, nature and percentage of significant impurities, presence and amount of additives, and n-octanol/water partition coefficient) should be taken into consideration in both the planning of the test and interpretation of the results.

1.2. DEFINITIONS AND UNITS

Cell density: the number of cells per millilitre;

Growth: the increase in cell density over the test period;

Growth rate: the increase in cell density per unit time;

EC_{50}: in this method, that concentration of test substance which results in a 50 % reduction in either growth (E_bC_{50}) or growth rate (E_rC_{50}) relative to the control;

NOEC (no observed effect concentration): in this method, the highest tested concentration at which no significant inhibition of growth is observed relative to the control.

All concentrations of the test substance are given in weight by volume (milligrams per litre). They may also be expressed as weight by weight (mg.kg^{-1}).

1.3 REFERENCE SUBSTANCES

A reference substance may be tested as a means of demonstrating that under the laboratory test conditions the sensitivity of the test species has not changed significantly.

If a reference substance is used, the results should be given in the test report. Potassium dichromate can be used as a reference substance, but its colour may affect the light quality and intensity available to the cells and also the spectrophotometric determinations if used. Potassium dichromate has been used in an international inter-laboratory test (see ref. (3) and Appendix 2).

1.4. PRINCIPLE OF THE TEST METHOD

A limit test may be performed at 100 mg per litre in order to demonstrate that the EC_{50} is greater than this concentration.

Exponentially-growing cultures of selected green algae are exposed to various concentrations of the test substance over several generations under defined conditions.

The test solutions are incubated for a period of 72 hours, during which the cell density in each solution is measured at least every 24 hours. The inhibition of growth in relation to a control culture is determined.

1.5. QUALITY CRITERIA

The quality criteria shall apply to the limit test as well as the full test method.

The cell density in the control cultures should have increased by a factor of at least 16 within three days.

The concentrations of the test substance shall be maintained to within 80 % of the initial concentrations/ throughout a time corresponding to the duration of the test.

For substances which dissolve easily in the test medium, yielding stable solutions i.e. those which will not to any significant extent volatilize, degrade, hydrolyze or adsorb, the initial concentration can be taken as being equivalent to the nominal concentration. Evidence shall be presented that the concentrations have been maintained throughout the test and that the quality criteria have been satisfied.

For substances that are:

(i)   poorly soluble in the test medium, or

(ii)   capable of forming stable emulsions or dispersions, or

(iii)   not stable in aqueous solutions,

the initial concentration shall be taken as the concentration measured at the start of the test. The concentration shall be determined after a period of equilibration.

In any of these cases, further measurements must be made during the test to confirm the actual exposure concentrations or that the quality criteria have been met.

It is recognized that significant amounts of the test substance may be incorporated into the algal biomass during the period of the test. Therefore, for the purpose of demonstrating compliance with the above quality criteria, both the amount of the substance incorporated into the algal biomass and the substance in solution (or, if not technically possible measured in the

water column) should be taken into account. However, as determination of the substance concentration in the algal biomass may pose significant technical problems, compliance with the quality criteria may be demonstrated by running a test vessel at the highest substance concentration but without algae and measuring concentrations in solution (or, if not technically possible in the water column) at the beginning and at the end of the test period.

1.6. DESCRIPTION OF THE TEST PROCEDURE

1.6.1. Reagents

1.6.1.1. Solutions of test substances

Stock solutions of the required strength are prepared by dissolving the substance in deionized water or water according to 1.6.1.2.

The chosen test concentrations are prepared by adding suitable aliquots to algal pre-cultures (see Appendix 1). Substances should normally only be tested up to the limit of solubility. For some substances (e.g. substances having low solubility in water, or high P_{ow} or those forming stable dispersion rather than true solution in water), it is acceptable to run a test concentration above the solubility limit of the substance to ensure that the maximum soluble stable concentration has been obtained. It is important, however, that this concentration will not otherwise disturb the test system (e.g. film of the substance on the water surface preventing the oxygenation of the water, etc.).

Ultrasonic dispersion, organic solvents, emulsifiers or dispersants may be used as an aid to prepare stock solutions of substances with low aqueous solubility or to help to disperse these substances in the test medium. When such auxiliary substances are used, all test concentrations should contain the same amount of auxiliary substances, and additional controls should be exposed to the same concentration of the auxiliary substance as that used in the test series. The concentration of such auxiliaries should be minimized, but in no case should exceed 100 mg per litre in the test medium.

The test should be carried out without adjustment of the pH. If there is evidence of marked change in the pH, it is advised that the test should be repeated with pH adjustment and the results reported. In that case, the pH value of the stock solution should be adjusted to the pH value of the solution water unless there are specific reasons not to do so. HCl and NaOH are preferred for this purpose. This pH adjustment should be made in such a way that the concentration of test substance in the stock solution is not changed to any significant extent. Should any chemical reaction or physical precipitation of the test compound be caused by the adjustment, this should be reported.

1.6.1.2. Test medium

The water should be good-quality distilled water, or deionized water with a conductivity less than 5 μS.cm^{-1}. The apparatus for distillation of water must not contain any part made of copper.

The following medium is recommended.

Four stock solutions are prepared, according to the following table. The stock solutions are sterilised by membrane filtration or by autoclaving, and stored in the dark at 4 °C. Stock

solution no.4 should be sterilised only by membrane filtration. These stock solutions are diluted to achieve the final nutrient concentrations in the test solutions.

Nutrient	Concentration in the stock solution	Final concentration in the test solution
Stock solution 1: macro-nutrients		
NH_4Cl	1,5 g/l	15 mg/l
$MgCl_2.6H_2O$	1,2 g/l	12mg/l
$CaCl_2.2H_2O$	1,8 g/l	18 mg/l
$MgSO_4.7H_2O$	1,5 g/l	15 mg/l
KH_2PO_4	0,16 g/l	1,6 mg/l
Stock solution 2: Fe-EDTA		
$FeCl_3.6H_2O$	80 mg/l	0,08 mg/l
$Na_2EDTA.2H_2O$	100 mg/l	0,1 mg/l
Stock solution 3: trace elements		
H_3BO_3	185 mg/l	0,185 mg/l
$MnCl_2.4H_2O$	415 mg/l	0,415 mg/l
$ZnCl_2$	3 mg/l	3×10^{-3} mg/l
$CoCl_2.6H_2O$	1,5 mg/l	$1,5 \times 10^{-3}$ mg/l
$CuCl_2.2H_2O$	0,01 mg/l	10^{-5} mg/l
$Na_2MoO_4.2H_2O$	7 mg/l	7×10^{-3} mg/l
Stock solution 4: $NaHCO_3$		
$NaHCO_3$	50 g/l	50 mg/l

The pH of the medium after equilibration with air is approximately 8.

1.6.2. Apparatus

– Normal laboratory equipment,

– Test flasks of suitable volume (e.g. 250 ml conical flasks are suitable when the volume of the test solution is 100 ml). All test flasks should be identical as regards to material and dimensions.

- Culturing apparatus: cabinet or chamber in which a temperature in the range 21 °C to 25 °C can be maintained at ± 2 °C, and continuous uniform illumination provided in the spectral range 400 to 700 nm. If algae in control cultures have achieved the recommended growth rates, it can be assumed that the conditions for growth, including light intensity, have been adequate.

  It is recommended to use, at the average level of the test solutions, a light intensity in the range 60 to 120 $\mu E.m^{-2}.s^{-1}$ (35 to 70 x 10^{18} photons.m^{-2}.s^{-1}) when measured in the range 400 to 700 nm using an appropriate receptor. For light measuring instruments calibrated in lux, an equivalent range of 6000 to 10000 lx is acceptable.

  The light intensity could be obtained using four to seven 30 W fluorescent lamps of the universal white type (colour temperature of approximately 4300 K), at a distance of 0,35 m from the algal culture.

- Cell density measurements should be made using a direct counting method of living cells, e.g. a microscope with counting chambers. However, other procedures (photometry, turbidimetry,...) may be used if sufficiently sensitive and if shown to be sufficiently well correlated with cell density .

1.6.3. Test organisms

It is suggested that the species of green algae used be a fast-growing species that is convenient for culturing and testing. The following species are preferred:

- *Selenastrum capricornutum*, e.g. ATCC 22662 or CCAP 278/4,

- *Scenedesmus subspicatus*, e.g. 86.81 SAG,

Note:

ATCC = American Type Culture Collection (U.S.A.)

CCAP = Culture Centre of Algae and Protozoa (U.K.)

SAG = Collection of algal culture (Gottingen, F.R.G.)

If other species are used, the strain should be reported.

1.6.4. Test procedure

The concentration range in which effects are likely to occur is determined on the basis of results from range-finding tests.

The two measures of growth (biomass and growth rate) may result in widely disparate measures of growth inhibition; both should be used in the range finding test to ensure that the geometric progression of concentrations will allow estimation of both the E_bC_{50} and the E_rC_{50}.

Initial cell density

It is recommended that the initial cell density in the test cultures be approximately 10^4 cells/ml for *Selenastrum capricornutum* and *Scenedesmus subspicatus*. When other species are used the biomass should be comparable.

Concentrations of test substance

For the test, at least five concentrations are made up in a geometric series at a concentration ratio not exceeding 2,2. The lowest concentration tested should have no observed effect on the growth of the algae. The highest concentration tested should inhibit growth by at least 50 % relative to the control and, preferably, stop growth completely.

Replicates and controls

The test design should include three replicates at each test concentration. Three controls without test substance are run and, if relevant, three controls containing the auxiliary substance are also run. If justified, the test design may be altered to increase the number of concentrations and reduce the number of replicates per concentration.

Performance of the test

Test cultures containing the desired concentrations of test substance and the desired quantity of algal inoculum are prepared by adding aliquots of stock solutions of the test substance to suitable amounts of algal pre-cultures (see Appendix 1).

The culture flasks are shaken and placed in the culturing apparatus. The algal cells are kept in suspension by shaking, stirring or bubbling with air, in order to improve gas exchange and reduce pH variation in the test solutions. The cultures should be maintained at a temperature in the range of 21 to 25 °C, controlled at ± 2 °C.

The cell density in each flask is determined at least at 24, 48 and 72 hours after the start of the test. Filtered algal medium containing the appropriate concentration of the test chemical is used to determine the background when using cell density measurements other than direct counting methods.

The pH is measured at the beginning of the test and at 72 hours.

The pH of the controls should not normally deviate by more than 1,5 units during the test.

Testing volatile substances

There is to date no generally accepted way to test volatile substances. When a substance is known to have a tendency to vaporize, closed test flasks with increased head-space may be used. The possibility of shortage of CO_2 should be considered when calculating the head-space of the closed flasks. Variations to this method have been proposed (see reference (4)).

Attempts should be made to determine the amount of the substance which remains in solution, and extreme caution is advised when interpreting results of tests with volatile chemicals using closed systems.

Limit test

Using the procedures described in this method, a limit test may be performed at 100 mg per litre in order to demonstrate that the EC_{50} is greater than this concentration.

If the nature of the substance is such that a concentration of 100 mg per litre in the test water cannot be attained, the limit test should be performed at a concentration equal to the solubility

of the substance (or the maximum concentration forming a stable dispersion) in the medium used (see also point 1.6.1.1).

The limit test should be performed at least in triplicate, with the same number of controls. The two measures of growth (biomass and growth rate) should be used for the limit test.

If, in a limit test, a mean decrease of 25 % or more is found in either biomass or growth rate between the limit test and the control, a full test should be carried out.

2. DATA AND EVALUATION

The measured cell density in the test cultures and controls are tabulated together with the concentrations of the test substance and the times of measurements. The mean value of the cell density for each test substance concentration and for the controls is plotted against time (0-72 h) to produce growth curves.

To determine the concentration/ effect relationship, the two following approaches should be used. Some substances can stimulate the growth at low concentrations. Only data points indicating inhibition between 0 and 100 % should be considered.

2.1. COMPARISON OF AREAS UNDER THE GROWTH CURVES

The area between the growth curves and the horizontal line $N = N_0$ may be calculated according to the formula:

$$A = (N_1 - N_0) / 2 \times t_1 + (N_1 + N_2 - 2N_0) / 2 \times (t_2 - t_1) + ... + (N_{n-1} + N_n - 2N_0) / 2 (t_n - t_{n-1})$$

Where

A = area,

N_o = number of cells/ml at time t_0 (beginning of the test),o,

N_1 = measured number of cells/ml at t_1,

N_n = measured number of cells/ml at time,

t_1 = time of first measurement after beginning of test,

t_n = time of n_{th} measurement after beginning of test.

n = number of measurements taken after the beginning of the test.

The percentage inhibition of the cell growth at each test substance concentration (I_A) is calculated according to the formula:

$$I_A = (A_c - A_t) / A_c \times 100$$

Where

A_c = area between the control growth curve and the horizontal line $N = N_o$.

A_t = area between the growth curve at the concentration t and the horizontal line N = N_o.

I_A = values are plotted on semilogarithmic paper or on semilogarithmic probit paper against the corresponding concentrations. If plotted on probit paper, the points are fitted by a straight line, either by eye or by a computed regression.

The EC_{50} is estimated from the regression line by reading off the concentration that is equivalent to a 50 % inhibition (I_A = 50%). To denote this value unambiguously in relation to this method of calculation, it is proposed to use the symbol E_bC_{50}. It is essential that the E_bC_{50} is quoted with the appropriate exposure period, e.g. E_bC_{50} (0-72h).

2.2. COMPARISON OF GROWTH RATES

The average specific growth rate (μ) for exponentially growing cultures can be calculated as

$$\mu = (\ln N_n - \ln N_0) / (t_n - t_0)$$

where t_o is the time at the beginning of the test.

Alternatively, the average specific growth rate may be derived from the slope of the regression line in a plot of ln N *versus* time.

The percentage inhibition of specific growth rate at each test substance concentration ($I_\mu t$) is calculated according to the formula:

$$I_\mu t = (\mu_c - \mu_t) / \mu_c \times 100$$

Where

μ_c = mean control specific growth rate

μ_t = mean specific growth rate for the test concentration t

The percentage reduction in average specific growth rate at each test substance concentration compared to the control value is plotted against the logarithm of the concentration. The EC_{5o} may be read from the resulting graph. To denote unambiguously the EC_{50} derived by this method it is proposed to use the symbol E_rC_{50}. The times of measurement must be indicated, e.g. if the value relates to times 0 and 72 hours, the symbol becomes E_rC_{50} (0-72h).

Note: specific growth rate is a logarithmic term, and small changes in growth rate may lead to great changes in biomass. E_bC and E_rC values are therefore not numerically comparable.

2.3. CALCULATION OF THE NOEC

The No Observed Effect Concentration is determined by a suitable statistical procedure for multisample comparison (e.g. analysis of variance and Dunnett's test), using the individual replicates values of the areas under the growth curves A (see point 2.1) or the specific growth rates μ (see point 2.2).

3.   REPORTING

The test report shall, if possible, include the following information:

–   test substance: chemical identification data;

–   test organisms: origin, laboratory culture, strain number, method of cultivation;

–   test conditions:

  –   date of the start and the end of the test and its duration,

  –   temperature,

  –   composition of medium,

  –   culturing apparatus,

  –   pH of solutions at the start and end of the test (an explanation should be provided if pH deviations of more than 1,5 unit are observed),

  –   vehicle and method used for solubilizing the test substance and concentration of the vehicle in the test solutions,

  –   light intensity and quality,

  –   concentrations tested (measured or nominal).

–   results:

  –   cell density for each flask at each measuring point and method for measuring cell density,

  –   mean values of cell density,

  –   growth curves,

  –   graphical presentation of the concentration effect relationship,

  –   EC values and method of calculation,

  –   NOEC,

  –   other observed effects.

4.   REFERENCES

1)   OECD, Paris, 1981, Test Guideline 201, Decision of the Council C(81) 30 Final.

2)   Umweltbundesamt, Berlin, 1984, Verfahrensvorschlag 'Hemmung der Zellvermehrung bei der Grünalge Scenedesmus subspicatus', in: Rudolph/Boje: bkotoxikologie, ecomed, Landsberg, 1986.

3)   ISO 8692- Water quality -Fresh water algal growth inhibition test with *Scenedesmus subspicatus* and *Selenastrum capricornutum.*

4)   S.Galassi and M.Vighi -Chemosphere, 1981, vol.10, 1123-1126.

APPENDIX 1: EXAMPLE OF A PROCEDURE FOR THE CULTURING OF ALGAE

General observations

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

Suitable methods should be used to ensure that the algal cultures are not infected with bacteria (ISO 4833). Axenic cultures may be desirable but unialgal cultures are essential.

All operations should be carried out under sterile conditions in order to avoid contamination with bacteria and, other algae. Contaminated cultures should be rejected.

Procedures for obtaining algal cultures

Preparation of nutrient solutions (media):

The medium can be prepared by diluting concentrated stock solutions of nutrients. For solid medium, 0,8 % of agar is added. The medium used should be sterile. Sterilisation by autoclaving may lead to a loss of NH_3.

Stock culture:

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

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

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

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

Pre-culture:

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

APPENDIX 2

The ISO 8692 -Water quality -Fresh water algal growth inhibition test with *Scenedesmus subspicatus* and *Selenastrum capricornutum* reports the following results for an inter-laboratory test among 16 laboratories, testing potassium dichromate:

	Means (mg/l)	Range (mg/l)
E_rC_{50} (0 - 72h)	0,84	0,60 to 1,03
E_bC_{50} (0 - 72 h)	0,53	0,20 to 0,75

C.4. DETERMINATION OF 'READY' BIODEGRADABILITY

PART I. GENERAL CONSIDERATIONS

I.1.   INTRODUCTION

Six test methods are described that permit the screening of chemicals for ready biodegradability in an aerobic aqueous medium:

(a)   Dissolved Organic Carbon (DOC) Die-Away (Method C.4-A)

(b)   Modified OECD Screening -DOC Die-Away (Method C.4-B)

(c)   Carbon dioxide (CO_2) Evolution (Modified Sturm Test) (Method C.4-C)

(d)   Manometric Respirometry (Method C.4-D)

(e)   Closed Bottle (Method C.4-E)

(f)   MITI (Ministry of International Trade and Industry -Japan) (Method C.4-F)

General and common considerations to all six tests are given in Part I of the method. Items specific for individual methods are given in Parts II to VII. The annexes contain definitions, formulas and guidance material.

An OECD inter-laboratory comparison exercise, done in 1988, has shown that the methods give consistent results. However, depending on the physical characteristics of the substance to be tested, one or other of the methods may be preferred.

I.2.   SELECTION OF THE APPROPRIATE METHOD

In order to select the most appropriate method, information on the chemical's solubility, vapour pressure and adsorption characteristics is essential. The chemical structure or formula should be known in order to calculate theoretical values and/or check measured values of parameters, e.g. ThOD, $ThCO_2$, DOC, TOC, COD (see Annexes I and II).

Test chemicals which are soluble in water to at least 100 mg/l may be assessed by all methods, provided they are non-volatile and non-adsorbing. For those chemicals which are poorly soluble in water, volatile or adsorbing, suitable methods are indicated in Table 1. The manner in which poorly water-soluble chemicals and volatile chemicals can be dealt with is described in Annex III. Moderately volatile chemicals may be tested by the DOC Die-Away method if there is sufficient gas space in the test vessels (which should be suitably stoppered). In this case, an abiotic control must be set up to allow for any physical loss.

Table 1: Applicability of test methods

Test	Analytical Method	Suitability for substances which are:		
		poorly soluble	volatile	adsorbing
DOC Die-Away	Dissolved organic carbon	—	—	+ / −
Mod. OECD Die-Away	Dissolved organic carbon	—	—	+ / −
CO_2 Evolution	Respirometry: CO_2 evolution	+	—	+
Manometric Respirometry	Manometric respirometry: oxygen consumption	+	+ / −	+
Closed Bottle	Respirometry: dissolved oxygen	+ / −	+	+
MITI	Respirometry: oxygen consumption	+	+ / −	+

Information on the purity or the relative proportions of major components of the test material is required to interpret the results obtained, especially when the results are low or marginal.

Information on the toxicity of the test chemical to bacteria (Annex IV) may be very useful for selecting appropriate test concentrations and may be essential for the correct interpretation of low biodegradation values.

I.3. REFERENCE SUBSTANCES

In order to check the procedure, reference chemicals which meet the criteria for ready biodegradability are tested by setting up an appropriate flask in parallel to the normal test runs.

Suitable chemicals are aniline (freshly distilled), sodium acetate and sodium benzoate. These reference chemicals all degrade in these methods even when no inoculum is deliberately added.

It was suggested that a reference chemical should be sought which was readily biodegradable but required the addition of an inoculum. Potassium hydrogen phthalate has been proposed but more evidence needs to be obtained with this substance before it can be accepted as a reference substance.

In the respirometric tests, nitrogen-containing compounds may affect the oxygen uptake because of nitrification (see Annexes II and V).

I.4. PRINCIPLE OF THE TEST METHODS

A solution, or suspension, of the test substance in a mineral medium is inoculated and incubated under aerobic conditions in the dark or in diffuse light. The amount of DOC in the test solution due to the inoculum should be kept as low as possible compared to the amount of DOC due to the test substance. Allowance is made for the endogenous activity of the inoculum by running parallel blank tests with inoculum but without test substance, although the endogenous activity of cells in the presence of the substance will not exactly match that in the endogenous control. A reference substance is run in parallel to check the operation of the procedures.

In general, degradation is followed by the determination of parameters, such as DOC, CO_2 production and oxygen uptake, and measurements are taken at sufficiently frequent intervals to allow the identification of the beginning and end of biodegradation. With automatic respirometers the measurement is continuous. DOC is sometimes measured in addition to another parameter but this is usually done only at the beginning and the end of the test. Specific chemical analysis can also be used to assess primary degradation of the test substance, and to determine the concentration of any intermediate substances formed (obligatory in the MITI test).

Normally, the test lasts for 28 days. Tests however may be ended before 28 days, i.e. as soon as the biodegradation curve has reached a plateau for at least 3 determinations. Tests may also be prolonged beyond 28 days when the curve shows that biodegradation has started but that the plateau has not been reached day 28.

I.5. QUALITY CRITERIA

I.5.1. Reproducibility

Because of the nature of biodegradation and of the mixed bacterial populations used as inocula, determinations should be carried out at least in duplicate.

It is common experience that the larger the concentration of micro-organisms initially added to the test medium, the smaller will be the variation between replicates. Ring tests have also shown that there can be large variations between results obtained by different laboratories, but good agreement is normally obtained with easily biodegradable compounds.

I.5.2. Validity of the test

A test is considered valid if the difference of extremes of replicate values of the removal of test chemical at the plateau, at the end of the test or at the end of the 10-day window, as appropriate, is less than 20% and if the percentage degradation of the reference substance has reached the level for ready biodegradability by 14 days. If either of these conditions is not met, the test should be repeated. Because of the stringency of the methods, low values do not necessarily mean that the test substance is not biodegradable under environmental conditions, but indicates that more work will be necessary to establish biodegradability.

If in a toxicity test, containing both the test substance and a reference chemical, less than 35% degradation (based on DOC) or less than 25 % (based on ThOD or $ThCO_2$) occurred in 14 days, the test chemicals can be assumed to be inhibitory (see also Annex IV). The test series should be repeated, if possible using a lower concentration of test chemical and/or a higher concentration of inoculum, but not greater than 30 mg solids/litre.

I.6. GENERAL PROCEDURES AND PREPARATIONS

General conditions applying to the tests are summarised in Table 2. Apparatus and other experimental conditions pertaining specifically to an individual test are described later under the heading for that test.

Table 2: Test conditions

Test	DOC Die-Away	CO_2Evolution	Manometric Respirometry	Modified OECD Screeing	Closed Bottle	MITI (I)
Concentration of Test Substance as mg/l mg DOC/l mg ThOD/l	10-40	10-20	100 50-100	10-40	2-10 5-10	100
Concentration of Inoculum (in cells/l, approximatively)	$\leq$ 30 mg/l SS or $\leq$ 100 ml effluent/l ($10^7 - 10^8$)			0,5 ml secondary effluent/l (10^5)	$\leq$ 5 ml of effluent/l ($10^4 - 10^6$)	30 mg/l SS ($10^7 - 10^8$)
Concentration of elements in mineral medium (in mg/l) P N Na K Mg Ca Fe	116 1,3 86 122 2,2 9,9 0,05-0,1				11,6 0,13 8,6 12,2 2,2 9,9 0,05-0,1	29 1,3 17,2 36,5 6,6 29,7 0,15
pH	7,4 $\pm$ 0,2					preferably 7,0
Temperature	22 $\pm$ 2 °C					25 $\pm$ 1 °C

DOC = Dissolved organic Carbon   ThOD =Theoretical Oxygen Demand   SS = Suspended Solids

I.6.1. Dilution water

Deionized or distilled water, free from inhibitory concentrations of toxic substances (e.g. Cu^{++} ions) is used. It must contain no more than 10% of the organic carbon content introduced by the test material. The high purity of the test water is necessary to eliminate high blank values. Contamination may result from inherent impurities and also from the ion-exchange resins and lysed material from bacterial and algae. For each series of tests use only one batch of water, checked beforehand by DOC analysis. Such a check is not necessary for the closed bottle test, but the oxygen consumption of the water must be low.

I.6.2. Stock solutions of mineral components

To make up the test solutions, stock solutions of appropriate concentrations of mineral components are made up. The following stock solutions may be used (with different dilution factors) for the methods DOC Die-Away, Modified OECD Screening, CO_2 Evolution, Manometric Respirometry, Closed Bottle test.

The dilution factors and, for the MITI test, the specific preparation of the mineral medium are given under the headings of the specific tests.

Stock solutions:

Prepare the following stock solutions, using analytical grade reagents.

(a) Monopotassium dihydrogen orthophosphate, KH_2PO_4 8,50 g
     Dipotassium monohydrogen orthophosphate, K_2HPO_4 21,75 g
     Disodium monohydrogen orthophosphate dihydrate $Na_2HPO_4. 2 H_2O$ 33,40 g
     Ammonium chloride, NH_4Cl 0,50 g

     Dissolve in water and make up to 1 litre The pH of the solution should be 7,4.

(b) Calcium chloride, anhydrous, $CaCl_2$ 27,50 g
     or Calcium chloride dihydrate, $CaCl_2, 2 H_2O$ 36,40 g

     Dissolve in water and make up to 1 litre

(c) Magnesium sulphate heptahydrate, $MgSO_4. 7 H_2O$ 22,50 g

     Dissolve in water and make up to 1 litre.

(d) Iron (III) chloride hexahydrate, $FeCl_3. 6H_2O$ 0,25 g

     Dissolve in water and make up to 1 litre.

Note: in order to avoid having to prepare this solution immediately before use add one drop of conc. HCL or 0,4 g ethylenediaminetetra-acetic acid disodium salt (EDTA) par litre.

I.6.3. Stock solutions of chemicals

For example, dissolve 1-10 g, as appropriate, of test or reference chemical in deionized water and make up to 1 litre when the solubility exceeds 1 g/l. Otherwise, prepare stock solutions in the mineral medium or add the chemical direct to the mineral medium. For the handling of less soluble chemicals, see Annex III, but in the MITI test (Method C.4-F), neither solvents nor emulsifying agents are to be used.

I.6.4. Inocula

The inoculum may be derived from a variety of sources: activated sludge, sewage effluents (unchlorinated), surface waters and soils or from a mixture of these. For the DOC Die-Away, CO_2 Evolution and Manometric Respirometry tests, if activated sludge is used, it should be taken from a treatment plant or laboratory-scale unit receiving predominantly domestic sewage. Inocula from other sources have been found to give higher scattering of results. For the Modified OECD Screening and the Closed Bottle tests a more dilute inoculum without sludge fiocs is needed and the preferred source is a secondary effluent from a domestic waste water treatment plant or laboratory-scale unit. For the MITI test the inoculum is derived from a mixture of sources and is described under the heading of this specific test.

I.6.4.1. Inoculum from activated sludges

Collect a sample of activated sludge freshly from the aeration tank of a sewage treatment plant or laboratory-scale unit treating predominantly domestic sewage. Remove coarse particles if necessary by filtration through a fine sieve and keep the sludge aerobic thereafter.

Alternatively, settle or centrifuge (e.g. at 1 l00 g for 10 min.) after removal of any coarse particles. Discard the supernatant. The sludge may be washed in the mineral medium. Suspend the concentrated sludge in mineral medium to yield a concentration of 3-5 g suspended solids/l and aerate until required.

Sludge should be taken from a properly working conventional plant. If sludge has to be taken from a high rate treatment plant, or is thought to contain inhibitors, it should be washed. Settle or centrifuge the re-suspended sludge after thorough mixing, discard the supernatant and again re-suspend the washed sludge in a further volume of mineral medium. Repeat this procedure until the sludge is considered to be free from excess substrate or inhibitor.

After complete re-suspension is achieved, or with untreated sludge, withdraw a sample just before use for the determination of the dry weight of the suspended solids.

A further alternative is to homogenise activated sludge (3-5 g suspended solids/l). Treat the sludge in a mechanical blender for 2 min. at medium speed. Settle the blended sludge for 30 min. or longer if required and decant liquid for use as inoculum at the rate of 10 mill of mineral medium.

I.6.4.2. Other sources of inoculum

It can be derived from the secondary effluent of a treatment plant or laboratory-scale unit receiving predominantly domestic sewage. Collect a fresh sample and keep it aerobic during

transport. Allow to settle for 1 h. or filter through a coarse filter paper and keep the decanted effluent or filtrate aerobic until required. Up to 100 ml of this type of inoculum may be used per litre of medium.

A further source for the inoculum is surface water. In this case, collect a sample of an appropriate surface water, e.g. river, lake, and keep aerobic until required. If necessary, concentrate the inoculum by filtration or centrifugation.

I.6.5.   Pre-conditioning of inocula

Inocula may be pre-conditioned to the experimental conditions, but not pre-adapted to the test chemical. Pre-conditioning consists of aerating activated sludge in mineral medium or secondary effluent for 5-7 days at the test temperature. Pre-conditioning sometimes improves the precision of the test methods by reducing blank values. It is considered unnecessary to pre-condition MITI inoculum.

I.6.6.   Abiotic controls

When required, check for the possible abiotic degradation of the test substance by determining the removal of DOC, oxygen uptake or carbon dioxide evolution in sterile controls containing no inoculum. Sterilize by filtration through a membrane (0,2-0,45 micrometre) or by the addition of a suitable toxic substance at an appropriate concentration. If membrane filtration is used, take samples aseptically to maintain sterility. Unless adsorption of the test chemical has been ruled out beforehand, tests which measure biodegradation as the removal of DOC, especially with activated sludge inocula, should include an abiotic control which is inoculated and poisoned.

I.6.7.   Number of flasks

The number of flasks in a typical run is described under the headings of each tests.

The following type of flask may be used:

–   Test suspension: containing test substance and inoculum

–   Inoculum blank: containing only inoculum

–   Procedure control: containing reference substance and inoculum

–   Abiotic sterile control: sterile, containing test substance (see 1.6.6)

–   Adsorption control: containing test substance, inoculum and sterilising agent

–   Toxicity control: containing test substance, reference substance and inoculum

It is mandatory that determination in test suspension and inoculum blank is made in parallel. It is advisable to make the determinations in the other flasks in parallel as well.

This may, however, not always be possible. Ensure that sufficient samples or readings are taken to allow the percentage removal in the 10-day window to be assessed.

I.7.   DATA AND EVALUATION

In the calculation of D_t, percentage degradation, the mean values of the duplicate measurement of the parameter in both test vessels and inoculum blank are used. The formulas are set out in the sections below on specific tests. The course of degradation is displayed graphically and the 10-day window is indicated. Calculate and report the percentage removal achieved at the end of the 10-day window and the value at the plateau or at the end of the test, whichever is appropriate.

In respirometric tests nitrogen-containing compounds may affect the oxygen uptake because of nitrification (see Annexes II and V).

I.7.1.   Degradation measured by means of DOC determination

The percentage degradation D_t at each time a sample was taken should be calculated separately for the flasks containing test substance using mean values of duplicate DOC measurements in order that the validity of the test can be assessed (see 1.5.2.). It is calculated using the following equation:

$$ThOD_{NH4} = \frac{16\left[2c + 1/2(h - cl - 3n) + 3s + 5/2p + 1/2na - o\right]}{MW} \, mg/mg$$

where:

D_t = % degradation at time t,

C_o = mean starting concentration of DOC in the inoculated culture medium containing the test substance (mg DOC/l),

C_t = mean concentration of DOC in the inoculated culture medium containing test substance at time t (mg DOC/l),

C_{bo} = mean starting concentration of DOC in blank inoculated mineral medium (mg DOC/l),

C_{bt} = mean concentration of DOC blank inoculated mineral medium at time t (mg DOC/l).

All concentrations are measured experimentally.

I.7.2.   Degradation measured by means of specific analysis

When specific analytical data are available, calculate primary biodegradation from:

$$D_t = \frac{S_b - S_a}{S_b} \times 100$$

where:

D_t = % degradation at time t, normally 28 days,

S_a = residual amount of test substance in inoculated medium at end of test (mg),

S_b = residual amount of test substance in the blank test with water/medium to which only the test substance was added (mg).

1.7.3. Abiotic degradation

When an abiotic sterile control is used, calculate the percentage abiotic degradation using:

$$\% \text{ abiotic degradation} = \frac{C_{s(o)} - C_{s(t)}}{C_{s(o)}} \times 100$$

Where:

$C_{s(o)}$ = DOC Concentration in sterile control at day 0

$C_{s(t)}$ = DOC Concentration in sterile control at day t

I.8. REPORTING

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

– test and reference chemicals, and their purity;

– test conditions;

– inoculum: nature and sampling site(s), concentration and any pre-conditioning treatment;

– proportion and nature of industrial waste present in sewage if known;

– test duration and temperature;

– in the case of poorly soluble test chemicals, treatment given;

– test method applied; scientific reasons and explanation should be given for any change of procedure;

– data sheet;

– any observed inhibition phenomena;

– any observed abiotic degradation;

– specific chemical analytical data, if available;

– analytical data on intermediates, if available;

–   the graph of percentage degradation against time for the test and reference substances; the lag phase, degradation phase, l0-day window and slope should be clearly indicated (Annex I). If the test has complied with the validity criteria, the mean of the degradation percentages of the flasks containing test substance may be used for the graph.

–   percentage removal after l0-day window, and at plateau or at end of the test.

PART II. DOC DIE-AWAY TEST (METHOD C.4-A)

II.1.   PRINCIPLE OF THE METHOD

A measured volume of inoculated mineral medium containing a known concentration of the test substance (10-40 mg DOC/l) as the nominal sole source of organic carbon is aerated in the dark or diffused light at 22 ± 2 °C.

Degradation is followed by DOC analysis at frequent intervals over a 28-day period. The degree of biodegradation is calculated by expressing the concentration of DOC removed (corrected for that in the blank inoculum control) as a percentage of the concentration initially present. The degree of primary biodegradation may also be calculated from supplemental chemical analysis made at the beginning and end of incubation.

II.2.   DESCRIPTION OF THE METHOD

II.2.1.   Apparatus

(a)   Conical flasks, e. g. 250 ml to 2 l, depending on the volume needed for DOC analysis;

(b)   Shaking machine to accommodate the conical flasks, either with automatic temperature control or used in a constant temperature room; and of sufficient power to maintain aerobic conditions in all flasks;

(c)   Filtration apparatus, with suitable membranes;

(d)   DOC analyser;

(e)   Apparatus for determining dissolved oxygen;

(f)   Centrifuge.

II.2.2.   Preparation of mineral medium

For the preparation of the stock solutions, see I.6.2.

Mix 10 ml of solution (a) with 800 ml dilution water, add 1 ml of solutions (b) to (d) and make up to 1 l with dilution water .

II.2.3.   Preparation and pre-conditioning of inoculum

The inoculum may be derived from a variety of sources: activated sludge; sewage effluents; surface waters; soils or from a mixture of these.

See I.6.4., I.6.4.1., I.6.4.2. and I.6.5.

II.2.4.   Preparation of flasks

As an example, introduce 800 ml portions of mineral medium into 2 l conical flasks and add sufficient volumes of stock solutions of the test and reference substances to separate flasks to give a concentration of chemical equivalent to 10-40 mg DOC/l. Check the pH values and adjust, if necessary, to 7,4. Inoculate the flasks with activated sludge or other source of inocula (see I.6.4.), to give a final concentration not greater than 30 mg suspended solids/l. Also prepare inoculum controls in the mineral medium but without test or reference chemical.

If needed, use one vessel to check the possible inhibitory effect of the test chemical by inoculating a solution containing, in the mineral medium, comparable concentrations of both the test and a reference chemical.

Also, if required, set up a further, sterile flask to check whether the test chemical is degraded abiotically by using an uninoculated solution of the chemical (see I.6,6.).

Additionally, if the test chemical is suspected of being significantly adsorbed on to glass, sludge, etc. , make a preliminary assessment to determine the likely extent of adsorption and thus the suitability of the test for the chemical (see Table 1). Set up a flask containing the test substance, inoculum and sterilizing agent.

Make up the volumes in all flasks to 11 with mineral medium and, after mixing, take a sample from each flask to de.termine the initial concentration of DOC (see Annex II.4). Cover the openings of the flasks, e. g. with aluminium foil, in such a way as to allow free exchange of air between the flask and the surrounding atmosphere. Then insert the vessels into the shaking machine for starting the test.

II.2.5.   Number of flasks in typical run

Flasks 1 and 2: Test suspension

Flasks 3 and 4: Inoculum blank

Flask 5: Procedure control

preferably and when necessary:

Flask 6: Abiotic sterile control

Flask 7: Adsorption control

Flask 8: Toxicity control

See also I.6.7.

II.2.6.   Performance of the test

Throughout the test, determine the concentrations of DOC in each flask in duplicate at known time intervals, sufficiently frequently to be able to determine the start of the 10-day window and

the percentage removal at the end of the 10-day window. Take only the minimal volume of test suspension necessary for each determination.

Before sampling make good evaporation losses from the flasks by adding dilution water (I.6.1) in the required amount if necessary. Mix the culture medium thoroughly before withdrawing a sample and ensure that material adhering to the walls of the vessels is dissolved or suspended before sampling. Membrane-filter or centrifuge (see Annex II.4) immediately after the sample has been taken. Analyse the filtered or centrifuged samples on the same day, otherwise store at 2-4 °C for a maximum of 48 h, or below -18 °C for a longer period.

II.3. DATA AND REPORTING

II.3.1. Treatment of results

Calculate the percentage degradation at time t as given under I.7.1. (DOC determination) and, optionally, under I.7.2. (specific analysis).

Record all results on the data sheets provided.

II.3.2. Validity of results

See I.5.2.

II.3.3. Reporting

See I.8.

II.4. DATA SHEET

An example of a data sheet is given hereafter.

DOC DIE-AWAY TEST

1. LABORATORY

2. DATE AT START OF TEST

3. TEST SUBSTANCE

Name:

Stock solution concentration: mg/1 as chemical

Initial concentration in medium, to: mg/1 as chemical

4. INOCULUM

Source:

Treatment given:

Pre-conditioning, if any:

Concentration of suspended solids in reaction mixture: mg/1

5. CARBON DETERMINATIONS

Carbon analyser:

	Flask nr		DOC after n days (mg/l)				
			0	n_1	n_2	n_3	n_x
Test chemical plus inoculum	1	a_1					
		a_2					
		a,mean $C_{a(t)}$					
	2	b_1					
		b_2					
		b, mean $C_{b(t)}$					
Blank inoculum without test chemical	3	c_1					
		c_2					
		c, mean $C_{c(t)}$					
	4	d_1					
		d_2					
		d,mean $C_{d(t)}$					
	$C_{bl(t)} = \dfrac{C_{c(t)} + C_{d(t)}}{2}$						

6. EVALUATION OF RAW DATA

Flask nr		% degradation after n days				
		0	n_1	n_2	n_3	n_x
1	$D_1 = \left(1 - \dfrac{C_{a(t)} - C_{bl(t)}}{C_{a(o)} - C_{bl(o)}}\right) \times 100$	0				
2	$D_2 = \left(1 - \dfrac{C_{b(t)} - C_{bl(t)}}{C_{b(o)} - C_{bl(o)}}\right) \times 100$	0				
Mean (*)	$D = \dfrac{D_1 - D_2}{2}$	0				

(*) D_1 and D_2 should not be averaged if there is a considerable difference.

Note: similar formats may be used for the reference chemical and toxicity controls.

7. ABIOTIC CONTROL (optional)

	Time (days)	
	0	t
DOC conc. (mg/l) in sterile control	$C_{s(o)}$	$C_{s(t)}$

$$\% \text{ abiotic degradation} = \frac{C_{s(o)} - C_{s(t)}}{C_{s(o)}} \times 100$$

8. SPECIFIC CHEMICAL ANALYSIS (optional)

	residual amount of test chemical at end of test (mg/l)	% primary degradation
Sterile control	S_b	
Inoculated test medium	S_a	

PART III. MODIFIED OECD SCREENING TEST (Method C.4-B)

III.1.   PRINCIPLE OF THE METHOD

A measured volume of mineral medium containing a known concentration of the test substance (10-40 mg DOC/litre) as the nominal sole source of organic carbon is inoculated with 0,5 ml effluent per litre of medium. The mixture is aerated in the dark or diffused light at 22 ± 2 °C.

Degradation is followed by DOC analysis at frequent intervals over a 28-day period. The degree of biodegradation is calculated by expressing the concentration of DOC removed (corrected for that in the blank inoculum control) as a percentage of the concentration initially present. The degree of primary biodegradation may also be calculated from supplemental chemical analysis made at the beginning and end of incubation.

III.2.   DESCRIPTION OF THE METHOD

III.2.1. Apparatus

(a)   Conical flasks, e.g. 250 ml to 2 litres, depending on the volume needed for DOC analysis;

(b)   Shaking machine - to accommodate the conical flasks, either with automatic temperature control or used in a constant temperature room, and of sufficient power to maintain aerobic conditions in all flasks;

(c)   Filtration apparatus, with suitable membranes;

(d)   DOC analyser;

(e)   Apparatus for determining dissolved oxygen;

(f)   Centrifuge.

III.2.2. Preparation of mineral medium

For the preparation of the stock solutions, see I.6.2.

Mix 10 ml of solution (a) with 80 ml dilution water, add 1 ml of solutions (b) to (d) and make up to 1 litre with dilution water.

This method uses only 0,5 ml effluent/litre as inoculum and therefore the medium may need to be fortified with trace elements and growth factors. This is done by adding 1 ml each of the following solutions per litre of final medium:

Trace element solution:

Manganese sulfate tetrahydrate, $MnSO_4 . 4H_2O$ 39,9 mg

Boric acid, H_3BO_3	57,2 mg
Zinc sulfate heptahydrate, $ZnSO_4 . 7H_2O$	42,8 mg
Ammonium heptamolybdate $(NH_4)_6 Mo_7O_{24}$	34,7 mg
Fe-chelate ($FeCl_3$ ethylenediamine-tetra-acetic acid)	100,0 mg

Dissolve in, and make up to 1000 ml with dilution water

Vitamin solution:

Yeast extract	15,0 mg

Dissolve the yeast extract in 100 ml water. Sterilise by passage through a 0,2 micron membrane, or make up freshly.

III.2.3. Preparation and pre-conditioning of inoculum

The inoculum is derived from the secondary effluent of a treatment plant or laboratory scale unit receiving predominantly domestic sewage. See I.6.4.2. and I.6.5.

0,5 ml per litre of mineral medium is used.

III.2.4. Preparation of flasks

As an example, introduce 800 ml portions of mineral medium into 2-litre conical flasks and add sufficient volumes of stock solutions of the test and reference substances to separate flasks to give a concentration of chemical equivalent to 10-40 mg DOC/litre. Check the pH value and adjust, if necessary, to 7,4. Inoculate the flasks with sewage effluent at 0,5 ml/litre (see I.6.4.2.). Also prepare inoculum controls in the mineral medium but without test or reference chemical.

If needed, use one vessel to check the possible inhibitory effect of the test chemical by inoculating a solution containing, in the mineral medium, comparable concentrations of both the test and a reference chemical.

Also, if required, set up a further, sterile flask to check whether the test chemical is degraded abiotically by using an uninoculated solution of the chemical (see I.6.6.).

Additionally, if the test chemical is suspected of being significantly adsorbed on to glass, sludge, etc., make a preliminary assessment to determine the likely extent of adsorption and thus the suitability of the test for the chemical (see Table 1). Set up a flask containing the test substance, inoculum and sterilizing agent.

Make up the volumes in all flasks to 1 litre with mineral medium and, after mixing, take a sample from each flask to determine the initial concentration of DOC (see Annex II.4). Cover the openings of the flasks, e.g. with aluminium foil, in such a way as to allow free exchange of air between the flask and the surrounding atmosphere. Then insert the vessels into the shaking machine for starting the test.

III.2.5. Number of flasks in typical run

Flasks 1 and 2: Test suspension

Flasks 3 and 4: Inoculum blank

Flask 5: Procedure control

and preferably and when necessary:

Flask 6: Abiotic sterile control

Flask 7: Adsorption control

Flask 8: Toxicity control

See also 1.6.7.

III.2.6. Performance of the test

Throughout the test, determine the concentrations of DOC in each flask in duplicate at known time intervals, sufficiently frequently to be able to determine the start of the 10-day window and the percentage removal at the end of the 10-day window. Take only the minimal volume of test suspension necessary for each determination.

Before sampling make good evaporation losses from the flasks by adding dilution water (I.6.1) in the required amount if necessary. Mix the culture medium thoroughly before withdrawing a sample and ensure that material adhering to the walls of the vessels is dissolved or suspended before sampling. Membrane-filter or centrifuge (see Annex II.4) immediately after the sample has been taken. Analyse the filtered or centrifuged samples on the same day, otherwise store at 2-4 °C for a maximum of 48 h, or below -18 °C for a longer period.

III.3. DATA AND REPORTING

III.3.1. Treatment of results

Calculate the percentage degradation at time t as given under I.7.1. (DOC determination) and, optionally, under I.7.2. (specific analysis).

Record all results on the data sheets provided.

III.3.2. Validity of results

See I.5.2.

III.3.3. Reporting

See I.8.

III.4. DATA SHEET

An example of a data sheet is given hereafter .

MODIFIED OECD SCREENING TEST

1. LABORATORY

2. DATE AT START OF TEST

3. TEST SUBSTANCE

Name:

Stock solution concentration: mg/litre as chemical

Initial concentration in medium, to: mg/litre as chemical

4. INOCULUM

Source:

Treatment given:

Pre-conditioning, if any:

Concentration of suspended solids in reaction mixture: mg/1

5. CARBON DETERMINATIONS

Carbon analyser:

	Flask nr		DOC after n days (mg/l)				
			0	n_1	n_2	n_3	n_x
Test chemical plus inoculum	1	a_1					
		a_2					
		a,mean $C_{a(t)}$					
	2	b_1					
		b_2					
		b, mean $C_{b(t)}$					
Blank inoculum without test chemical	3	c_1					
		c_2					
		c, mean $C_{c(t)}$					
	4	d_1					
		d_2					
		d,mean $C_{d(t)}$					
	$C_{bl(t)} = \dfrac{C_{c(t)} + C_{d(t)}}{2}$						

6. EVALUATION OF RAW DATA

Flask nr		% degradation after n days				
		0	n_1	n_2	n_3	n_x
1	$D_1 = \left(1 - \dfrac{C_{a(t)} - C_{bl(t)}}{C_{a(o)} - C_{bl(o)}}\right) \times 100$	0				
2	$D_2 = \left(1 - \dfrac{C_{b(t)} - C_{bl(t)}}{C_{b(o)} - C_{bl(o)}}\right) \times 100$	0				
Mean (*)	$D = \dfrac{D_1 - D_2}{2}$	0				

(*) D_1 and D_2 should not be averaged if there is a considerable difference.

Note: similar formats may be used for the reference chemical and toxicity controls.

7. ABIOTIC CONTROL (optional)

	Time (days)	
	0	t
DOC conc.(mg/litre) sterile control	$C_{s(o)}$	$C_{s(t)}$

$$\% \text{ abiotic degradation} = \frac{C_{s(o)} - C_{s(t)}}{C_{s(o)}} \times 100$$

8. SPECIFIC CHEMICAL ANALYSIS (optional)

	residual amount of test chemical at end of test	% primary degradation
Steril control	S_b	
Inoculated test medium	S_a	$\dfrac{S_b - S_a}{S_b} \times 100$

PART IV. CO$_2$ EVOLUTION TEST (METHOD C.4-C)

IV.1.   PRINCIPLE OF THE METHOD

A measured volume of inoculated mineral medium containing a known concentration of the test chemical (10-20 mg DOC or TOC/l) as the nominal sole source of organic carbon is aerated by the passage of carbon, dioxide-free air at a controlled rate in the dark or in diffuse light. Degradation is followed over 28 days by determining the carbon dioxide produced, which is trapped in barium or sodium hydroxide and which is measured by titration of the residual hydroxide or as inorganic carbon. The amount of carbon dioxide produced from the test chemical (corrected for that derived from the blank inoculum) is expressed as a percentage of ThCO$_2$. The degree of biodegradation may also be calculated from supplemental DOC analysis made at the beginning and end of incubation.

IV.2.   DESCRIPTION OF THE METHOD

IV.2.1. Apparatus

(a)   Flasks, 2-5 litres, each fitted with an aeration tube reaching nearly the bottom of the vessel and an outlet;

(b)   magnetic stirrers, when assessing poorly soluble chemicals;

(c)   Gas-absorption bottles;

(d)   Device for controlling and measuring airflow;

(e)   Apparatus for carbon dioxide scrubbing, for preparation of air which is free from carbon dioxide; alternatively, a mixture of CO$_2$-free oxygen and CO$_2$-free nitrogen, from gas cylinders, in the correct proportions (20% O$_2$: 80% N$_2$) may be used;

(f)   Device for determination of carbon dioxide, either titrimetrically or by some form of inorganic carbon analyser;

(g)   Membrane filtration device (optional);

(h)   DOC analyser (optional).

IV .2.2. Preparation of mineral medium

For the preparation of the stock solutions, see I.6.2.

Mix 10 ml of solution (a) with 800 ml dilution water, add 1 ml of solutions (b) to (d) and make up to 11 with dilution water.

IV .2.3. Preparation and pre-conditioning of inoculum

The inoculum may be derived from a variety of sources: activated sludge; sewage effluents; surface waters; soils or from a mixture of these.

See I.6.4., I.6.4.1., I.6.4.2. and I.6.5.

IV .2.4. Preparation of flasks

As an example the following volumes and weights indicate the values for 5-litre flasks containing 3 l of suspension. If smaller volumes are used modify the values accordingly, but ensure that the carbon dioxide formed can be measured accurately.

To each 5-litre flask add 2400 ml mineral medium. Add an appropriate volume of the prepared activated sludge (see I.6.4.1. and I.6.5.) to give a concentration of suspended solids of not more than 30 mg/l in the final 3 1 of inoculated mixture. Alternatively first dilute the prepared sludge to give a suspension of 500-1000 mg/l in the mineral medium before adding an aliquot to the contents of the 5 litre flask to attain a concentration of 30 mg/l; this ensures greater precision. Other sources of inoculum may be used (see I.6.4.2.).

Aerate these inoculated mixtures with CO_2-free air overnight to purge the system of carbon dioxide.

Add the test material and reference substance, separately, as known volume of stock solutions, to replicate flasks to yield concentrations, contributed by the added chemicals, of 10 to 20 mg DOC or TOC/l; leave some flasks without addition of chemicals as inoculum controls. Add poorly soluble test substances directly to the flasks on a weight or volume basis or handle as described in Annex III.

If required, use one flask to check the possible inhibitory effect of the test chemical by adding both the test and reference chemicals at the same concentrations as present in the other flasks.

Also, if required, use a sterile flask to check whether the test chemical is degraded abiotically by using an uninoculated solution of the chemical (see I.6.6.). Sterilise by the addition of a toxic substance at an appropriate concentration.

Make up the volumes of suspensions in all flasks to 3 1 by the addition of mineral medium previously aerated with CO_2-free air. Optionally, samples may be withdrawn for analysis of DOC (see Annex II.4.) and/or specific analysis. Connect the absorption bottles to the air outlets of the flasks.

If barium hydroxide is used, connect three absorption bottles, each containing 100 ml of 0,0125 M barium hydroxide solution, in series to each 5-litre flask. The solution must be free of precipitated sulphate and carbonate and its strength must be determined immediately before use. If sodium hydroxide is used, connect two traps, the second acting as a control to demonstrate that all the carbon dioxide was absorbed in the first. Absorption bottles fitted with serum bottle closures are suitable. Add 200 ml 0,05 M sodium hydroxide to each bottle, which is sufficient to absorb the total quantity of carbon dioxide evolved when the test chemical is completely

degraded. The sodium hydroxide solution, even when freshly prepared, will contain traces of carbonates; this is corrected by deduction of the carbonate in the blank.

IV.2.5. Number of flasks in a typical run

Flasks 1 and 2: Test suspension

Flasks 3 and 4: Inoculum blank

Flask 5: Procedure control

and, preferably and when necessary:

Flask 6: Abiotic sterile control

Flask 7: Toxicity control

See also I.6.7.

IV.2.6. Performance of the test

Start the test by bubbling CO_2-free air through the suspensions at a rate of 30-100 ml/min. Take samples of the carbon dioxide absorbent periodically for analysis of the CO_2-content. During the first ten days it is recommended that analyses should be made every second or third day and then every fifth day until the 28th day so that the 10-day window period can be identified.

On the 28th day, withdraw samples (optionally) for DOC and/or specific analysis, measure the pH of the suspensions and add 1 ml of concentrated hydrochloric acid to each flask; aerate the flasks overnight to drive off the carbon dioxide present in the test suspensions. On day 29 make the last analysis of evolved carbon dioxide.

On the days of measurement of CO_2, disconnect the barium hydroxide absorber closest to the flask and titrate the hydroxide solution with HCl 0,05 M using phenolphthalein as the indicator. Move the remaining absorbers one place closer to the flask and place a new absorber containing 100 ml fresh 0,0125 M barium hydroxide at the far end of the series. Make titrations as needed, for example, when substantial precipitation is seen in the first trap and before any is evident in the second, or at least weekly. Alternatively, with NaOH as absorbent, withdraw with a syringe a small sample (depending on the characteristics of the carbon analyser used) of the sodium hydroxide solution in the absorber nearer to the flask. Inject the sample into the IC part of the carbon analyser for analysis of evolved carbon dioxide directly.

Analyse the contents of the second trap only at the end of the test to correct for any carryover of carbon dioxide.

IV.3. DATA AND REPORTING

IV.3.1. Treatment of results

The amount of CO_2 trapped in an absorber when titrated is given by:

$$mgCO_2 = (100 \times C_B - 0.5 \times V \times C_A) \times 44$$

where:

V = volume of HCl used for titration of the 100 ml in the absorber (ml),

C_B = concentration of the barium hydroxide solution (M),

C_A = concentration of the hydrochloric acid solution (M),

if C_B is 0,0125 M and C_A is 0,05 M, the titration for 100 ml barium hydroxide is 50 ml and the weight of CO_2 is given by:

$$\frac{0.05}{2} \times 44 \times ml\ HCI\ titrated = 1.1 \times ml\ HCI$$

Thus, in this case, to convert volume of HCl titrated to mg CO_2 produced the factor is 1,1.

Calculate the weights of CO_2 produced from the inoculum alone and from the inoculum plus test chemical using the respective titration values and the difference is the weight of CO_2 produced from the test chemical alone.

For example, if the inoculum alone gives a titration of 48 ml and inoculum plus test chemical gives 45 ml,

CO_2 from inoculum = 1,1 x (50-48) = 2,2 mg

CO_2 from inoculum plus test chemical = 1,1 x (50-45) = 5,5 mg

and thus the weight of CO_2 produced from the test chemical is 3,3 mg.

The percentage biodegradation is calculated from:

–   % degradation = (mg CO_2 produced x 100) / (ThCO_2 x mg test chemical added)

or,

–   % degradation = (mg CO2 produced x 100) / (mg TO added in test x 3.67)

3,67 being the conversion factor (44/12) for carbon to carbon dioxide.

Obtain the percentage degradation after any time interval by adding the percentage of ThCO_2 values calculated for each of the days, up to that time, on which it was measured.

For sodium hydroxide absorbers, calculate the amount of carbon dioxide prodllced, expressed as IC (mg), by multiplying the concentration of IC in the absorbent by the volume of the absorbent.

Calculate the percentage degradation from :

– % of ThCO2 = (mg IC flask - mg IC blank x 100) / (MG TOC added as test chemical)

Calculate DOC removals (optional) as described under I.7. Record these and all other results on the data sheets provided.

IV.3.2. Validity of results

The IC content of the test chemical suspension in the mineral medium at the beginning of the test must be less than 5 % of the TC, and the total CO_2 evolution in the inoculum blank at the end of the test should not normally exceed 40 mg/1 medium. If values greater than 70 mg CO_2/litre are obtained, the data and experimental technique should be examined critically.

See also I.5.2.

IV.3.3. Reporting

See I.8.

IV.4.   DATA SHEET

An example of a data sheet is given hereafter .

CARBON DIOXIDE EVOLUTION TEST

1. LABORATORY

2. DATE AT START OF TEST

3. TEST SUBSTANCE

Name:

Stock solution concentration: mg/litre as chemical

Initial conc. in medium: mg/litre as chemical

Total C added to flask: mg C

$ThCO_2$: mg CO_2

4. INOCULUM

Source:

Treatment given:

Pre-conditioning if any:

Concentration of suspended solids in reaction mixture: mg/litre

Time (day)	CO₂ formed Test (mg) 1 / 2	CO₂ formed Test (mg) mean	CO₂ formed blank (mg) 3 / 4	CO₂ formed blank (mg) mean	CO₂ formed cumulative (mg) (test minus blank mean) 1	CO₂ formed cumulative (mg) (test minus blank mean) 2	ThCO₂ cumulative CO₂ / ThCO₂ × 100 1	ThCO₂ cumulative CO₂ / ThCO₂ × 100 2	ThCO₂ cumulative CO₂ / ThCO₂ × 100 mean
0									
n_1									
n_2									
n_3									
28									

Note: similar formats may be used for the reference chemical and toxicity controls.

6. **CARBON ANALYSIS** (optional)

Carbon analyser:

Time (day)	Blank mg/l	Test chemical mg/l
0	$C_{b(o)}$	C_o
28 (*)	$C_{b(t)}$	C_t
(*) or at end of incubation		

$$\% \text{ DOC removed} = \left(1 - \frac{C_t - C_{b(t)}}{C_o - C_{b(o)}}\right) \times 100$$

7. **ABIOTIC DEGRADATION** (optional)

$$\% \text{ abiotic degradation} = \frac{CO_2 \text{ formation in sterile flask after 28 day (mg)}}{ThCO_2 \text{ (mg)}} \times 100$$

PART V. MANOMETRIC RESPIROMETRY TEST (METHOD C.4-D)

V.1.   PRINCIPLE OF THE METHOD

A measured volume of inoculated mineral medium, containing a known concentration of test chemical (100 mg/litre of the test substance, to give at least 50-100 mg ThOD/litre) as the nominal sole source of organic carbon, is stirred in a closed flask at a constant temperature (± 1° C or closer) for up to 28 days. The consumption of oxygen is determined either by measuring the quantity of oxygen (produced electrolytically) required to maintain constant gas volume in the respirometer flask, or from the change in volume or pressure (or a combination of the two) in the apparatus. Evolved carbon dioxide is absorbed in a solution of potassium hydroxide or another suitable absorbent. The amount of oxygen taken up by the test chemical (corrected for uptake by blank inoculum, run in parallel) is expressed as a percentage of ThOD or COD. Optionally, primary biodegradation may also be calculated from supplemental specific analysis made at the beginning and end of incubation, and ultimate biodegradation by DOC analysis.

V.2.   DESCRIPTION OF THE METHOD

V.2.1.   Apparatus

(a)   suitable respirometer;

(b)   temperature control, maintaining ± 1 °C or better;

(c)   membrane-filtration assembly (optional);

(d)   carbon analyser (optional).

V .2.2.   Preparation of mineral medium

For the preparation of the stock solutions, see I.6.2.

Mix 10 ml of solution (a) with 800 ml dilution water, add 1 ml of solutions (b) to (d) and make up to 1 litre with dilution water.

V.2.3.   Preparation and pre-conditioning of inoculum

The inoculum may be derived from a variety of sources: activated sludge; sewage effluents; surface waters and soils or from a mixture of these.

See I.6.4., I.6.4.1., I.6.4.2. and I.6.5.

V.2.4.   Preparation of flasks

Prepare solutions of the test and reference chemicals, in separate batches, in mineral medium equivalent to a concentration, normally, of 100 mg chemical/litre (giving at least 50-100 mg ThOD/litre), using stock solutions.

Calculate the ThOD on the basis of formation of ammonium salts unless nitrification is anticipated, when the calculation should be based on nitrate formation (see Annex II.2.)

Determine the pH values and if necessary adjust to $7,4 \pm 0,2$.

Poorly soluble substances should be added at a later stage (see below).

If the toxicity of the test chemical is to be determined, prepare a further solution in mineral medium containing both test and reference chemicals at the same concentrations as in the individual solutions.

If measurement of the physico-chemical uptake of oxygen is required, prepare a solution of the test chemical at, normally, 100 mg ThOD/litre which has been sterilised by the addition of a suitable toxic substance (see I.6,6.).

Introduce the requisite volume of solutions of test and reference chemicals, respectively, into at least duplicate flasks. Add to further flasks mineral medium only (for inoculum controls) and, if required, the mixed test/ reference chemical solution and the sterile solution.

If the test chemical is poorly soluble, add it directly at this stage on a weight or volume basis or handle it as described in Annex III. Add potassium hydroxide, soda lime pellets or other absorbent to the CO_2-absorber compartments.

V.2.5.   Number of flasks in a typical run

Flasks 1 and 2: Test suspension

Flasks 3 and 4: Inoculum blank

Flask 5: Procedure control

preferably, and when necessary:

Flask 6: Sterile control

Flask 7: Toxicity control

See also I.6.7.

V.2.6.   Performance of the test

Allow the vessels to reach the desired temperature and inoculate appropriate vessels with prepared activated sludge or other source of inoculum to give a concentration of suspended solids not greater than 30 mg/litre. Assemble the equipment, start the stirrer and check for air-tightness, and start the measurement of oxygen uptake. Usually no further attention is required other than taking the necessary readings and making daily checks to see that the correct temperature and adequate stirring are maintained.

Calculate the oxygen uptake from the readings taken at regular and frequent intervals, using the methods given by the manufacturer of the equipment. At the end of incubation, normally 28 days,

measure the pH of the contents of the flasks, especially if oxygen uptakes are low or greater than ThODNH₄ (for nitrogen-containing compounds).

If required, withdraw samples from the respirometer flasks, initially and finally, for analysis of DOC or specific chemical (see Annex II.4). At the initial withdrawal, ensure that the volume of test suspension remaining in the flask is known. 'When oxygen is taken up by N-containing test substance, determine the increase in concentration of nitrite and nitrate over 28 days and calculate the correction for the oxygen consumed by nitrification (Annex V).

V.3.   DATA AND REPORTING

V.3.1.   Treatment of results

Divide the oxygen uptake (mg) of the test chemical after a given time (corrected for that by the blank inoculum control after the same time) by the weight of the test chemical used. This yields the BOD expressed as mg oxygen/mg test chemical, that is

BOD =   (mg O_2 uptake by test chemical -mg O_2 uptake by blank) / (mg test chemical in flask)

    =   mg O_2 per mg test chemical

calculate the percentage biodegradation either from:

  % biodegradation = % ThOD = BOD (mg O_2/mg chemical) x 100 / ThOD (mg O_2 chemical)

or form

        % COD = BOD (mg O_2/mg chemical) x 100 / COD (mg O_2 chemical)

It should be noted that these two methods do not necessarily give the same value; it is preferable to use the former method.

For test substances containing nitrogen, use the appropriate ThOD (NH_4 or NO_3) according to what is known or expected about the occurrence of nitrification (Annex II.2). If nitrification occurs but is not complete, calculate a correction for the oxygen consumed by nitrification from the changes in concentration of nitrite and nitrate (Annex V).

When optional determinations of organic carbon and/or specific chemical are made, calculate the percentage degradation, as described under I.7.

Record all results on the data sheets attached.

V.3.2.   Validity of results

The oxygen uptake of the inoculum blank is normally 20-30 mg O_2/ litre and should not be greater than 60 mg/litre in 28 days. Values higher than 60 mg/litre require critical examination of the data and experimental techniques. If the pH value is outside the range 6-8,5 and the oxygen consumption by the test chemical is less than 60% , the test should be repeated with a lower concentration of test chemical.

See also I.5.2.

V.3.3. Reporting

See I.8.

V.4. DATA SHEET

An example of a data sheet is given hereafter .

MANOMETRIC RESPIROMETRY TEST

1. LABORATORY

2. DATE AT START OF TEST

3. TEST SUBSTANCE

Name:

Stock solution concentration: mg/litre

Initial concentration in medium, C_o: mg/litre

Volume in test flask (V): ml

ThOD or COD: mg O_2/mg test substance (NH_4 or NO_3)

4. INOCULUM

Source:

Treatment given:

Pre-conditioning, if any:

Concentration of suspended solids in reaction mixture: mg/1

5. OXYGEN UPTAKE: BIODEGRADABILITY

		Zeit (Tage)											
		0		7		14			21			28	
O_2 upt. (mg) test chemical	1												
	2												
	a, mean												
O_2 upt. (mg) blank	3												
	4												
	b, mean												
Corrected BOD (mg)	$(a_1 - b_m)$												
	$(a_2 - b_m)$												
BOD per mg test chemical	$\dfrac{(a_1-b)}{C_oV}$												
	$\dfrac{(a_2-b)}{C_oV}$												
% degradation	$D_1\,(a_1)$												
$\dfrac{BOD}{ThOD} \times 100$	$D_2\,(a_2)$												
	Mean *												
V = volume of medium in test flask													

* D_1 and D_2 should not be averaged if there is a considerable difference.

N.B.: Similar formats may be used for the reference chemical and the toxicity controls.

6. CORRECTION FOR NITRIFICATION (see Annex V)

Day	0	28	Difference
(i) Concentration of nitrate (mg N/litre)			(N)
(ii) Oxygen equivalent (4,57 × N × V) (mg)	—	—	
(iii) Concentration of nitrite (mg N/litre)			(N)
(iv) Oxygen equivalent (3,43 × N × V) (mg)	—	—	
(ii + iv) Total oxygen equivalent	—	—	

7. CARBON ANALYSIS (optional)

Carbon analyser:

Time (day)	Blank mg/litre	Test chemical mg/litre
0	(C_{blo})	(C_o)
28*	(C_{blt})	(C_t)
* or at end of incubation		

$$\% \text{ DOC removed} = \left(1 - \frac{C_t - C_{blt}}{C_0 - C_{blo}}\right) x100$$

8. SPECIFIC CHEMICAL (optional)

S_b = concentration in physico-chemical (sterile) control at 28 days

S_a = concentration in inoculated flask at 28 days,

$$\% \text{ biodegradation} = \frac{S_b - S_a}{S_b} x100$$

9. ABIOTIC DEGRADATION (optional)

a = oxygen consumption in sterile flasks after 28 days, (mg)

$$\text{oxygen consumption per mg test chemical} = \frac{ax100}{C_oV}$$

(see sections 1 and 3)

$$\% \text{ abiotic degradation} = \frac{ax100}{C_oVxThOD}$$

PART VI. CLOSED BOTTLE TEST (METHOD C.4-E)

VI.1 PRINCIPLE OF THE TEST METHOD

The solution of the test chemical in mineral medium, usually at 2-5 mg/litre, is inoculated with a relatively small number of micro-organisms from a mixed population and kept in completely full, closed bottles in the dark at constant temperature. Degradation is followed by analysis of dissolved oxygen over a 28-day period. The amount of oxygen taken up by the test chemical, corrected for uptake by the blank inoculum run in parallel, is expressed as a percentage of ThOD or COD.

VI.2 DESCRIPTION OF THE METHOD

VI.2.1. Apparatus

a)   BOD bottles, with glass stoppers, e.g. 250-300 ml;

b)   Water bath or incubator, for keeping bottles at constant temperature ($\pm$ 1 °C or better) with the exclusion of light;

c)   Large glass bottles (2-5 litres) for the preparation of media and for filling the BOD bottles;

d)   Oxygen electrode and meter, or equipment and reagents for Winkler titration.

VI.2.2. Preparation of mineral medium

For the preparation of the stock solutions, see I.6.2.

Mix 1 (one) ml of solution (a) to (d) and make up to 1 litre with dilution water.

VI.2.3. Preparation of the inoculum

The inoculum is normally derived from the secondary effluent of a treatment plant or laboratory-scale unit receiving predominantly domestic sewage. An alternative source for the inoculum is surface water. Normally use from one drop (0,05 ml) to 5 ml of filtrate per litre of medium; trials may be needed to discover the optimum volume for a given effluent (See I.6.4.2. and I.6.5.).

VI.2.4. Preparation of flasks

Strongly aerate mineral medium for at least 20 min. Carry out each test series with mineral medium derived from the same batch. Generally, the medium is ready for use after standing for 20 h, at the test temperature. Determine the concentration of dissolved oxygen for control purposes; the value should be about 9 mg/litre at 20 °C. Conduct all transfer and filling operations of the air-saturated medium bubble-free, for example, by the use of siphons.

Prepare parallel groups of BOD bottles for the determination of the test and reference chemicals in simultaneous experimental series. Assemble a sufficient number of BOD bottles, including inoculum blanks, to allow at least duplicate measurements of oxygen consumption to be made at the desired test intervals, for example, after 0, 7, 14, 21 and 28 days. To ensure being able to identify the 10-day window, more bottles may be required.

Add fully aerated mineral medium to large bottles so that they are about one-third full. Then add sufficient of the stock solutions of the test chemical and reference chemical to separate large bottles so that the final concentration of the chemicals is normally not greater than 10 mg/litre. Add no chemicals to the blank control medium contained in a further large bottle.

In order to ensure that the inoculum activity is not limited, the concentration of dissolved oxygen must not fall below 0,5 mg/litre in the BOD bottles. This limits the concentration of test chemical to about 2 mg/litre. However, for poorly degradable compounds and those with a low ThOD, 5-10 mg/litre can be used. In some cases, it would be advisable to run parallel series of test chemical at two different concentrations, for example, 2 and 5 mg/litre. Normally, calculate the ThOD on the basis of formation of ammonium salts but, if nitrification is expected or known to occur, calculate on the basis of the formation of nitrate (ThOD$_{NO3}$: see Annex II.2). However, if nitrification is not complete but does occur, correct for the changes in concentration of nitrite and nitrate, determined by analysis, (see Annex V).

If the toxicity of the test chemical is to be investigated (in the case, for example, of a previous low biodegradability value having been found), another series of bottles is necessary.

Prepare another large bottle to contain aerated mineral medium (to about one-third of its volume) plus test chemical and reference chemical at final concentrations normally the same as those in the other large bottles.

Inoculate the solutions in the large bottles with secondary effluent (one drop or about 0,05 ml, to 5 ml/litre) or with another source such as river water (see I.6.4.2.). Finally, make up the solutions to volume with aerated mineral medium using a hose which reaches down to the bottom of the bottle to achieve adequate mixing.

VI.2.5. Number of flasks in a typical run

In a typical run the following bottles are used:

–   at least 10 containing test chemical and inoculum (test suspension),

–   at least 10 containing only inoculum (inoculum blank),

–   at least 10,containing reference chemical and inoculum (procedure control),

–   and, when necessary, 6 bottles containing test chemical, reference chemical and inoculum (toxicity control). However, to ensure being able to identify the 10-day window, about twice as many bottles would be necessary.

VI.2.6. Performance of the test

Dispense each prepared solution immediately into the respective group of BOD bottles by hose from the lower quarter (not the bottom) of the appropriate large bottle, so that all the BOD bottles are completely filled. Tap gently to remove any air bubbles. Analyse the zero-time bottles immediately for dissolved oxygen by the Winkler or electrode methods. The contents of the bottles can be preserved for later analysis by the Winkler method by adding manganese (II) sulfate and sodium hydroxide (the first Winkler reagent). Store the carefully stoppered bottles, containing the oxygen fixed as brown manganese (III) hydrated oxide, in the dark at 10-20 °C for no longer than 24 hours before proceeding with the remaining steps of the Winkler method. Stopper the remaining replicate bottles ensuring that no air bubbles are enclosed, and incubate at 20 °C in the dark. Each series must be accompanied by a complete parallel series for the determination of the inoculated blank medium. Withdraw at least duplicate bottles of all series for dissolved oxygen analysis at time intervals (at least weekly) over the 28 days incubation.

Weekly samples should allow the assessment of percentage removal in a 14-day window, whereas sampling every 3-4 days should allow the 10-day window to be identified, which would require about twice as many bottles.

For N-containing test substances, corrections for uptake of oxygen by any nitrification occurring should be made. To do this, use the O_2-electrode method for determining the concentration of dissolved oxygen and then withdraw a sample from the BOD bottle for analysis for nitrite and nitrate. From the increase in concentration of nitrite and nitrate, calculate the oxygen used (see Annex V).

VI.3.   DATA AND REPORTING

VI.3.1. Treatment of results

First calculate the BOD exerted after each time period by subtracting the oxygen depletion (mg O_2/litre) of the inoculum blank from that exhibited by the test chemical. Divide this corrected depletion by the concentration (mg/litre) of the test chemical, to obtain the specific BOD as mg oxygen per mg test chemical. Calculate the percentage biodegradability by dividing the specific BOD by the specific ThOD (calculated according to Annex II.2) or COD (determined by analysis, see Annex II.3), thus:

BOD =   (mg O_2 uptake by test chemical -mg O_2 uptake by blank) / (mg test chemical in flask)

   =   mg O_2 per mg test chemical

–   % degradation = BOD (mg O2/ mg test chemical x 100) / ThOD (mg O_2/mg test chemical)

Or

–   % degradation = BOD (mg O2/ mg test chemical x 100) / COD (mg O2/mg test

chemical)

It should be noted that these two methods do not necessarily give same value; it is preferable to use the former method.

For test substances containing nitrogen, use the appropriate ThOD (NH_4 or NO_3) according to what is known or expected about the occurrence of nitrification (Annex II.2). If nitrification occurs but is not complete, calculate a correction for the oxygen consumed by nitrification from the changes in concentration of nitrite and nitrate (Annex V).

VI.3.2. Validity of results

Oxygen depletion in the inoculum blank should not exceed 1,5 mg dissolved oxygen/litre after 28 days. Values higher than this require investigation of the experimental techniques. The residual concentration of oxygen in the test bottles should not fall below 0,5 mg/litre at any time. Such low oxygen levels are valid only if the method of determining dissolved oxygen used is capable of measuring such levels accurately.

See also I.5.2.

VI.3.3. Reporting

See I.8.

VI.4. DATA SHEET

An example of a data sheet is given hereafter.

CLOSED BOTTLE TEST

1. LABORATORY

2. DATE AT START OF TEST

3. TEST SUBSTANCE

Name:

Stock solution concentration: mg/litre

Initial concentration in bottle: mg/litre

ThOD or COD: mg O_2/mg test substance

4. INOCULUM

Source:

Treatment given:

Pre-conditioning if any:

Concentration in the reaction mixture: mg/litre

5. DO DETERMINATION

Method: Winkler/electrode

Flask Analyses

Time of incubation (d)			DO (mg/l)			
			0	n_1	n_2	
Blank (without chemical)	1	C_1				
	2	C_2				
Mean		$m_b = \dfrac{C_1 + C_2}{2}$				
Test chemical	1	a_1				
	2	a_2				
Mean		$m_t = \dfrac{a_1 + a_2}{2}$				

Note: Similar format may be used for reference compound and toxicity control.

6. CORRECTION FOR NITRIFICATION (see Annex V)

Time of incubation (d)	0	n_1	n_2	n_3
(i) Concentration of nitrate (mg N/litre)				
(ii) Change in nitrate concentration (mg N/litre)	—			
(iii) Oxygen equivalent (mg/litre)	—			
(iv) Concentration of nitrite (mg N/litre)				
(v) Change in nitrite concentration (mg N/litre)	—			
(vi) Oxygen equivalent (mg/litre)	—			
(iii + vi) Total oxygen equivalent (mg/litre)	—			

7. DO DEPLETION: % DEGRADATION

	Depletion after n days (mg/litre)			
	n_1	n_2	n_3	
FLASK 1: $(m_{to} - m_{tx}) - (m_{bo} - m_{bx})$				
FLASK 2: $(m_{to} - m_{tx}) - (m_{bo} - m_{bx})$				
FLASK 1: $\% D_1 = \dfrac{[(m_{to} - m_{tx}) - (m_{bo} - m_{bx})] \times 100}{\text{conc. of test} \times \text{ThOD chemical}}$				
FLASK 2: $\% D_2 = \dfrac{[(m_{to} - m_{tx}) - (m_{bo} - m_{bx})] \times 100}{\text{conc. of test} \times \text{ThOD chemical}}$				
$\% D \text{ mean } * = \dfrac{D_1 + D_2}{2}$				

(*) Do not take mean if there is considerable difference between duplicates.

m_{to} = value in the flask at time 0

m_{tx} = value in the flask at time x

m_{bo} = mean blank value at time 0

m_{bx} = mean blank value at time x

Apply also correction for nitrification from iii + vi in section 6.

8. BLANK DO DEPLETIONS

Oxygen consumption by blank: $(m_{bo} - m_{b28})$ mg/litre. This consumption is important for the validity of the test. It should be less than 1,5 mg/litre.

PART VII. M.I.T.I. TEST (METHOD C.4-F)

VII.1.   PRINCIPLE OF THE METHOD

The oxygen uptake by a stirred solution, or suspension, of the test chemical in a mineral medium, inoculated with specially grown, unadapted micro-organisms, is measured automatically over a period of 28 days in a darkened, enclosed respirometer at 25 ± 1 °C. Evolved carbon dioxide is absorbed by soda lime. Biodegradability is expressed as the percentage oxygen uptake (corrected for blank uptake) of the theoretical uptake (ThOD). The percentage of primary biodegradability is also calculated from supplemental specific chemical analysis made at the beginning and end of incubation and, optionally, by DOC analysis.

VII.2.   DESCRIPTION OF THE METHOD

VII.2.1. Apparatus

Automatic electrolytic BOD meter or respirometer normally equipped with 6 bottles, 300 ml each and equipped with cups to contain CO2 absorbent;

(b)   Constant temperature room and/ or water-bath at 25 °C $\pm$ 1 °C or better;

(c)   Membrane-filtration assembly (optional);

(d)   Carbon analyser (optional).

VII.2.2. Preparation of mineral medium

Prepare the following stock solutions, using analytical grade reagents and water (I.6.1.):

(a)   Monopotassium dihydrogen ortho phosphate, KH_2PO_4 8,50 g

Dipotassium monohydrogen ortho phosphate, K_2HPO_4 21,75 g

Disodium monohydrogen ortho phosphate dodecahydrate Na_2HPO_4 12 H_2O 44,60 g

Ammonium chloride, NH_4Cl 1,70 g

Dissolve in water and make up to 1 litre

The pH value of the solution should be 7,2

(b)   Magnesium sulphate heptahydrate, $MgSO_4$ 7 H_2O 22,50 g

Dissolve in water and make up to 1 litre

(c)   Calcium chloride anhydrous, $CaCl_2$ 27 ,50 g

Dissolve in water and make up to 1 litre

(d)   Iron (III) chloride hexahydrate, $FeCl_3$ 6 H_2O 0,25 g

Dissolve in water and make up to 1 litre

Take 3 ml of each solution (a), (b), (c) and (d) and make up to 1 litre.

VII.2.3. Preparation of inoculum

Collect fresh samples from no fewer than ten sites, mainly in areas where a variety of chemicals are used and discharged. From sites such as sewage treatment works, industrial waste-water treatment, rivers, lakes, seas, collect 11 samples of sludge, surface soil, water, etc. and mix thoroughly together. After removing floating matter and allowing to stand, adjust the supernatant to pH 7 ± 1 with sodium hydroxide or phosphoric acid.

Use an appropriate volume of the filtered supernatant to fill a fill-and-draw activated sludge vessel and aerate the liquid for about 23 1/2 h. Thirty minutes after stopping aeration, discard about one third of the whole volume of supernatant and add an equal volume of a solution (pH 7) containing 0,1 % each of glucose, peptone and monopotassium ortho phosphate, to the settled material and recommence aeration. Repeat this procedure once per day. The sludge unit must be operated according to good practice: effluents should be clear , temperature should be kept at 25 ± 2° C, pH should be 7 ± 1, sludge should settle well, sufficient aeration to keep the mixture aerobic at all times, protozoa should be present and the activity of the sludge should be tested against a reference substance at least every three months. Do not use sludge as inoculum until after at least one month's operation, but not after more than four months. Thereafter, sample from at least 10 sites arregular intervals, once every three months.

In order to maintain fresh and old sludge at the same activity, mix the filtered supernatant of an activated sludge in use with an equal volume of the filtered supernatant of a freshly collected ten-source mixture and culture the combined liquor as above. Take sludge for use as inoculum 18-24 h after the unit has been fed.

VII.2.4. Preparation of flasks

Prepare the following six flasks:

Nr. 1: test chemical in dilution water at 100 mg/1

Nr. 2,3 and 4: test chemical in mineral medium at 100 mg/1

Nr. 5: reference chemical (e.g. aniline) in mineral medium at 100 mg/1

Nr. 6: mineral medium only

Add poorly soluble test chemicals directly on a weight or volume basis or handle as described in Annex III, except that neither solvents nor emulsifying agents should be used. Add the CO_2 absorbent to all flasks in the special cups provided. Adjust the pH in flasks nr. 2, 3 and 4 to 7,0.

VII.2.5. Performance of the test

Inoculate flasks nr. 2, 3 and 4 (test suspensions), nr. 5 (activity control) and nr. 6 (inoculum blank) with a small volume of the inoculum to give a concentration of 30 mg/1 suspended solids. No inoculum is added to flask nr. 1 which serves as an abiotic control. Assemble the equipment, check for air-tightness, start the stirrers, and start the measurement of oxygen uptake under conditions of darkness. Daily check the temperature, stirrer and coulometric oxygen uptake recorder, and note any changes in colour of the contents of the flasks. Read the oxygen uptakes for the six flasks directly by an appropriate method, for example, from the six-point chart recorder, which produces a BOD curve. At the end of incubation, normally 28 days, measure the pH of the contents of the flasks and determine the concentration of the residual test chemical and any intermediate and, in the case of water soluble substance, the concentration of DOC {Annex II.4). Take special care in the case of volatile chemicals. If nitrification is anticipated, determine nitrate and nitrite concentration, if possible.

VII.3.   DATA AND REPORTING

VII.3.1. Treatment of results

Divide the oxygen uptake (mg) by the test chemical after a given time, corrected for that taken up by the blank inoculum conttol after the same time, by the weight of the test chemical used. This yields the BOD expressed as mg oxygen/mg test chemical, that is:

BOD =   (mg O_2 uptake by test chemical -mg O_2 uptake by blank) / (mg test chemical in flask)

=   mg O_2 per mg test chemical

The percentage biodegradation is then obtained from:

–   % biodegradation = % ThOD = BOD (mg O_2/mg chemical) x 100 / ThOD (mg O_2/mg chemical)

For mixtures, calculate the ThOD from the elemental analysis, as for simple compound. Use the appropriate ThOD ($ThOD_{NH4}$ or $ThOD_{NO3}$) according to whether nitrification is absent or complete (Annex II.2). If however, nitrification occurs but is incomplete, make a correction for the oxygen consumed by nitrification calculated from the changes in concentrations of nitrite and nitrate (Annex V).

Calculate the percentage primary biodegradation from loss of specific (parent) chemical (see 1.7,2).

$$D_t = \frac{S_b - S_a}{S_b} x100\%$$

If there has been a loss of test chemical in the flask nr. 1 measuring physico-chemical removal, report this and use the concentration of test chemical (S_b) after 28 days in this flask to calculate the percentage biodegradation.

When determinations of DOC are made (optional), calculate the percentage ultimate biodegradation from:

$$D_t = \left(1 - \frac{C_t - C_{bt}}{C_o - C_{bo}}\right) x100\%$$

as described under point I.7.1. If there has been a loss of DOC in the flask nr .1, measuring physico-chemical removal, use the DOC concentration in this flask to calculate the percentage biodegradation.

Record all results on the data sheets attached.

VII.3.2. Validity of results

The oxygen uptake of the inoculum blank is normally 20-30 mg O_2/1 and should not be greater than 60 mgl/l in 28 days. Values higher than 60 mgl/l require critical examination of the data and experimental techniques. If the pH value is outside the range 6-8,5 and the oxygen consumption by the test chemical is less than 60 % , the test should be repeated with a lower concentration of test chemical.

See also I.5.2.

If the percentage degradation of aniline calculated from the oxygen consumption does not exceed 40% after 7 days and 65% after 14 days, the test is regarded as invalid.

VII.3.3. Reporting

See I.8.

VII.4.   DATA SHEET

An example of a data sheet is given below.

MITI (I) TEST

1. LABORATORY

2. DATE AT START OF TEST

3. TEST SUBSTANCE

Name:

Stock solution concentration: mg/1 as chemical

Initial concentration in medium, C_o: mg/1 as chemical

Volume of reaction mixture, V: ml

ThOD: mg O_2/1

4. INOCULUM

Sludge sampling sites:

1)… 6)…

2)… 7)…

3)… 8)…

4)… 9)…

5)… 10)…

Concentration of suspended solids in activated sludge after acclimatization with synthetic sewage = ... mg/1

Volume of activated sludge per litre of final medium = ...ml

Concentration of sludge in final medium = ...mg/1

5. OXYGEN UPTAKE: BIODEGRADABILITY

Type of respirometer used:

		Zeit (Tage)				
		0	7	14	21	28
O_2 upt. (mg) test chemical	a_1					
	a_2					
	a_3					
O_2 upt. (mg) blank	b					
Corrected O_2 upt. (mg)	$(a_1 - b)$ $(a_2 - b)$ $(a_3 - b)$					
BOD per mg test chemical	$\dfrac{(a - b)}{C_o V}$	Flask 1					
		Flask 2					
		Flask 3					
% degradation $\dfrac{BOD}{ThOD} \times 100$		1					
		2					
		3					
		mean *					

Note: Similar formats may be used for the reference compound.

* Do not take a mean if there are considerable differences betwen replicates.

6. **CARBON ANALYSIS** (optional):

Carbon analyser:

Flask	DOC				% DOC removed	Mean
	Measured		Corrected			
Water + test substance	a				—	—
Sludge + test substance	b1		b1 – c			
Sludge + test substance	b2		b2 – c			
Sludge + test substance	b3		b3 – c			
Control blank	c		—		—	—

$$\% \text{ DOC removed: } \frac{a - (b - c)}{a} \times 100$$

7. **SPECIFIC CHEMICAL ANALYTICAL DATA**

	Residual amount of test chemical at end of test	% degradation
blank test with water	S_b	
inoculated medium	S_{a1}	
	S_{a2}	
	S_{a3}	

$$\% \text{ degradation} = \frac{S_b - S_a}{S_b} \times 100$$

Calculate % degradation for flasks a1, a2 and a3 respectively

8. **REMARKS**

BOD curve against time, if available, should be attached.

ABBREVIATIONS AND DEFINITIONS

DO: Dissolved oxygen (mg/l) is the concentration of oxygen dissolved in an aqueous sample.

BOD: Biochemical oxygen demand (g) is the amount of oxygen consumed by micro-organisms when metabolizing a test compound; also expressed as g oxygen uptake per g test compound. (See method C.5).

COD: Chemical oxygen demand (g) is the amount of oxygen consumed during oxidation of a test compound with hot, acidic dichromate; it provides a measure of the amount of oxidisable matter present; also expressed as 9 oxygen consumed per g test compound. (See method C.6).

DOC: Dissolved organic carbon is the organic carbon present in solution or that which passes through a 0,45 micrometre filter or remains in the supernatant after centrifuging at 40000 m.s^{-2} (± 4000 g) for 15 min.

ThOD: Theoretical oxygen demand (mg) is the total amount of oxygen required to oxidise a chemical completely; it is calculated from the molecular formula (see Annex II.2) and is also expressed as mg oxygen required per mg test compound.

$ThCO_2$: Theoretical carbon dioxide (mg) is the quantity of carbon dioxide calculated to be produced from the known or measured carbon content of the test compound when fully mineralized; also expressed as mg carbon dioxide evolved per mg test compound.

TOC: Total organic carbon of a sample is the sum of the organic carbon in solution and in suspension.

IC: Inorganic carbon

TC: Total carbon, is the sum of the organic and inorganic carbon present in a sample.

Primary Biodegradation:

is the alteration in the chemical structure of a substance, brought about by biological action, resulting in the loss of specific property of that substance.

Ultimate Biodegradation (aerobic):

is the level of degradation achieved when the test compound is totally utilised by micro-organisms resulting in the production of carbon dioxide, water, mineral salts and new microbial cellular constituents (biomass).

Readily Biodegradable:

an arbitrary classification of chemicals which have passed certain specified screening tests for ultimate biodegradability; these tests are so stringent that it is assumed that such compounds will rapidly and completely biodegrade in aquatic environments under aerobic conditions.

Inherently Biodegradable:

a classification of chemicals for which there is unequivocal evidence of biodegradation (primary or ultimate) in any recognized test of biodegradability .

Treatability:

is the amenability of compounds to removal during biological wastewater treatment without adversely affecting the normal operation of the treatment processes. Generally, readily biodegradable compounds are treatable but not all inherently biodegradable compounds are. Abiotic processes may also operate.

Lag time

is the time from inoculation, in a die-away test, until the degradation percentage has increased to at least 10%. The lag time is often highly variable and poorly reproducible.

Degradation time

is the time from the end of the lag time till the time that 90% of maximum level of degradation has been reached.

l0-day window

is the 10 days immediately following the attainment of 10% degradation.

ANNEX II
CALCULATION AND DETERMINATION OF SUITABLE SUMMARY PARAMETERS

Depending on the method chosen, certain summary parameters will be required. The following section describes the derivation of these values. The use of these parameters is described in the individual methods.

1. Carbon Content

The carbon content is calculated from the known elemental composition or determined by elemental analysis of the test substance.

2. Theoretical oxygen demand (ThOD)

The theoretical oxygen demand (ThOD) may be calculated if the elemental composition is known or derermined by elemental analysis. It is for the compound:

$$C_cH_hCl_{cl}N_nNa_{na}O_oP_pS_s$$

without nitrification,

$$\text{ThOD}_{NH4} = \frac{16[2c + 1/2(h - cl - 3n) + 3s + 5/2p + 1/2na - o]}{MW}\,mg/mg$$

or with nitrification

$$\text{ThOD}_{\text{NO3}} = \frac{16[2c + 1/2(h - cl) + 5/2n + 3s + 5/2p + 1/2na - o]}{\text{MW}} \, \text{mg/mg}$$

3. Chemical Oxygen Demand (COD)

The Chemical oxygen demand (COD) is determined according to method C.6.

4. Dissolved Organic Carbon (DOC)

Dissolved organic carbon (DOC) is by definition the organic carbon of any chemical or mixture in water passing through a 0,45 micrometre filter.

Samples from the test vessels are withdrawn and filtered immediately in the filtration apparatus using an appropriate membrane filter. The first 20 ml (amount can be reduced when using small filters) of the filtrate are discarded. Volumes of 10-20 ml or lower, if injected (volume depending on the amount required for carbon analyser) are retained for carbon analysis. The DOC-concentration is determined by means of an organic carbon analyser which is capable of accurately measuring a carbon concentration equivalent or lower than 10% of the initial DOC-concentration used in the test.

Filtered samples which cannot be analysed on the same working day can be preserved by storage in a refrigerator at 2-4 °C for 48 h, or below -18 °C for longer periods.

Remarks:

Membrane filters are often impregnated with surfactants for hydrophilisation. Thus the filter may contain up to several mg of soluble organic carbon which would interfere in the biodegradability determinations. Surfactants and other soluble organic compounds are removed from the filters by boiling them in deionised water for three periods each of one hour. The filters may then be stored in water for one week. If disposable filter cartridges are used each lot must be checked to confirm that it does not release soluble organic carbon.

Depending on the type of membrane filter the test chemical may be retained by adsorption. It may therefore be advisable to ensure that the test chemical is not retained by the filter.

Centrifugation at 40000 m.sec^{-2} (4000 g) for 15 min may be used for differentiation of TOC versus DOC instead of filtration. The method is not reliable at initial concentration of < 10 mg DOC/l since either not all bacteria are removed or carbon as part of the bacterial plasma is redissolved.

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– Gerike, P. , The biodegradability testing of poorly water soluble compounds. Chemosphere, 1984, vol 13 (1), 169.

ANNEX III
EVALUATION OF THE BIODEGRADABILITY OF POORLY SOLUBLE SUBSTANCES

In biodegradability tests with poorly soluble substances the following aspects should receive special attention.

While homogeneous liquids will seldom present sampling problems, it is recommended that solid materials be homogenised by appropriate means to avoid errors due to non-homogeneity. Special care must be taken when representative samples of a few milligrams are required from mixtures of chemicals or substances with large amounts of impurities.

Various forms of agitation during the tests may be used. Care should be taken to use only sufficient agitation to keep the chemical dispersed, and to avoid overheating, excessive foaming and excessive shear forces.

An emulsifier which gives a stable dispersion of the chemical may be used. It should not be toxic to bacteria and must not be biodegraded or cause foaming under test conditions.

The same criteria apply to solvents as to the emulsifiers.

It is not recommended that solid carriers be used for solid test substances but they may be suitable for only substances.

When auxiliary substances such as emulsifiers, solvents and carriers are used, a blank run containing the auxiliary substance should be performed.

Any of the three respirometric tests CO_2, BOD, MITI can be used to study the biodegradability of poorly soluble compounds.

BIBLIOGRAPHY

– de Morsier, A. et al., Biodegradation tests for poorly soluble compounds. Chemosphere, 1987, vol. 16, 833.

– Gerike, P ., The Biodegradability testing of poorly water soluble compounds. Chemosphere, 1984, vol. 13, 169.

ANNEX IV
EVALUATION OF THE BIODEGRADABILITY OF CHEMICALS SUSPECTED TO BE TOXIC TO THE INOCULUM

When a chemical is subjected to ready biodegradability testing and appears to be non-biodegradable, the following procedure is recommended if a distinction between inhibition and inertness is desired (Reynolds et al., 1987).

Similar or identical inocula should be used for the toxicity and biodegradation tests.

To assess the toxicity of chemicals studied in ready biodegradability tests, the application of one or a combination of the inhibition of Sludge Respiration rate (activated sludge respiration inhibition test -Dir 87/302/EEC), BOD and/or Growth Inhibition methods would seem appropriate.

If inhibition due to toxicity is to be avoided, it is suggested that the test substance concentrations used in ready biodegradability testing should be less than 1/10 of the EC_{50} values (or less than EC_{20} values) obtained in toxicity testing. Compounds with an EC_{50} value of greater than 300 mg/1 are not likely to have toxic effects in ready biodegradability testing.

EC_{50} values of less than 20 mg/1 are likely to pose serious problems for the subsequent testing. Low test concentrations should be employed, necessitating the use of the stringent and sensitive Closed Bottle test or the use of ^{14}C -labelled material. Alternatively, an acclimatised inoculum may permit higher test substance concentrations to be used. In the latter case, however, the specific criterion of the ready biodegradability test is lost.

BIBLIOGRAPHY

Reynolds, L. et al. , Evaluation of the toxicity of substances to be assessed for biodegradability. Chemosphere, 1987, vol. 16, 2259.

ANNEX V
CORRECTION FOR OXYGEN UPTAKE FOR INTERFERENCE BY NITRIFICATION

Errors due to not considering nitrification in the assessment by oxygen uptake of the biodegradability of test substances not containing N are marginal (not greater than 5 %), even if oxidation of the ammonium-N in the medium occurs erratically as between test and blank vessels. However, for test substances containing N, serious errors can arise.

If nitrification has occurred but is not complete the observed oxygen uptake by the reaction mixture may be corrected for the amount of oxygen used in oxidising ammonium to nitrite and nitrate, if the changes in concentration during incubation of nitrite and nitrate are determined by consideration of the following equations:

$$2\,NH_4Cl + 3O_2 = 2\,HN\,O_2 + 2\,HCI + 2\,H_2O \tag{1}$$

$$2\,HNO_2 + O_2 = 2\,HNO_3 \tag{2}$$

Overall:

$$2\,NH_4Cl + 4\,O_2 = 2\,HNO_3 + 2\,HCl + 2\,H_2O \tag{3}$$

From equation (1), the oxygen uptake by 28 g of nitrogen contained in ammonium chloride (NH_4Cl) in being oxidised to nitrite is 96 g, i.e. a factor of 3,43 (96/28). In the same way, from

equation (3) the oxygen uptake by 28 g of nitrogen in being oxidised to nitrate is 128 g, i.e. a factor of 4,57 (128/28).

Since the reactions are sequential, being carried out by distinct and different bacterial species, it is possible for the concentration of nitrite to increase or decrease; in the latter case an equivalent concentration of nitrate would be formed. Thus, the oxygen consumed in the formation of nitrate is 4,57 multiplied by the increase in concentration of nitrate, whereas the oxygen associated with the formation of nitrite is 3,43 multiplied by the increase in the concentration of nitrite or with the decrease in its concentration the oxygen loss is -3,43 multiplied by the decrease in concentration.

That is:

O_2 consumed in nitrate formation = 4,57 x increase in nitrate concentration   (4)

and

O_2 consumed in nitrite formation = 3,43 x increase in nitrite concentration   (5)

and

O_2 lost in nitrite disappearance = -3,43 x decrease in nitrate concentration   (6)

So that

O_2 uptake due to nitrification = ± 3,43 x change in nitrite conc. + 4,57 x increase in   (7)
nitrate conc.

and thus

O_2 uptake due to C oxidation = total observed uptake uptake due to nitrification   (8)

Alternatively, if only total oxidized N is determined, the oxygen uptake due to nitrification may be taken to be, as a first approximation, 4,57 x increase in oxidised N

The corrected value for oxygen consumption due to C oxidation is then compared with ThOD NH_3, as calculated in Annex II.

C.5 DEGRADATION -BIOCHEMICAL OXYGEN DEMAND

1.   METHOD

1.1.   INTRODUCTION

The purpose of the method is the measurement of the biochemical oxygen demand (BOD) of solid or liquid organic substances.

Data elaborated with this test pertain to water-soluble compounds; however, volatile compounds and those of low water solubility may also, at least in principle, be tested.

The method is applicable only to those organic test materials which are not inhibitory to bacteria at the concentration used in the test. If the test material is not soluble at the test concentration, special measures, such as the use of ultrasonic dispersion, may have to be employed to achieve good dispersion of test material.

Information on the toxicity of the chemical may be useful to the interpretation of low results and in the selection of appropriate test concentrations.

1.2.   DEFINITION AND UNITS

The BOD is defined as the mass of dissolved oxygen required by a specified volume of solution of the substance for the process of biochemical oxidation under prescribed conditions.

The results are expressed as grams of BOD per gram of tested substance.

1.3.   REFERENCE SUBSTANCES

The use of a suitable reference substance to check the activity of the inoculum is desirable.

1.4.   PRINCIPLE OF THE TEST METHOD

A predetermined amount of the substance, dissolved or dispersed in a well-aerated suitable medium, is inoculated with micro-organisms and incubated at a constant defined ambient temperature in the dark.

The BOD is determined by the difference in dissolved oxygen content at the beginning and at the end of the test. The duration of the test must be at least five days and not more than 28 days.

A blank must be determined in a parallel assay containing no test substance.

1.5. QUALITY CRITERIA

The BOD determination cannot be considered as a valid determination of the biodegradability of a substance. This test may only be regarded as a screening test.

1.6. DESCRIPTION OF THE TEST METHOD

A preliminary solution or dispersion of the substance is prepared to obtain a BOD concentration compatible with the method used. The BOD is then determined following any suitable national or international standardized method.

2.   DATA AND EVALUATION

The BOD contained in the preliminary solution is calculated according to the selected normalized method, and converted into grams of BOD per gram of tested substance.

3.   REPORTING

The method used shall be stated.

The biochemical oxygen demand should be a mean of at least three valid measurements.

All information and remarks relevant for the interpretation of results have to be reported, especially with regard to impurities, physical state, toxic effects and inherent composition of the substance which would affect the results.

The use of an additive to inhibit biological nitrification must be reported.

4.   REFERENCES

List of standardized methods, for example:

NF T 90 -103: Determination of the biochemical oxygen demand.

NBN 407: Biochemical oxygen demand.

NEN 32355.4: Bepaling van het biochemish zuurstofverbruik (BZV).

The determination of biochemical oxygen demand, Methods for the examination of water and associated materials, HMSO, London.

ISO 5815: Determination of biochemical oxygen demand after n days.

C.6. DEGRADATION - CHEMICAL OXYGEN DEMAND

1. METHOD

1.1. INTRODUCTION

The purpose of the method is the measurement of the chemical oxygen demand (COD) of solid or liquid organic substances in a standard, arbitrary manner, under fixed laboratory conditions.

Information on the formula of the substance will be useful to conduct this test and interpret the result obtained (e.g. halogen salts, ferrous salts of organic compounds, organochlorine compounds).

1.2. DEFINITIONS AND UNITS

The chemical oxygen demand is a measure of the oxidizability of a substance, expressed as the equivalent amount in oxygen of an oxidizing reagent consumed by the substance under fixed laboratory conditions.

The result is expressed in grams of COD per gram of tested substance.

1.3. REFERENCE SUBSTANCES

Reference substances do not need to be employed in all cases when investigating a new substance. They should serve primarily to calibrate the method from time to time and to allow comparison of results when another method is applied.

1.4. PRINCIPLE OF THE TEST METHOD

A predetermined amount of the substance, dissolved or dispersed in water, is oxidized by potassium dichromate in a strong sulphuric acid medium with silver sulphate as a catalyst, under reflux for two hours. The residual dichromate is determined by titration with standardized ferrous ammonium sulphate.

In case of chlorine-containing substances, mercuric sulphate () is added to reduce chloride interference.

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[1] After use, solutions containing mercury salts should be treated to avoid dissemination of mercury in the environment.

1.5. QUALITY CRITERIA

Because of the arbitrary manner of determination, COD is an 'oxidizability indicator' and as such is used as a practical method to measure organic matter.

Chloride can interfere in this test; inorganic reducing or oxidizing agents may also interfere with the COD determination.

Some cyclic compounds and many volatile substances (e.g. lower fatty acids) are not fully oxidized by this test.

1.6. DESCRIPTION OF THE TEST METHOD

A preliminary solution or dispersion of the substance is prepared to obtain a COD between 250 and 600 mg per litre.

Remarks:

In the case of poorly soluble and non-dispersible substances, an amount of finely powdered substance or liquid substance corresponding to about 5 mg of COD can be weighed and put in the experimental apparatus with water.

The chemical oxygen demand (COD) is often and especially in case of poorly soluble substances determined advantageously in a variant of the method, i.e., in a closed system with a pressure equaliser (H. Kelkenberg, 1975). In this modification compounds which are only with difficulty determined by the conventional method - e.g. acetic acid - may often be successfully quantified. The method also fails, however, in the case of pyridine. If the potassium dichromate concentration, as prescribed in ref.(1), is raised to 0,25 N (0,0416 M), the direct weighing-in of 5-10 mg of substance is facilitated which is essential for the COD determination of poorly water soluble substances (ref. (2)).

Otherwise, the COD is then determined following any suitable national or international standardized method.

2. DATA AND EVALUATION

The COD contained in the experimental flask is calculated following the selected normalized method, and converted to grams of COD per gram of tested substance.

3. REPORTING

The reference method used should be stated.

The chemical oxygen demand should be a mean of at least three measurements. All information and remarks relevant to the interpretation of the results have to be reported, especially with regard to impurities, physical state and inherent properties of the substance (if known) which would affect the results.

The use of mercuric sulphate to minimize the chloride interference must be reported.

4.   REFERENCES

(1)   Kelkenberg, H.,Z. von Wasser und Abwasserforschung, 1975, vol. 8, 146.

(2)   Gerike, P. The biodegradability testing of poorly water soluble compounds. Chemosphere, 1984, vol. 13,169.

List of standardized methods, for example:

NBN T 91-201 Determination of the chemical oxygen demand.

ISBN O 11 7512494 Chemical oxygen demand (dichromate value) of polluted and waste waters.

NF T 90-101 Determination of the chemical oxygen demand.

DS 217 = water analysis Determination of the chemical oxygen demand.

DIN 38409-H-41 Determination of the chemical oxygen demand (COD) within the range above 15 mg per litre.

NEN 3235 5.3 Bepaling van het chemisch zuurstofverbruik.

ISO 6060 Water quality: chemical oxygen demand dichromate methods.

C.7. DEGRADATION -
ABIOTIC DEGRADATION HYDROLYSIS AS A FUNCTION OF pH

1.   METHOD

This method is based on the OECD Test Guideline (1).

1.1.   INTRODUCTION

Hydrolysis is an important reaction controlling abiotic degradation. This reaction is particularly relevant for substances with low biodegradability; and it can influence the persistence of a substance in the environment.

Most hydrolysis reactions are of pseudo first-order and, therefore, half-life times are independent of concentration. This usually allows the extrapolation of results found at laboratory concentration to environmental conditions.

Furthermore, several examples have been reported (2), showing a satisfactory agreement between the results found in pure and natural waters for several types of chemicals.

It is useful to have preliminary information on the vapour pressure of the substance to perform this test.

This method is applicable only to water-soluble substances. Impurities may affect the results.

Hydrolytic behaviour of chemicals should be examined at pH values more commonly found in the environment (pH 4 to 9).

1.2.   DEFINITIONS AND UNITS

Hydrolysis refers to a reaction of a chemical RX with water. This reaction may be represented by the net exchange of the group X with OH:

$$RX + HOH \rightarrow ROH + HX \qquad\qquad [1]$$

The rate at which the concentration of RX decreases is given by:

$$rate = k\,[H2O]\,.[RX] \qquad\qquad [2]$$

Because water is present in great excess compared to the chemical, this type of reaction is usually described as a pseudo-first order reaction in which the observed rate constant is given by the relationship:

$$kobs = k \times [H2O] \qquad\qquad [3]$$

This constant can be determined for one pH value and one temperature, T, using the expression:

$$k_{obs} = \frac{2.303}{t} \times \log \frac{C_o}{C_t} \qquad\qquad [4]$$

where:

t   = time,

Co   = the concentration of the substance at time 0,

Ct   = the concentration of the substance at time t, and

2,303   = the conversion factor between natural and base 10 logarithms.

The concentrations are expressed in grams per litre or moles per litre.

The dimension of this constant kobs is (time)-1.

'The half-life period' t1/2, is defined as the time required to reduce the concentration of the test substance by 50%, that is:

$$Ct = 1/2.Co \qquad\qquad [5]$$

From the expressions (4) and (5) one can demonstrate that:

$$t1/2 = 0,693/kobs \qquad\qquad [6]$$

1.3.   REFERENCE SUBSTANCES

It is not necessary to use reference substances in all cases when investigating a new substance. They should serve primarily to check the performance of the method from time to time and to allow comparison with results from another method.

The following substances have been used as reference substances (1):

Acetylsalicylic acid (aspirin)

Phosphorothioic acid O,O-diethyl 0-(6-methyl-2-(1-methylethyl)4-pyrimidinyl) ester. (Dimpylate, Diazinon)

1.4.   PRINCIPLE OF THE TEST METHOD

The substance is dissolved in water at a low concentration; the pH and the temperature are controlled.

The decrease of the concentration of the substance with time is followed by any suitable analytical procedure.

The logarithm of the concentration is plotted against time and, if the plot is a straight line, the first-order rate constant may be obtained from its slope (see point 2).

When it is not practical to determine a rate constant directly for a particular temperature, it is usually possible to estimate the constant through the use of the Arrhenius relationship, which gives the temperature dependence of the rate constant. From the linear plot of the logarithm of the rate constant, as determined at appropriate temperature, as a function of the reciprocal of the absolute temperature, K, it is possible to extrapolate the rate constant value which was not directly obtainable.

1.5.   QUALITY CRITERIA

It is reported in reference (2) that measurements of hydrolysis rate-constants on 13 classes of organic structures can be of high precision.

The repeatability depends in particular on the control of the pH and the temperature and might be affected by the presence of micro-organisms and in special cases by the dissolved oxygen concentration.

1.6.   DESCRIPTION OF THE TEST METHOD

1.6.1.   Reagents

1.6.1.1. Buffer solutions

The test is carried out at three pH values: 4,0, 7,0 and 9,0.

For this purpose, buffer solutions should be prepared using reagent grade chemicals and distilled or deionized, sterile water. Some examples of buffer systems are presented in the Appendix.

The buffer system used may influence the rate of hydrolysis; if there is evidence of this, an alternative buffer system should be employed. The use of borate or acetate buffers is recommended in reference (2) instead of phosphate.

If the pH value of the buffer solutions is not known at the temperature used during the test, this can be determined with a calibrated pH meter at the selected temperature with a precision of ± 0,1 pH units.

1.6.1.2. Test solutions

The test substance should be dissolved in the selected buffer and the concentration should not exceed 0,01 M or half the saturation concentration, whichever is the lower.

The use of water-miscible organic solvents is recommended only for substances of low water solubility.

The amount of solvent should be less than 1%, and should not interfere with the hydrolytic process.

1.6.2. Apparatus

Stoppered glass flasks should be used, but grease must be avoided on the ground joint.

If the chemical or the buffer system is volatile, or if the test is being conducted at elevated temperatures, sealed or septum-closed tubes are preferred and head space should be avoided.

1.6.3. Analytical method

The method must be specific to allow determination of the test substance at the test solution concentrations and may well consist of some combination of suitable analytical techniques.

The analytical method used will depend on the nature of the substance and must be sufficiently precise and sensitive to detect a reduction of 10% of the initial concentration.

1.6.4. Test conditions

The tests will be carried out using a thermostatically controlled enclosure or a constant-temperature bath set at ± 0,5 °C of the chosen temperature. The temperature will be kept and measured to within ± 0,1 °C. Photolytic interference should be avoided by appropriate means.

For substances which are easily oxidizable, it will be necessary to exclude dissolved oxygen (e.g. by bubbling with nitrogen or argon for five minutes before preparation of the solution).

1.6.5. Test Procedure

1.6.5.1. Preliminary test

For all substances a preliminary test should be performed at 50 ± 0,5 °C at three pH values: 4,0, 7,0 and 9,0. A sufficient number of measurements are made, in order to be able to estimate whether, for each pH value and at 50 °C, the half-life time (t1/2) is lower than 2,4 hours or less than 10% of hydrolysis is observed after five days. (One can estimate that these values correspond respectively to half-life times lower than one day or higher than one year under conditions more representative of those of the environment (25 °C)). If the preliminary test indicates that 50% or more of the test substance has been hydrolyzed in 2,4 hours at 50 °C, or less than 10 % has been hydrolyzed after five days at each of the three pH values (4, 7 and 9), no further testing is necessary.

In other cases, and for individual pH values for which this condition has not been fulfilled, test 1 is carried out.

1.6.5.2. Test 1

Test 1 is carried out at one temperature; preferably at 50 ± 0,5 °C, and, if possible, under sterile conditions at those pH values for which the preliminary test has shown the necessity for further testing.

A sufficient number of samples (not less than four) should be chosen to cover the range 20 to 70% of hydrolysis to test for pseudo-first order behaviour at the specified pH values.

For each pH value at which test 1 is performed the order of reaction is determined.

Estimation of rate constant at 25 °C:

The decision on how to proceed experimentally depends on whether it may be concluded from test 1 that the reaction is pseudo-first order or not.

If it cannot be concluded with certainty from test 1 that the reaction is pseudo-first order, further experiments ,must be carried out as described in test 2.

If it can be safely concluded from test 1 that the reaction is pseudo-first order, further experiments should be carried out as described in test 3. (Alternatively, it may, under special circumstances, be possible to calculate the rate constants at 25 °C from constants at 50 °C, calculated using the results from test 1, (see 3.2)).

1.6.5.3. Test 2

This test is performed, at each pH value for which the results of test 1 have shown the necessity to do so:

–   either at one temperature lower than 40 °C,

–   or at two temperatures above 50 °C differing from each other by at least 10 °C.

For each pH value and temperature where test 2 is carried out, at least six adequately spaced data points should be taken so that the degrees of hydrolysis are in the range 20 to 70%.

For one pH value and one temperature, a determination is carried out in duplicate. When test 2 is done at two temperatures above 50 °C, the duplicate is preferably performed at the lower of these two temperatures.

For each pH value and temperature where test 2 is carried out, a graphical estimation of the half-life time (t1/2) will be given when possible.

1.6.5.4. Test 3

This test is carried out, at each pH value for which the results of test 1 have shown the necessity to do so.

–   either at one temperature lower than 40 °C,

–   or at two temperatures above 50 °C differing from each other by at least 10 °C.

For each pH and temperature where test 3 is performed, three data points are chosen, the first at time 0 and the second and third when the degree of hydrolysis is greater than 30%; the constant kobs and t1/2 should be calculated.

2. DATA

For pseudo-first order behaviour the values of kobs for each pH value and each temperature of the tests can be obtained from the plots of the logarithms of the concentration versus time using the expression:

$$kobs = -slope \times 2,303 \qquad [7]$$

Furthermore t1/2 can be calculated according to equation [6].

Evaluate k 25 °C by applying the Arrhenius equation where appropriate.

For non-pseudo-first order behaviour see 3.1.

3. REPORT

3.1 REPORTING

The test report shall, if possible, include the following information:

– specification of the substance;

– any results obtained with reference substances;

– the principle and details of the analytical method used;

– for each test: the temperature, pH value, buffer composition and a table of all concentration-time data points;

– for pseudo-first order reaction, the values of kobs of t1/2 and its calculation procedure;

– for non-pseudo-first order reaction, plot results as logarithm of concentration versus time;

– all information and observations necessary for the interpretation of the results.

3.2. INTERPRETATION OF RESULTS

It may be possible to calculate acceptable values of the rate constant (at 25 °C) of test substances, provided that experimental values of the activation energy already exist for homologues of the test substance and provided that it can be reasonably assumed that the activation energy of the test substance is of the same order of magnitude.

4. REFERENCES

(1) OECD, Paris, 1981, Test Guideline 111, Decision of the Council C(81) 30 final.

(2)   W. Mabey and T. Mill, 'Critical Review of Hydrolysis of Organic Compounds in Water Under Environmental Conditions,' J. Phys. Chem. Ref. Data, 1978, vol. 7 (2),383-415.

APPENDIX: BUFFER MIXTURES

A.   CLARK AND LUBS

The pH values reported in these tables have been calculated from the potential measurements using Sorensen standard equations. The actual pH value are 0,04 unit higher than the tabulated values.

Composition	pH
0,1 M potassium hydrogen phthalate + 0,1 N HCI at 20 °C	
2,63 ml 0,1 N HCI + 50 ml phthalate to 100 ml	3,8
0,1 M potassium hydrogen phthalate + 0,1 N NaOH at 20 °C	
0,40 ml 0,1 N NaOH + 50 ml phthalate to 100 ml	4,0
3,70 ml 0,1 N NaOH + 50 ml phthalate to 100 ml	4,2
0,1 M monopotassium phosphate + 0,1 N NaOH at 20 °C	6,8
29,63 ml 0,1 N NaOH + 50 ml phosphate to 100 ml	7,0
0,1 M H3BO3 in 0,1 M KCI + 0,1 N NaOH at 20 °C	
16,30 ml 0,1 N NaOH + 50 ml boric acid to 100 ml	8,8
21,30 ml 0,1 N NaOH + 50 ml boric acid to 100 ml	9,0
26,70 ml 0,1 N NaOH + 50 ml boric acid to 100 ml	9,2

B.   KOLTHOFF AND VLEESHOUWER

Composition	pH
0,1 M monopotassium citrate and 0,1 N NaOH at 18 °C (add tiny crystal of thymol to prevent growth of moulds)	
2,0 ml 0,1 N NaOH + 50 ml citrate to 100 ml	3,8
9,0 ml 0,1 N NaOH + 50 ml citrate to 100 ml	4,0
16,3 ml 0,1 N NaOH + 50 ml citrate to 100 ml	4,2

C.   SÖRENSEN

0,05 M borax + 0,1 N HCI

Composition		pH			
ml Borax	ml HCl	Sörensen 18 °C	Walbum		
			10 °C	40 °C	70 °C
8,0	2,0	8,91	8,96	8,77	8,59
8,5	1,5	9,01	9,06	8,86	8,67
9,0	1,0	9,09	9,14	8,94	8,74
9,5	0,5	9,17	9,22	9,01	8,80
10,0	0,0	9,24	9,30	9,08	8,86

0,05 M borax + 0,1 N NaOH

Composition		pH			
ml Borax	ml NaOH	Sörensen 18 °C	Walbum		
			10 °C	40 °C	70 °C
10,0	0,0	9,24	9,30	9,08	8,86
9,0	1,0	9,36	9,42	9,18	8,94
8,0	2,0	9,50	9,57	9,30	9,02
7,0	3,0	9,68	9,76	9,44	9,12

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 $6,0 \pm 0,5$.

(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: 1000; 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,]. R., 1977, Biology of Earthworms, Chapman and Hall, london, 331 pp.

3) Bouche. M. B., 1972, Lombriciens de France, Ecologie et Systematique, Institut National de la Recherche Agronomique, 671 pp.

4) Litchfield, J. T. andWilcoxon, F., A simplified method of evaluation dose effect experiments. I. 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 maybe 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 shaIlow 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 1000 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':

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

where:

DT   = biodegradation (%) at time T,

CA   = 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),

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

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

CBA   = 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, NH4Cl, A.R.:	38,5 g
sodium dihydrogenphosphate, NaH2PO4.2H2O, A.R.:	33,4 g,
potassium dihydrogenphosphate, KH2PO4, A.R.:	8,5 g,
di-potassium mono-hydrogenphosphate, K2HPO4, 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 3550 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 1000 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 arid 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 under 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.

Further 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 14C-labelled test substance may allow greater sensitivity. In the case of 14C test compound, the recovery of the 14CO2 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/1
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 1000 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
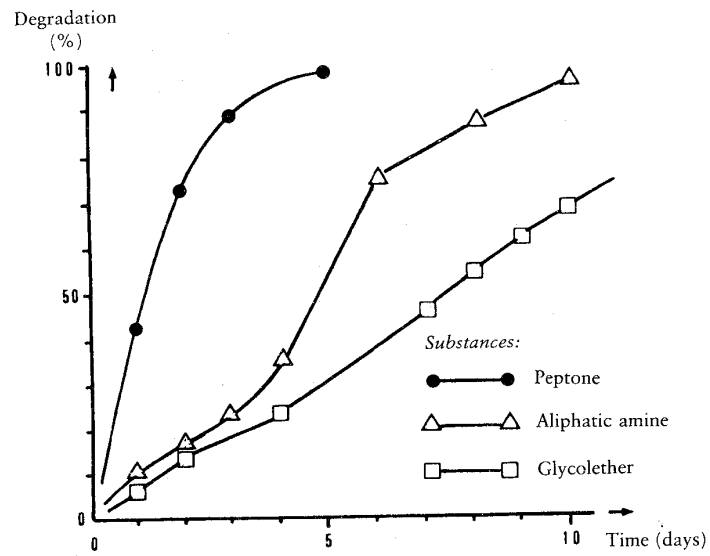
Figure 2

Examples of sludge adaptation
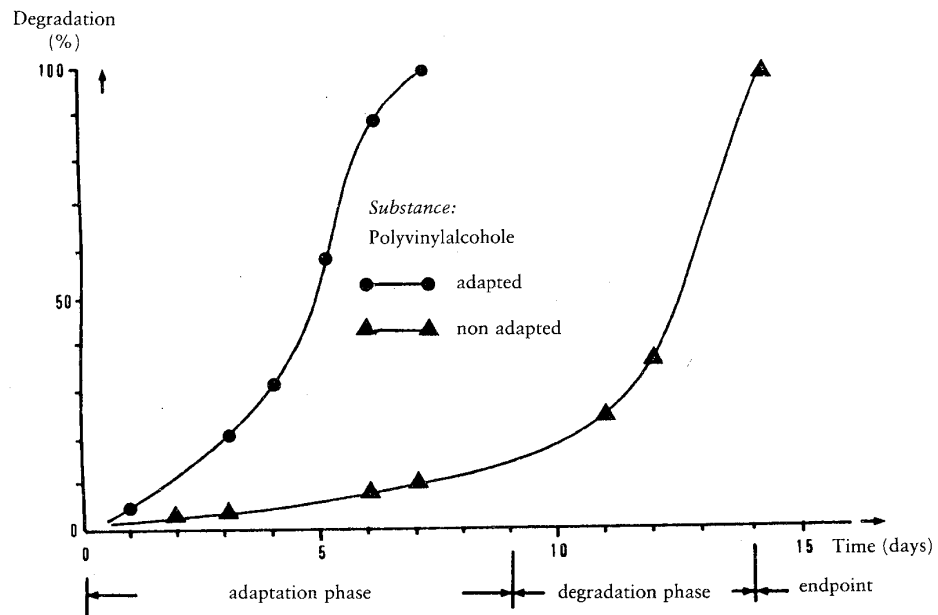
C. 10. BIODEGRADATION

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:

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

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:

$$R_W = \frac{C_I - C_o}{C_I} \times 100\% \quad [1(b)]$$

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 regulariy 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:	10 mg,
Urea:	30 mg,
NaCl:	7 mg,
$CaCl_2 .2H_2O$:	4 mg,
$MgSO_4 .7H_2O$:	2 mg,

K$_2$HPO$_4$:   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 1000 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 usually 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 or40 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.

$$DRc = \frac{4}{3}DR - \frac{100}{3} \quad [2]$$

$$DRc = \frac{8}{7}DR - \frac{100}{7} \quad [3]$$

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

$$^{s}DRc = \sqrt{\frac{\sum_{i=1}^{n}\left(\overline{DRc} - DRc_{i}\right)^{2}}{n-1}} \qquad [4]$$

where:

^{S}DRc = standard deviation of the series of DRc values,

$\overline{DR}_{c}$ = 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

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

where:

$t_{n-1\;;\alpha}$ = table value of t for n value pairs of E and E_{o} and statistical confidence P (P =1-α) 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:

$$\text{percentage removal of COD or DOC} = \frac{CODorDOCsewage - CODorDOCeffluent}{CODorDOCsewage} \times 100$$

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:

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

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-α) 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 analyses 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, FeCl3 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 COP 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.

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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 7Y404/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 Ausreßertests, insbesondere bei Ringversuchen zur Oberprüfung analytisch-chemischer Untersuchungsmethoden, Fresenius-Zeitschrift für Analytische Chemie, 303 (1980), pp. 406 to 408.

air

air

A

B

C

D

E

F

G

H

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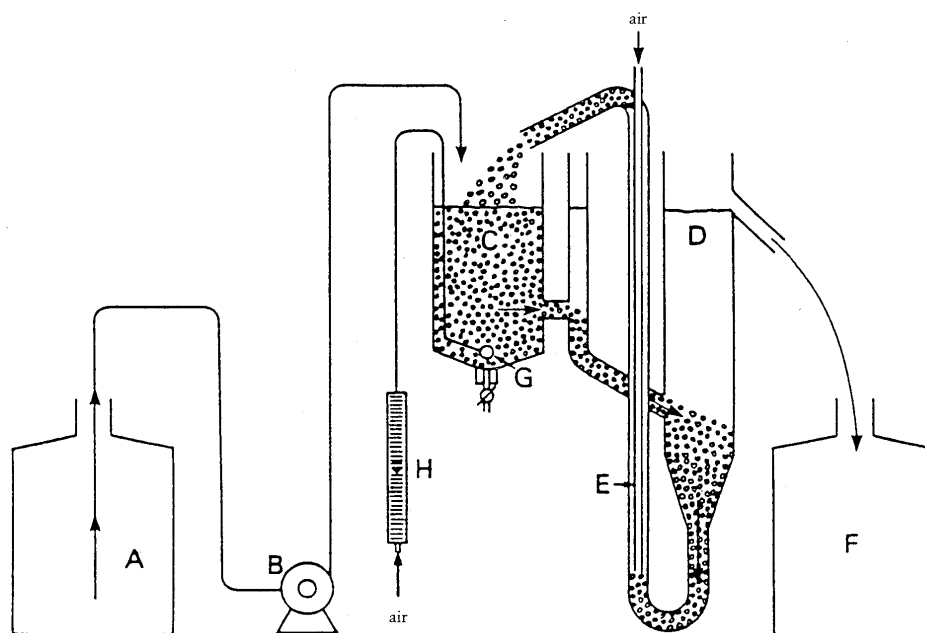
Key: A = storage vessel;   E = air lift;
         B = dosing device;   F = collector;
         C = aeration chamber (3 l capacity);   G = aerator;
         D = settling vessel;   H = air flow meter (optional).

Figure 2

Figure 1

Equipment used for assessing biodegradability

Key: A = storage vessel;   E = effluent collection vessel;
      B = dosing pump;   F = diffuser-stone aerator;
      C = porous aeration vessel;   G = rotameter (optional).
      D = outer impermeable vessel;
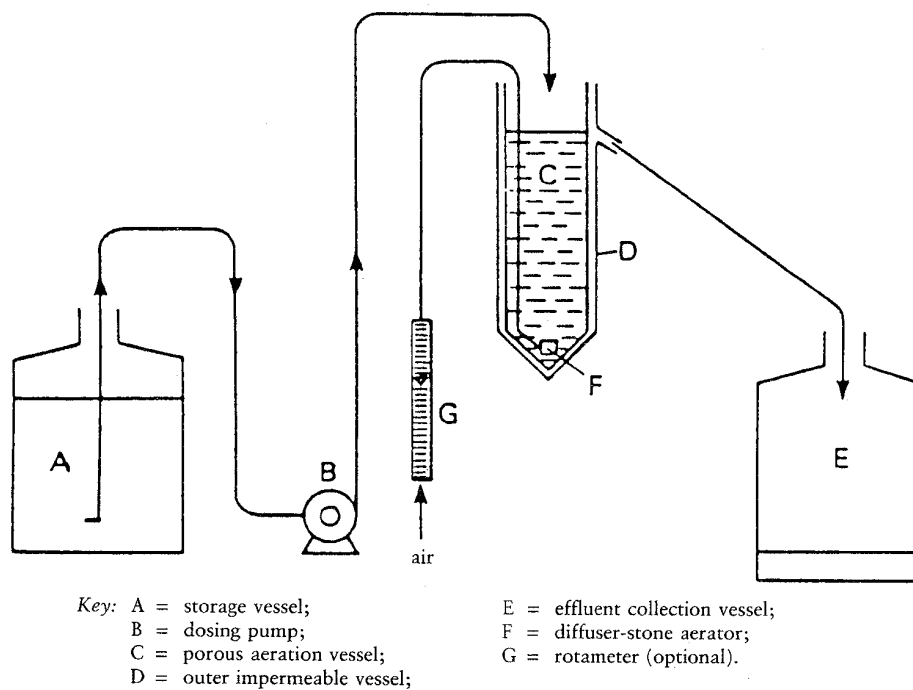
Figure 2

Details of three-litre porous-pot aeration vessel
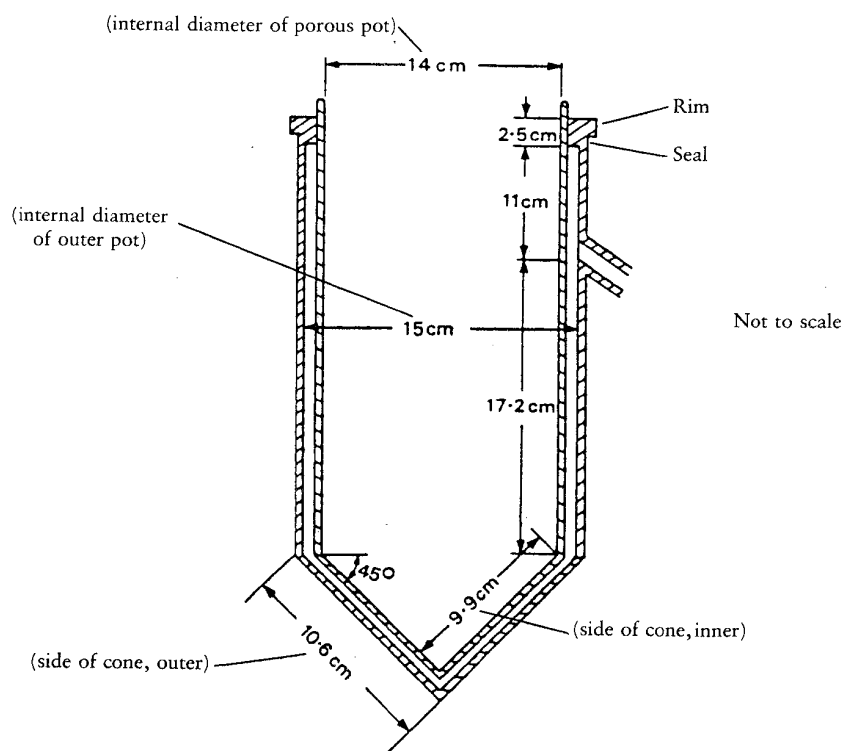
C. 11. BIODEGRADATION

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:

$$\left(1 - \frac{2R_s}{R_{c1} + R_{c2}}\right) \times 100 = \text{per cent inhibition}$$

where:

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

R_{c1} = oxygen-consumption rate, control 1,

R_{c2} = 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 5,5-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 measurement 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 Tox.icological Association of Dyestuffs Manufacturing Industries), Recommended Method No 103, also described by:

5) Robra, B., Wasserl 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:

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

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

where

$$D_{da} = \text{degradation/daily addition.}$$

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

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

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

where

$$D_{ssd} = \text{degradation/substance start of day;}$$

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 degrss 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.

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. 12. BIODEGRADATION

MODIFIED SCAS TEST

1. METHOD

1.1. INTRODUCTION

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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.

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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} - $C_{inorganic}$.

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

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–   are not inhibitory to bacteria,

–   do not significantly adsorb within the test system,

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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.

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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

$$D_{da} = \text{degradation/daily addition.}$$

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

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

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

where

$$D_{ssd} = \text{degradation/substance start of day;}$$

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.

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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).

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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.

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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.

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 $\mu g/g$ (ppm)) divided by the concentration of the chemical in the surrounding medium (C_w as $\mu g/ml$ (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 $\pm 20\%$ 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:

–   solubility in water

–   octanol-water partition coefficient P_{ow} (denoted also as K_{ow}, determined by an HPLC method in A.8)

–   hydrolysis

–   phototransformation in water determined under solar or simulated solar irradiation and under the irridiation conditions of the test for bioconcentration (3)

–   surface tension (i.e. for substances where the log P_{ow} cannot be determined)

–   vapour pressure

–   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 $\pm 2^oC$;

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

–   the concentration of the test substance in the chambers is maintained within $\pm 20\%$ 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 $\pm$ 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 ($\pm$ 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 should 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 appear 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.

2.3. 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;

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

–   the steady-state bioconcentration factor (BCFss) and/or kinetic concentration factor (BCFK) 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.

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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

(1) Meyer A., Orti G. (1993) Proc. Royal Society of London, Series B., Vol. 252, p. 231

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:

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

where

$$s = \text{solubility (moles/l) : (n=36)}$$

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:

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

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:

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

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):

$$0.80 = 1 \, e^{k_2 t \, 80} \quad \text{or} \quad t_{80} = \tfrac{1.6}{k_2} \qquad \text{[equation 5]}$$

Similarly 95 percent of steady-state is:

$$t_{95} = \tfrac{3.0}{k_2} \qquad\qquad\qquad \text{[equation 6]}$$

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):

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

or up (80 %) = 1.6/0.652, i.e. 2.45 days (59 hours)

up (95 %) = 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 %)	=	1.6/0.246, i.e. 6.5 days (156 hours)
or up (95 %)	=	3.0/0.246, i.e. 12.2 days (293 hours)

Alternatively, the expression :

$$t_{eq}=6.54 \times 10 \qquad\qquad = \qquad\qquad ^{3}\, P_{ow} + 55.31 \text{ (hours)}$$

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:

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

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

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

Similarly 95 % 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.

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.

$$k_2 = \frac{ln(C_{f1}/C_{f2})}{t_2 - t_1}$$
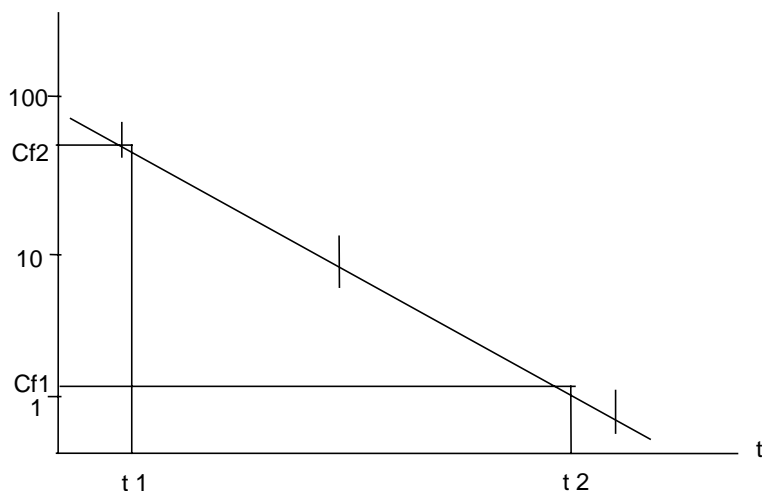
Note that deviations from straight line may indicate a more complex depuration pattern than first order kinetics. A graphical method may be applied for resolving types of depuration deviating from first order kinetics.

GRAPHICAL METHOD FOR DETERMINATION OF UPTAKE RATE CONSTANT k_1

Given K_2, calculate k_1 as follows:

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

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*) [2] (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.

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[2] 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. <u>252</u>, 231-236.

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

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 $\pm$ 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:

– oxygen and pH meters;

– equipment for determination of water hardness and alkalinity;

– adequate apparatus for temperature control and preferably continuous monitoring;

– 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);

– 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:

–   the objective of the study;

–   the method of statistical analysis that will be used;

–   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:

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.

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).

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

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 coloured 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 ECx 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 [3] 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

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

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).

1.6.6. 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$_x$s 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/ECx 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 ± 2 °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:

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.

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 (^{o}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 coxicity 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.

3.   REFERENCES

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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. $\leq$ 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 $25 \pm 2 \,^{\circ}C$. 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

(1) EPPO/Council of Europe (1993). Decision-Making Scheme for the Environmental Risk Assessment of Plant Protection Products - Honeybees. EPPO bulletin, vol. 23, N.1, 151-165. March ,1993.

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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 μm) 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	μg

	soil at adsorption equilibrium	
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	$\mu g \, cm^{-3}$
C_0	initial mass concentration of the test solution in contact with the soil	$\mu g \, cm^{-3}$
$C_{aq}^{ads}(t_i)$	mass concentration of the substance in the aqueous phase at the time t_i that the analysis is performed	$\mu g \, cm^{-3}$
$C_s^{ads}(eq)$	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	cm^3

	the serial method	
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[4]. The applicable relationship is obtained by rearranging equation (4) of the K_d in the form of equation (1):

$$\frac{V_0}{m_{soil}} = (\frac{m_0}{m_s^{ads}(eq)} - 1) K_d \qquad (1)$$

or in its logarithmic form assuming that $R = m_{soil}/V_0$ and $A_{eq}\%/100 = \frac{m_s^{ads}(eq)}{m_0}$:

$$\log R = -\log K_d + \log \left[\frac{(A_{eq}\%/100)}{(1-A_{eq}\%/100)}\right] \qquad (2)$$

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4        $C_s^{ads}(eq) = K_d \cdot C_{aq}^{ads}(eq)$
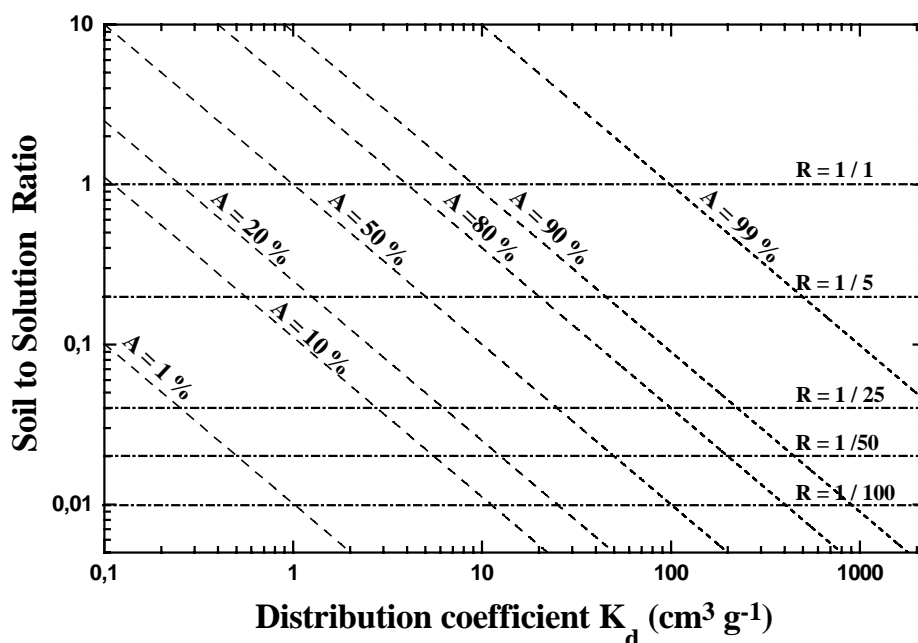
Fig. 1: Relationship between soil to solution ratios and K_d at various percentages of adsorbed test substance

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[5]. 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

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[5] 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).

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.

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[6] (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.

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

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[6]   DT-50: degradation time for 50% of the test substance.

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:

$$A_{t_i} = \frac{m_s^{ads}(t_i) \cdot 100}{m_0} \quad (\%)$$

(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 <u>distribution coefficient</u> 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.

$$K_d = \frac{C_s^{ads}(eq)}{C_{aq}^{ads}(eq)} = \frac{m_s^{ads}(eq)}{m_{aq}^{ads}(eq)} \frac{V_0}{m_{soil}} \quad (cm^3\,g^{-1})$$

(4)

where:

$C_s^{ads}(eq)$ = content of the substance adsorbed on the soil at adsorption equilibrium ($\mu g\,g^{-1}$);

$C_{aq}^{ads}(eq)$ = mass concentration of the substance in the aqueous phase at adsorption equilibrium ($\mu g\,cm^{-3}$).

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:

$$K_d = \frac{A_{eq}}{100 - A_{eq}} \cdot \frac{V_0}{m_{soil}} \quad (cm^3\,g^{-1})$$

(5)

where:

A_{eq} = percentage of adsorption at adsorption equilibrium, %.

The <u>organic carbon normalised adsorption coefficient</u> K_{oc} relates the distribution coefficient K_d to the content of organic carbon of the soil sample:

$$K_{oc} = K_d \cdot \frac{100}{\%OC} \quad (cm^3\,g^{-1})$$

(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 <u>Freundlich adsorption isotherms</u> 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:

$$C_s^{ads}(eq) = \frac{m_s^{ads}(eq)}{m_{soil}} = \frac{\left[C_0 - C_{aq}^{ads}(eq)\right] \cdot V_0}{m_{soil}} \quad (\mu g\ g^{-1})$$

$$(7)$$

The Freundlich adsorption equation is shown in (8):

$$C_s^{ads}(eq) = K_F^{ads} \cdot C_{aq}^{ads}(eq)^{1/n} \quad (\mu g\ g^{-1})$$

$$(8)$$

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.
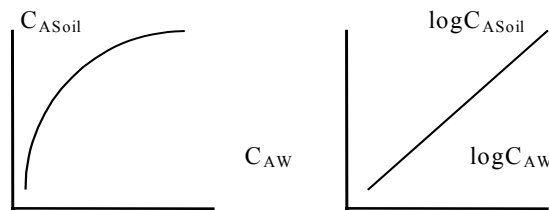
Fig. 2. Freundlich Adsorption Plot, normal and linearized

2.1.2. Mass balance

The <u>mass balance</u> (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:

$$MB = \frac{(V_{rec} \cdot C_{aq}^{ads}(eq) + m_E) \cdot 100}{V_0 \cdot C_0} \quad (\%)$$

$$(10)$$

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 (μg cm^{-3});

V_{rec} =   volume of the supernatant recovered after the adsorption equilibrium (cm^{-3}).

2.2.   DESORPTION

The <u>desorption</u> (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:

$$D_{t_i} = \frac{m_{aq}^{des}(t_i)}{m_s^{ads}(eq)} \cdot 100 \quad (\%)$$

$$(11)$$

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 <u>apparent desorption coefficient</u> (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:

$$K_{des} = \frac{m_s^{ads}(eq) - m_{aq}^{des}(eq)}{m_{aq}^{des}(eq)} \frac{V_T}{m_{soil}} \quad (cm^3 g^{-1})$$

(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".

<u>Remark</u>

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 <u>Freundlich desorption isotherms</u> 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:

$$C_s^{des}(eq) = \frac{m_s^{ads}(eq) - m_{aq}^{des}(eq)}{m_{soil}} \quad (\mu g\ g^{-1})$$

(13)

$m_{aq}^{des}(eq)$ is defined as :

$$m_{aq}^{des}(eq) = m_{m}^{des}(eq) \cdot \frac{V_0}{V_r^F} - m_{aq}^{A} \qquad \text{(µg)}$$

(14)

where:

$c_s^{des}(eq)$ = content of the test substance remaining adsorbed on the soil at desorption equilibrium (µg 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);

$$m_{aq}^{A} = m_{aq}^{ads}(eq) \cdot \left(\frac{V_0 - V_R}{V_0} \right)$$

(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):

$$c_s^{des}(eq) = K_F^{des} \cdot c_{aq}^{des}(eq)^{1/n} \qquad \text{(µg g}^{-1}\text{)}$$

(16)

or in the linear form:

$$\log c_s^{des}(eq) = \log K_F^{des} + 1/n \cdot \log c_{aq}^{des}(eq)$$

(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 (μg 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 CaCl2);

– 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

Prerequisite

Set up of a suitable analytical method

↓

Available ?

◇ Yes   ◇ No → No further test

↓

Preliminary study

↓

2 Soils

Tier 1

Selection of optimal soil/solution ratios

Determination of equilibrium time

Check for adsorption on the vessel surface

Check of the stability by means of parental mass balance

↓

Equilibrium plateau is achieved?

◇ Yes

If mass balance < 90%, test substance is unstable in the time scale of the test. The test can continue by analysing both phases (soil and aqueous solution).

Tier 2

Adsorption kinetics

↓

5 Soils

↓

$K_d * (m_{soi}/V_0) > 0.3$ (indirect method: measurement of solution depletion)
$K_d * (m_{soi}/V_0) > 0.1$ (direct method : measurement of both phases)

Tier 3

as appropriate

Adsorption isotherms

Desorption kinetics

↓

Desorption isotherms

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 |

	$m_{aq}^{ads}(eq)$	$c_{aq}^{ads}(eq)$	R	$m_s^{ads}(eq)_*$	$c_s^{ads}(eq)_*$	R‡	K_d*	R‡
	(µg)	(µg cm^{-3})		(µg)	(µg g^{-1})			
	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%

$$* \, m_s^{ads}(eq) = m_0 - m_{aq}^{ads}(eq), \quad C_s^{ads}(eq) = \frac{\left[C_0 - C_{aq}^{ads}(eq)\right] V_0}{m_{soil}}, \quad K_d = \frac{m_s^{ads}(eq)}{m_{aq}^{ads}(eq)} \frac{V_0}{m_{soil}}$$

$m_s^{ads}(eq)$ = 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‡ = 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:

$$K_{oc} = K_d \cdot \frac{100}{\%oc} \quad (cm^3 \, g^{-1}) \qquad = \qquad K_{om} = \frac{K_d}{1.724} \cdot \frac{100}{\%oc} \quad (cm^3 \, g^{-1})$$

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) |

CALCULATIONS FOR DEFINING THE CENTRIFUGATION CONDITIONS

1. The centrifugation time is given by the following formula, assuming spherical particles:

$$t = \frac{9}{2}\left[\frac{\eta}{\omega^2\, rp^2\,(\rho_s - \rho_{aq})}\right]\ln(Rb/Rt)$$

(1)

For simplification purposes, all parameters are described in non-SI units (g, cm).

where:

   ω = rotational speed ($=2\,\pi$ rpm/60), rad s^{-1};

   rp   = revolutions per minute;
   m

   η = viscosity of solution, g s^{-1} cm^{-1};

   r_p = particle radius, cm;

   ρ = soil density, g cm^{-3};

   s

   ρ_a = solution density, g cm^{-3};

   q

   R_t = distance from the centre of centrifuge rotor to top of solution in centrifuge tube, cm;

   R = distance from the centre of centrifuge rotor to bottom in
   b centrifuge tube, cm;

   R = length of the soil/solution mixture in the centrifuge
   b- tube, cm.
   R_t

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, $\eta = 8.95$ x 10^{-3}g s^{-1}cm^{-1} and $\rho_{aq} = 1.0$ g.cm^{-3}.

Then, the centrifugation time is given by the equation (2):

$$t = \frac{3.7}{(rpm)^2 \cdot rp^2\,(\rho s - 1)}\ln\frac{Rb}{Rt}$$

(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.
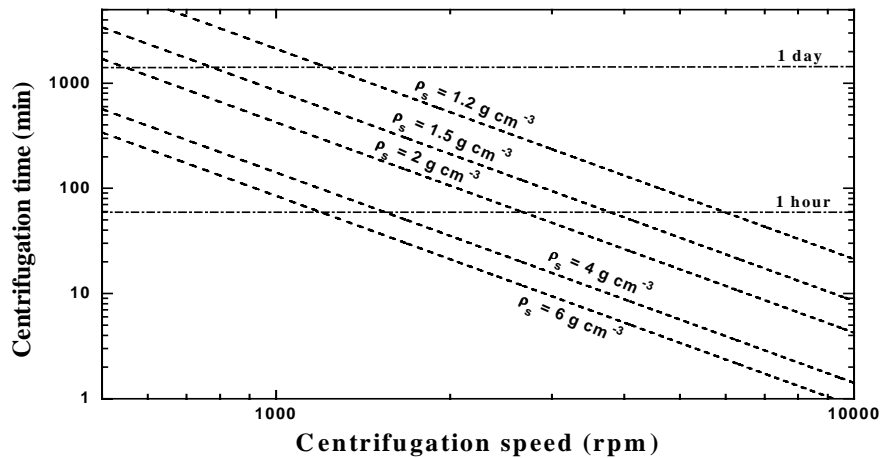
Fig. 1a. Variations of centrifugation time (t) versus centrifugation speed (rpm) for different soil densities

(ρ_s). $R_t = 10$ cm, $R_b - R_t = 10$ cm, $\eta = 8.95 \times 10^{-3}$ g s^{-1}cm^{-1} and $\rho_{aq} = 1.0$ g.cm^{-3} at 25 °C.
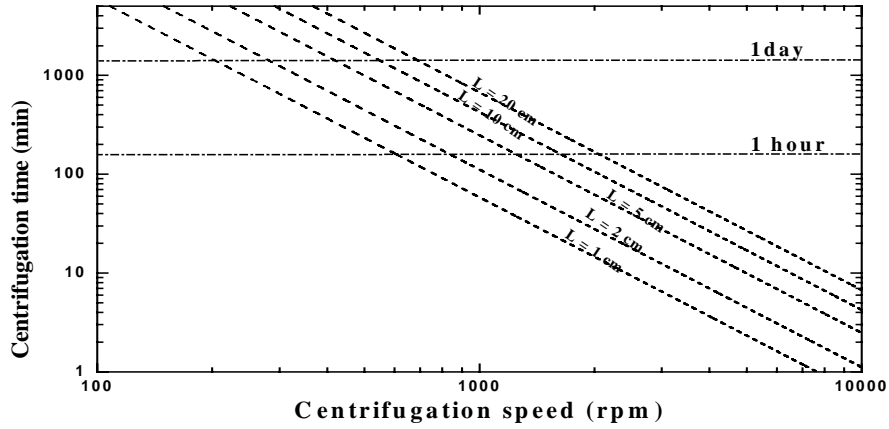
Fig. 1b. Variations of centrifugation time (t) versus centrifugation speed (rpm) for different lengths of the mixture in the centrifuge tube (Rb -Rt) = L; $R_t = 10$ cm, $\eta = 8.95 \times 10^{-3}$ g s^{-1}cm^{-1}, $\rho_{aq} = 1.0$ g.cm^{-3} at 25 °C and $\rho_s = 2.0$ g cm^{-3}.

ANNEX 5
CALCULATION OF ADSORPTION A (%) AND DESORPTION D (%)

The time scheme of the procedure is:
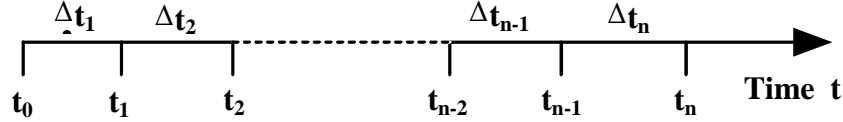
For all the calculations it is assumed that the test substance is stable and does not adsorb significantly to the container walls.

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:

$$A_{t_i} = \frac{m_s^{ads}(t_i) \cdot 100}{m_0} \quad (\%) \tag{1}$$

The terms of this equation may be calculated as follows:

$$m_0 = C_0 \cdot V_0 \quad (\mu g) \tag{2}$$

$$m_s^{ads}(t_i) = m_0 - C_{aq}^{ads}(t_i) \cdot V_0 \quad (\mu g) \tag{3}$$

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.
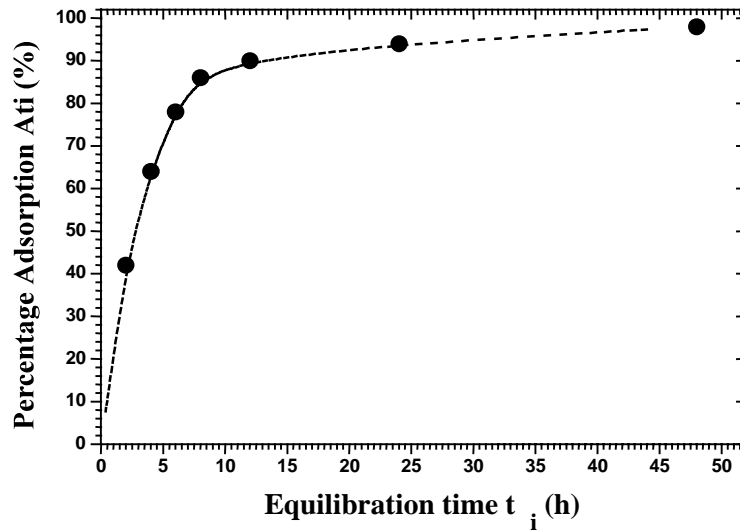
Fig. 1. Adsorption Equilibrium Plot
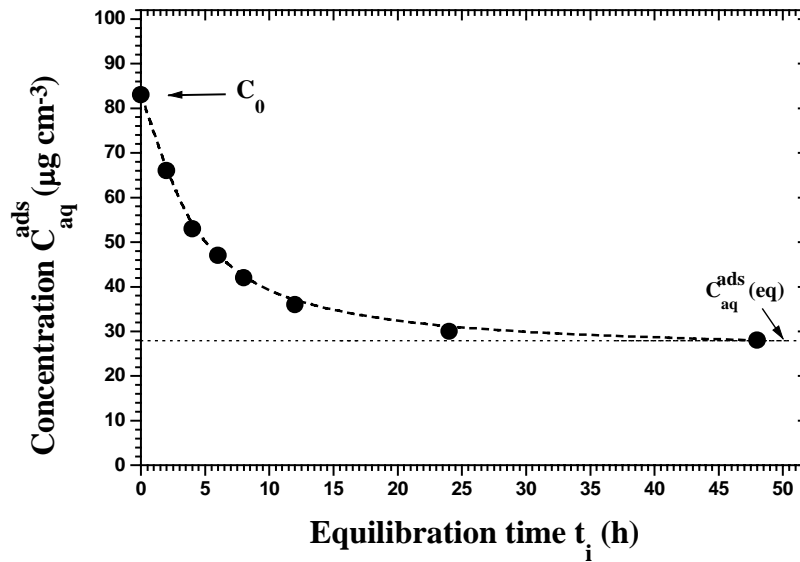
Fig.2. Mass concentration of the test substance in the aqueous phase (C_{aq}) versus time

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 $\Delta t_1 = t_1 - t_0$

$$m_s^{ads}(\Delta t_1) = m0 - m_m^{ads}(t_1) \cdot \left(\frac{V_0}{v_a^A} \right) \tag{4}$$

–   for the second time interval $\Delta t2 = t2 - t1$

$$m_s^{ads}(\Delta t_2) = m_m^{ads}(t_1) \cdot \left(\frac{V_0}{v_a^A} \right) - m_m^{ads}(t_2) \cdot \left(\frac{V_0 - v_a^A}{v_a^A} \right) \tag{5}$$

–   for the third time interval $\Delta t3 = t3 - t2$

$$m_s^{ads}(\Delta t_3) = m_m^{ads}(t_2) \cdot \left(\frac{V_0 - v_a^A}{v_a^A} \right) - m_m^{ads}(t_3) \cdot \left(\frac{V_0 - 2 \cdot v_a^A}{v_a^A} \right) \tag{6}$$

–   for the nth time interval $\Delta tn = tn - tn-1$

$$m_s^{ads}(\Delta t_n) = m_m^{ads}(t_{n-1}) \cdot \left(\frac{V_0 - (n-2) \cdot v_a^A}{v_a^A} \right) - m_m^{ads}(t_n) \cdot \left(\frac{\left(V_0 - (n-1) \cdot v_a^A \right)}{v_a^A} \right) \tag{7}$$

The percentage of adsorption at each time interval, $^A\!\Delta t_i$, is calculated using the following equation:

$$A_{\Delta t_i} = \frac{m_s^{ads}(\Delta t_i)}{m_0} \cdot 100 \quad (\%) \tag{8}$$

while the percentage of adsorption $^{(A_{t_i})}$ at a time point t_i is given by the equation:

$$A_{t_i} = \frac{\sum\limits_{j=\Delta t_1}^{\Delta t_i} m_s^{ads}(j)}{m_0} \cdot 100 \quad (\%) \tag{9}$$

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:

$$m_s^{ads}(eq) = \sum_{\Delta t_i = 1}^{n} m_s^{ads}(\Delta t_i)$$

(10)

-        the mass of the test substance in the solution is:

$$m_{aq}^{ads}(eq) = m_0 - \sum_{\Delta t_i = 1}^{n} m_s^{ads}(\Delta t_i)$$

(11)

–   and the percentage of adsorption at equilibrium is:

$$A_{eq} = \frac{m_s^{ads}(eq)}{m_0} \cdot 100 \quad (\%)$$

(12)

The parameters used above are defined as:

$m_s^{ads}(\Delta t_1), m_s^{ads}(\Delta t_2),..., m_s^{ads}(\Delta t_n)$ = mass of the substance adsorbed on the soil during the time intervals $\Delta_{t1}, \Delta_{t2},..., \Delta_{tn}$ respectively (μg);

$m_m^{ads}(t_1), m_m^{ads}(t_2),..., m_n^{ads}(t_n)$ = 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);

$$m_{aq}^{A} = m_{aq}^{ads}(eq) \cdot \left(\frac{V_0 - V_R}{V_0} \right)$$

(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:

$$V_T = V_o - \sum_{i=1}^{n} v_a^A (i) \quad (18)$$

At a time point t_i:

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:

$$m_{aq}^{des}(t_i) = m_m^{des}(t_i) \cdot \left(\frac{V_T}{v_a^D} \right) - m_{aq}^{A} \cdot \left(\frac{\left(V_T - (i-1) \cdot v_a^D \right)}{V_T} \right)$$

(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:

$$D_{t_i} = \frac{m_{aq}^{des}(t_i)}{m_s^{ads}(eq)} \cdot 100 \quad (\%)$$

(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 $\Delta t_1 = t_1 - t_0$

$$m_{aq}^{des}(\Delta t_1) = m_m^{des}(t_1) \cdot \left(\frac{V_T}{v_a^D}\right) - m_{aq}^A \quad \text{and} \quad m_s^{des}(t_1) = m_s^{aq}(eq) - m_{aq}^{des}(\Delta t_1)$$

(21)

for the second time interval $\Delta t_2 = t_2 - t_1$

$$m_{aq}^{des}(\Delta t_2) = m_m^{des}(t_2) \cdot \left(\frac{V_T}{v_a^D}\right) - m_{aq}^{des}(\Delta t_1) \cdot \left(\frac{\left(V_T - v_a^D\right)}{V_T}\right) - m_{aq}^A \cdot \left(\frac{\left(V_T - v_a^D\right)}{V_T}\right) \quad \text{and}$$

$$m_s^{des}(t_2) = m_s^{ads}(eq) - \left[m_{aq}^{des}(\Delta t_1) + m_{aq}^{des}(\Delta t_2)\right]$$

(22)

for the nth interval $\Delta t_n = t_n - t_{n-1}$

$$m_{aq}^{des}(\Delta t_n) = \left[m_m^{des}(t_n) \cdot \left(\frac{V_T}{v_a^D}\right) - m_{aq}^A \cdot \left(\frac{\left(V_T - (n-1) \cdot v_a^D\right)}{V_T}\right) - \sum_{i=1, n \neq 1}^{n-1} \left(\frac{\left(V_T - (n-i) \cdot v_a^D\right)}{V_T} \cdot m_{aq}^{des}(\Delta t_i)\right)\right] \quad \text{and}$$

$$m_s^{des}(t_n) = m_s^{ads}(eq) - \sum_{i=1, n \neq 1}^{n} m_{aq}^{des}(\Delta t_i)$$

(23)

Finally, the percentage of desorption at each time interval, $D_{\Delta t_i}$, is calculated using the following equation:

$$D_{\Delta t_i} = \frac{m_{aq}^{des}(\Delta t_i)}{m_s^{ads}(eq)} \cdot 100 \quad (\%)$$

(24)

while the percentage of desorption D_{t_i} at a time point t_i is given by the equation:

$$D_{t_i} = \frac{\sum_{j=\Delta t_1}^{\Delta t_i} m_{aq}^{des}(j)}{m_s^{ads}(eq)} \cdot 100 = \frac{m_{aq}^{des}(t_i)}{m_s^{ads}(eq)} \cdot 100 \, (\%)$$

(25)

where the above used parameters are defined as:

$m_s^{des}(\Delta t_1), m_s^{des}(\Delta t_2),... ,m_s^{des}(\Delta t_n)$ = mass of the substance remaining adsorbed on the soil after the time intervals Δt_1, Δt_2,..., Δt_n respectively (µg);

$m_{aq}^{des}(\Delta t_1), m_{aq}^{des}(\Delta t_2),... ,m_{aq}^{des}(\Delta t_n)$ = 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);

$$v_a^D \le 0.02 \cdot V_T \qquad (27)$$

ANNEX 6
ADSORPTION-DESORPTION IN SOILS: DATA REPORTING SHEETS

Substance tested:

Soil tested:

Dry mass content of the soil (105 °C, 12h):………………………………………………..%

Temperature:..°C

Suitability of the analytical method

Weighed soil	g	
Soil: dry mass	g	
Volume CaCl$_2$ sol.	cm^3	
Nominal conc. final sol.	µg cm^{-3}	
Analytical conc. final sol.	µg 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 Indirect ☐   Parallel ☐   Serial ☐
followed:

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 beginning 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 Nº								
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		μg cm^{-3}						
After agitation and centrifugation								
Concentration in aqueous phase		μg 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}	μg cm^{-3}				
Mass of substance extracted from soil and vessel walls	m_{E1}	μg				
2nd dilution with solvent						
Removed volume of solvent	V_s	cm^3				
Added volume of solvent	V	cm^3				
2nd extraction with solvent						
Signal analyzed in solvent phase	S_{E2}	var.				
Conc. test subst. in solvent	C_{E2}	μg 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 Indirect followed: 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 P M	V_0	cm^3				
in contact with soil S M	V_T	cm^3				
Mass test subst. left over the adsorption equilibrium due to incomplete volume	m_{aq}^A	μg				

replacement							
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	P M	v_r^i	cm^3				
the measurement of the test substance	S M	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.

$$K_d = \frac{C_{soil}}{C_{aq}} \; or \; \frac{C_{sludge}}{C_{aq}} \qquad (1)$$

where:

C_{soil} = concentration of test substance in soil at equilibrium ($\mu g \cdot$

$$g^{-1})$$

C_{slud} = concentration of test substance in sludge at equilibrium ($\mu g \cdot g^{-1}$)
ge

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.

$$\log \frac{x}{m} = \log K_f + \frac{1}{n} \cdot \log C_{aq} \qquad (1)$$

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.

$$K_{oc} = \frac{K_d}{f_{oc}} (\text{dimensionless or } ml \cdot g^{-1}) \text{ or } \frac{K_f}{f_{oc}} (\mu g \cdot g^{-1}) \qquad (3)$$

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.

$$Pow = \frac{C_{oc\,tan\,ol}}{C_{aq}}(= Kow) \qquad (5)$$

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:

$$-O \!-\! Si \quad -CH_2-CH_2-CH_2\!-\! -CN$$

silica　　　non-polar spacer　　　polar moiety
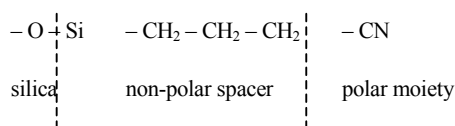
The principle of the test method is similar to Testing Method A.8 (Partition Coefficient, HPLC Method). While passing through the column along with the mobile phase the test substance interacts with the stationary phase. As a result of partitioning between mobile and stationary phases the test substance 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 correlation*s* 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 to

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 to 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 to 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 tR

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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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.

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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}

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.

<u>Solvents</u> 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.

<u>Dispersants</u> 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:

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.

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.

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.

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 ≤ 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:

$$Y = \frac{c}{1 + \left(\dfrac{x}{x_0}\right)^b}$$

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 ECx 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 mg/l dry weight on mg C/l.
Data from concentrated suspensions of semi continuous batch
cultured cells, re-suspended in distilled water.

1700
1600
1500
1400
1300
1200

mg/l dry weight
of concentrated
algal feed

1100
1000
900
800
700
600
500
400
300
200
100
0
-100

Correlation coefficient - 0,980

0   50   100   150   200   250   300   350   400   450   500   550   600   650   700   750

mg C/l of concentrated algal feed

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.

115
110
105
100
95
90
85
80
75

No cells/l (x 10^8)
of concentrated
algal feed

70
65
60
55
50
45
40
35
30
25
20
15
10
5
0

Correlation coefficient - 0,926

0   50   100   150   200   250   300   350   400   450   500   550   600   650   700   750

mg C/l of concentrated algal feed

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.

Absorbance at
440 nm of a
1/10 dilution
of concentrated
algal feed

Correlation coefficient - 0,998

mg C/l of concentrated algal feed

ANNEX 3
EXAMPLE DATA SHEET RECORDING MEDIUM RENEWAL, PHISICAL/CHEMICAL MONITORING DATA, FEEDING, DAPHNIA REPRODUCTION AND ADULT MORTALITY
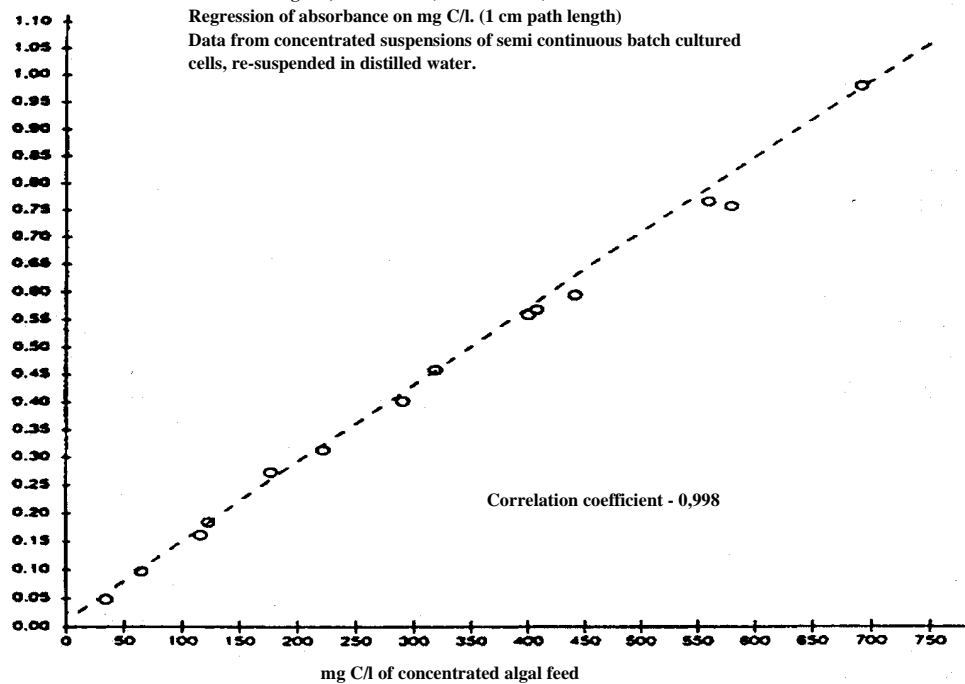
Experiment N^0:   Data starded: Clone: Medium: Type of food: Test Substance: Nominal conc:

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 (^{o}C) *																							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) <u>**Measured concentrations**</u>

Nominal conc.	Week 1 sample		Week 2 sample		Week 3 sample	
	Fresh	Old	Fresh	Old	Fresh	Old

(b) <u>**Measured concentrations as a percentage of nominal**</u>

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.
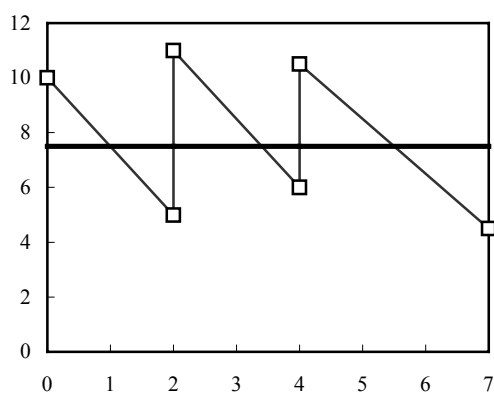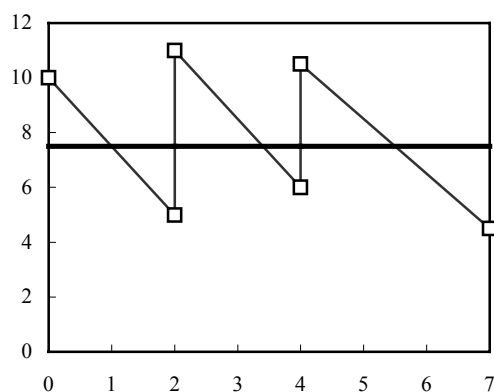
Days

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^o	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 :

$$Area = \frac{Conc0 - Conc1}{Ln(Conc0) - Ln(Conc1)} \times Days$$

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.