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. HCI 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**

(1) OECD, Paris, 1981, Test Guidelines 202, Decision of the Council C(81) 30 final and updates.

(2) International Standard ISO, Water Quality -Determination of inhibition of mobility of *Daphnia magna* Straus, ISO 6341-1989

(3) AFNOR Inhibition of mobility of *Daphnia magna* Straus (Cladocera -crustacea) NFT 90301 (January 1983).

(4) Verfahrensvorschlag des Umweltbundesamtes zum akuten Daphnien-Test. Rudolph, P. und Boje, R. Ökotoxikologie, Grundlagen fur die ökotoxikologische Bewertung von Umweltchemikalien nach dem Chemikaliengesetz, ecomed 1986.

(5) DIN Testverfahren rnit Wasserorganismen 38412 (L1) und (Lll).

(6) Finney, D.J. Statistical Methods in Biological Assay. Griffin, Weycombe, U.K., 1978.

(7) Litchfield, J .T .and Wilcoxon, F. A simplified method of evaluating dose-effect experiments. J. Pharmacol. and Exper. Ther., 1949, vol. 96, 99-113.

(8) Sprague, J.B. Measurement of pollutant toxicity to fish. I Bioassay methods for acute toxicity. Water Res., 1969, vol. 3,793-821.

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

(10) Stephan, C.E. Methods for calculating an LC_{50}. In Aquatic Toxicology and Hazard Evaluation (edited by F.I. Mayer and J.L. Hamelink). American Society for Testing and Materials. ASTM, 1977, STP 634, 65-84.

(11) Stephan, C.E., Busch, K.A., Smith, R, Burke, J. and Andrews, R.W. A computer program for calculating an LC_{50}. US EPA.

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

$CaC1_2.2\ H_2O$ (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 partecipating 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

Example of concentration: percentage immobilisation

Example of determination of EC_{50} using log-probit paper