The percentage inhibition of the cell growth at each test substance concentration (I_A) is calculated as the difference between the area under the control growth curve (A_c) and the area under the growth curve at each test substance concentration (A_t) as:

$$I_A = \frac{A_c - A_t}{A_c} \times 100$$

I_A values are plotted on semilogarithmic paper or on semilogarithmic probit paper against the corresponding concentrations. The points if plotted on probit paper are fitted by a straight line by eye, or, when a log-normal distribution of values can be assumed, a computed regression line may be drawn.

An EC 50 value results from the intercept of the (regression) line with the parallel drawn to the abscissa at 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. In relation to this guideline which specifies measurements at 24, 48 and 72 hours, the symbol becomes E_bC 50 (0-72h).

Other EC values, like E_bC 10, can also be derived from the plot of I_A versus log concentration.

(2) Comparison of growth rates

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

$$\mu = \frac{\ln N_2 - \ln N_1}{t_2 - t_1}$$

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 reduction in average growth rate at each test substance concentration compared to the control value is plotted against the logarithm of the concentration. The EC 50

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 observation times 24 and 48 hours the symbol becomes E_rC 50 (24-48h).

Note: 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.

- Test report

The test report should 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 one unit are observed]
- vehicle and method used for solubilising the test substance and concentration of the vehicle in the test solutions
- light intensity and quality
- concentrations tested (measured or nominal)

Results:

- cell concentration for each flask at each measuring point and method for measuring cell concentration

- mean values of cell concentrations
- growth curves
- graphical presentation of the concentration effect relationship
- EC values and method of calculation
- NOEC
- other observed effects

4. LITERATURE

• Standard Procedures

1. Draft International Standard ISO/TC 147/SC5/W5/N67: Toxicity with Respect to Algae.
2. NEN 6506: Water-Determination of Toxicity with Algae, (Dutch standard) Nederlands Normalisatie Instituut, Rijswijk (1979).
3. DIN 38 412, Teil 1: Testverfahren mit Wasserorganismen (Gruppe L) Allgemeine Hinweise zur Planung, Durchführung und Auswertung biologischer Testverfahren (German standard) Deutsches Institut für Normung e.V, Berlin (June 1982).
4. DIN *Draft* 38 412, Teil 9: Testverfahren mit Wasserorganismen (Gruppe L). Bestimmung der Wirkung von Wasserinhaltsstoffen auf Grünalgen (Scenedesmus-Zellvermehrungshemmtest) (German standard) Deutsches Institut für Normung e.V., Berlin (April 1982).
5. U.S. EPA: Algal Assay Procedure: Bottle Test, National Environmental Research Centre, Corvallis, Oregon (1971).
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7. AFNOR T 90 304 (French standard).

- Other

8. A.O. Hanstveit, in *Degradability, Ecotoxicity and Bioaccumulation: the Determination of the Possible Effects of Chemicals and Wastes on the Aquatic Environment*, Chapter 5, Government Publishing Office, the Hague (1980).

9. G. Bringmann and R. Kühn, *Water Research 14*, 231-241 (1980).

10. A.G. Payne and R.H. Hall, in *Aquatic Toxicology*, (edited by L.L. Marking and R.A. Kimble), ASTM STP 667, pp. 171-180 American Society for Testing and Materials, (1979).

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12. Standard Methods for Examination of Water and Waste Water, Part 800. 15th ed., American Public Health Association, Washington, D.C. (1980).

5. ANNEX

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

Equipment and materials

See under 2B: Preparations and Experimental Organisms.

Procedures for obtaining algal cultures

– Preparation of nutrient solutions (media):

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

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

– Stock culture:

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

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

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

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

– Pre-culture:

The pre-culture is intended to give an amount of algae suitable for the inoculation of test cultures. The preculture is incubated under the conditions of the test and used when still exponentially growing, normally after an incubation period of about 3 days. When the algal cultures contain deformed or abnormal cells, they must be discarded.

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Adopted:
4 April 1984

OECD GUIDELINE FOR TESTING OF CHEMICALS

"*Daphnia* sp., Acute Immobilisation Test and Reproduction Test"

The following Test Guideline includes two parts:
Part I - the 24h EC 50 acute immobilisation test
Part II - the reproduction test (at least 14 days).

PART I - 24 H EC 50 ACUTE IMMOBILISATION TEST

1. INTRODUCTORY INFORMATION

• Prerequisites

- Water solubility
- Vapour pressure

• Guidance information

- Structural formula
- Purity of the substance
- Methods of analysis for the quantification of the substance in water
- Chemical stability in water and light
- n-Octanol/water partition coefficient
- Results of a test on ready biodegradability (see Test Guideline 301)

• Qualifying statement

For chemicals of low solubility under test conditions, it may not be possible to quantitatively determine the EC 50.

• Standard documents

See references (1) to (6), Part II, Section 4, Literature.

2. METHOD

A. INTRODUCTION, PURPOSE, SCOPE, RELEVANCE, APPLICATION AND LIMITS OF TEST

- Definitions

24h EC 50 is the concentration estimated to immobilise 50 per cent of the Daphnia after 24 hours exposure. (If another definition is used, this must be reported, together with its reference.)

Immobilisation: those animals not able to swim within 15 seconds after gentle agitation of the test container are considered to be immobile. (If another definition is used, this must be reported, together with its reference.)

- Reference substances

In the acute immobilisation test a reference substance may be tested for EC 50 as a means of assuring that the test conditions are reliable.

- Principle of the test method

In the acute immobilisation test a range of concentrations of the substance investigated exerts different degrees of toxic effects on the swimming capability of Daphnia under otherwise identical test conditions. Certain concentrations result in certain percentages of Daphnia being no longer capable of swimming at 24 hours. The test can be extended to 48 hours if desired.

- Conditions for the validity of the test

- In the control, not more than 10 per cent of the Daphnia should have been immobilised or trapped at the surface of the water.

- The dissolved oxygen concentration at the end of the test should be ≥ 60 per cent of the air saturation value at the temperature used.

"*Daphnia* sp., Acute Immobilisation Test and Reproduction Test"

B. DESCRIPTION OF THE TEST PROCEDURE

• Preparations

Equipment

Equipment which will come into contact with the test solutions should preferably be all-glass; this glassware should be cleaned with solvents known to remove previously tested chemicals.

Holding and dilution water

Any water suitable for culturing Daphnia, either natural or reconstituted water, can be used in this test. To avoid the necessity of adaptation prior to the test, it is recommended that the water used in the test be similar to the culture water. Examples of reconstituted water are given in references (1), (2), (4) and (8).

• Experimental animals

Selection of species

Daphnia magna, or any other suitable Daphnia species, not more than 24 hours old at the beginning of the test, laboratory bred, apparently healthy and with a known history (breeding method, pretreatment) are used in this test. It is advisable to use the same species in Part I and Part II of this test.

• Performance of the test

- At least 20 animals, preferably divided into four groups of five animals each, should be used at each test concentration and for the controls.
- The Daphnia should not be fed during the test.
- Loading: at least 2 ml of test solution should be provided for each animal.
- The test temperature should be between 18 and 22°C, and for each single test it should be constant within ±1°C.

- A light-dark cycle is optional: complete darkness is acceptable.

- The concentrations tested are in a geometric series. The highest concentration tested should preferably result in 100 per cent immobilisation. It should, however, not exceed 1 g/l. The lowest concentration tested should preferably give no observable effect. The concentrations may be either measured or nominal, i.e. calculated, based upon the amount of material used in preparing the solution.

- Solutions are preferably made up without the use of a solubilising agent. Solutions of test substances of low aqueous solubility may be prepared by mechanical dispersion or, if necessary, by use of vehicles such as organic solvents, emulsifiers or dispersants of low toxicity to Daphnia. When such vehicles are used, one control should be exposed to the concentration of the vehicle used with the highest concentration of the test substance. The concentration of organic solvents, emulsifiers or dispersants should not exceed 100 mg/l. In evaluating the data it must be borne in mind that the results may be due to the combined effects of the substance itself and of the vehicle, which cannot normally be distinguished experimentally.

- The dilution water should be aerated prior to the addition of the test substance, and the oxygen concentration of the controls and the test solutions should be measured at the beginning and the end of the test.

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

- The pH of the controls and the test solutions should be measured at the beginning and the end of the test; the pH of the test solutions should not be adjusted.

"*Daphnia* sp., Acute Immobilisation Test and Reproduction Test"

3. DATA AND REPORTING

- Treatment of results

The percentage immobility at 24 hours and, if determined, at 48 hours is plotted against concentration on logarithmic-probability paper. Normal statistical procedures arethen employed to calculate the EC 50 for the appropriate exposure period (see references, Part II, Section 4, Literature). Confidence limits (p = 0.95) for the calculated EC 50 values can be determined using the standard procedures quoted.

Where the data obtained are inadequate for the use of standard methods of calculating the EC 50, the highest concentration causing no immobility and the lowest concentration producing 100 per cent immobility should be used as an approximation for the EC 50 (this being considered the geometric mean of these two concentrations). In this case, the ratio of the higher to the lower concentration should not exceed 2.

- Test report

See pages 12-14 of this Test Guideline.

PART II - REPRODUCTION TEST AT LEAST 14 DAYS

The results of the acute immobilisation test are to be used to determine, with judgement, the concentration levels to be used in the reproduction test. It is suggested that this reproduction test be carried out using a geometrical concentration series of at least five concentrations with a factor of at most 10, starting at about the 24h EC 50 and ending at about 1/100 of the 24h EC 50. If necessary, lower concentrations should be tested.

"*Daphnia* sp., Acute Immobilisation Test and Reproduction Test"

1. INTRODUCTORY INFORMATION

• Prerequisites

- Water solubility
- *Vapour pressure*
- Chemical stability in water and light
- Results of a test on ready biodegradability (see Test Guidelines 301 A-E)
- 24h EC 50 in Daphnia

• Guidance information

- Structural formula
- Purity of the test substance
- n-Octanol/water partition coefficient
- *Method of analysis for the quantification of the test substance in water*

• Qualifying statement

- For chemicals with low solubility under test conditions, it may not be possible to quantitatively determine the EC 50.

• Recommendations

- Instead of a test of two weeks duration in which three batches of young should be born per female, a test of three or four weeks may be preferred in order to obtain a more thorough judgement of the influence of the test substance on mortality and reproduction: in this period about six to nine batches of young should be born per female.

- It is recommended that a statistical test (such as an analysis of variance) be used to determine whether the test replications can be analysed together.

"*Daphnia* sp., Acute Immobilisation Test and Reproduction Test"

- Standard documents

See references (1) to (6), Section 4, Literature.

2. METHOD

A. INTRODUCTION, PURPOSE, SCOPE, RELEVANCE, APPLICATION AND LIMITS OF TEST

- Definitions

Semi-static test is a test without flow of solution, but with occasional batchwise renewal of test solutions after prolonged periods (e.g. 24 hours).

Flow-through test is a test in which water is renewed continuously in the test chambers, the test substance being transported with the water used to renew the test medium.

EC 50 is the concentration of the test substance in water estimated to result in a 50 per cent reduction in reproduction of the Daphnia within a particular period of exposure (which must be stated).

- Reference substances

No reference substances are recommended for the reproduction test. Nevertheless, if a reference substance has been tested in the acute test, the results should be given in the test report.

- Principle of the test method

In the reproduction test, effects on the mortality and the reproductive capacity and other signs of intoxication in Daphnia are determined to be used as indications of the toxicity of a substance dissolved in water. For this purpose, the test organisms are exposed to solutions containing the test substance in various concentrations for a period of not less than two weeks, and long enough for the development of at least three broods.

The mortality, the time of the first production of young, the number of young born and the signs of intoxication observed are compared with the corresponding parameters in the controls.

• Conditions for the validity of the test

- The mortality in the controls should not exceed 20 per cent at the end of the test.
- The dissolved oxygen concentration (throughout the test) must have been ≥ 60 per cent of the air saturation value.
- The pH of the controls, and of at least the most concentrated solutions, must be known throughout the test; the deviation from the initial value should be ≤ 0.3 units.
- There must be evidence that the concentration of the substance being tested has been satisfactorily maintained (e.g. at least 80 per cent of the nominal concentration) over the test period. The results should be based on measured concentrations if the deviation from the nominal concentration is greater than 20 per cent.
- The first young should have been born in the controls after a maximum of nine days.
- The average cumulative number of young per female in the controls after three broods, should be ≥ 20 at a temperature of 20 ± 1.0°C.
- If, at the highest concentration tested, no effect on reproduction is detected, this should be reported.

B. DESCRIPTION OF THE TEST PROCEDURE

• Preparations

"*Daphnia* sp., Acute Immobilisation Test and Reproduction Test"

Equipment and material

Normal laboratory apparatus and equipment should be used. Equipment which will come into contact with the test solutions should preferably be all-glass; this glassware should be cleaned with solvents known to remove previously tested chemicals.

Dilution water

Any water, either natural or reconstituted water, can be used, provided that it will sustain good reproduction in Daphnia (see Conditions for the validity of the test, above). In addition, the dilution water should meet the criteria given in reference (7). Examples of reconstituted water are given in references (1), (2), (4) and (8).

- Experimental animals

Selection of species

Daphnia magna less than 24 hours old at the beginning of the test, laboratory bred, apparently healthy and with a known history (breeding method, pretreatment) are used in this test. Other Daphnia species may be used provided that the relevant reproduction parameters are comparable to those of *Daphnia magna*.

Feeding

In the 14-day reproduction test food (of any kind and in any quantity) that results in meeting the criteria of reproduction given in Conditions for the validity of the test, above, is acceptable. Overloading of the test solutions with food should be avoided in order to minimise sorption of the test substance. Log-phase unicellular green algae are generally suitable.

- Performance of the test

– This reproduction test should not be carried out in a static test system: either a semi-static or flow-through system should be used. The renewal period should be guided by the chemical analysis and (if applicable) the oxygen level in the test solution; the solutions

should be renewed at least once every 48 hours (if desired, on Monday, Wednesday and Friday). If the renewal scheme is used, the glassware must be emptied and food residues removed at renewal. It is recommended that the glassware be rinsed with distilled water and kept in a coded series for the following renewal. Each test unit therefore consists of two vessels which are used alternately. If flow-through systems are used, these should be cleaned at intervals of at most twice a week.

- At least 40 animals, preferably divided into four groups of ten animals each, should be used at each test concentration and for the controls.
- The Daphnia should be fed at least daily.
- Loading: at least 40 ml of test solution should be provided for each animal.
- The test temperatue should be between 18 and 22°C, and for each single test it should be constant within ± 1.0°C.
- A light-dark cycle is necessary: 8 hours darkness and 16 hours light are recommended.
- The concentrations tested are made up in a geometric series. Substances should not be tested at concentrations exceeding 1 g/l.
- Samples of the test solutions should be taken at the beginning and during the test: the actual concentration must not drop below 80 per cent of the nominal concentration. Aeration of the test solutions is permissible, unless this would cause the actual concentration of the test substance to drop below 80 per cent of the nominal concentration.
- Solutions are preferably made up without the use of a solubilising agent like organic solvent, emulsifiers and dispersants.
- Solutions of test substances of low aqueous solubility may be prepared by mechanical dispersion or, if necessary, by use of vehicles such as organic solvents, emulsifiers or dispersants of low toxicity to Daphnia. When such vehicles are used, one control should be exposed to the concentration of the vehicle used with the highest concentration of the test substance.

- The concentration of organic solvents, emulsifiers or dispersants should not exceed 100 mg/l. In evaluating the data it must be borne in mind that the results may be due to the combined effects of the substance itself and of the vehicle, which cannot normally be dis- tinguished experimentally.

- The test solutions should be aerated prior to the introduction of the test substance and Daphnia.

- When more than 20 per cent of the test substance would be lost through volatility, the test should be carried out either in an almost-closed flow-through system or in an enclosed container of sufficient size to ensure that the oxygen level does not fall below 60 per cent of the saturation value.

- The dissolved oxygen concentration in all test solutions should be checked once every 48 hours (if desired, every Monday, Wednesday and Friday).

- The pH of the controls and of at least the most concentrated solutions is checked before and after each renewal: if necessay the pH of the other solutions should also be checked. The results of these measurements are recorded.

- The live and dead Daphnia of the "parental" generation (P) are counted and the dead specimens removed preferably daily, but at least three times a week (Monday, Wednesday and Friday).

- The presence of eggs in the brood pouch, males or winter eggs must be recorded. The condition and size of the P generation should be visually compared with the controls.

- When the parental animals are about seven days old, the first young Daphnia emerge from the brood pouch, after which a new batch appears every two to three days. These batches are called "broods" of the F_1 (filial 1) generation.

- The newborn young of the F_1 generation should be counted at least three times a week, with an interval of 48-72 hours (e.g. Monday, Wednesday and Friday) and their visually estimated condition recorded. After counting and examination, the young are poured away. The presence of eggs on the bottom of the test vessel from which no young have emerged is checked and recorded.

- Test duration: the minimum duration of the test is 14 days, in which period not less than three broods of the F1 generation must have appeared in the controls. If this is not the case, the test must be continued until the third brood in the control is complete, which is normally achieved within 21 days.

3. DATA AND REPORTING

- Test report

For both parts of the test, report:

- Test substance: chemical identification data

- Test organisms: source of Daphnia, any pre-treatment, breeding method (including source, kind and amount of food, feeding frequency)

- Test method: description of or reference to the method used

- Test conditions:

  - vehicles and/or additives used and their concentrations. If it is observed that the stability or homogeneity of the test solution cannot be maintained, then care should be taken in the interpretation of the results and note made that these may not be reproducible

  - dilution water: source and chemical and physical characteristics, including at least hardness, pH, Ca/Mg ratio, Na/K ratio, alkalinity

  - test temperature

- light quality, intensity and periodicity
- all measurements of pH and oxygen level made during the test, preferably in tabular form
- results and date of test performed with reference substance, if available
- description of test vessels: volume of solution, number of test organisms per vessel, number of test vessels per concentration, any treatment of the test vessels, the introduction of the test substance in the dilution water
- in case of renewal, the renewal procedure and scheme; in case of flow-through, the test substance delivery system and the flow-rate
- if measured, the actual concentrations of the test substance and the dates of measurement

– Number and percentage of Daphnia that showed any adverse effect in the controls and in each treatment group at each observation period and a description of the nature of the effects observed (e.g. immobilisation, mortality) in tabular form

– Description or reference to statistical procedures applied

– Any other effects differentiating organisms in tests and controls.

For the 24 hours EC 50 acute immobilisation test, also report:

– The 24h EC 50, preferably with 95 per cent confidence limits, determined by a suitable method

– If possible, the slope of the concentration response curve with its 95 per cent confidence limits

– The highest concentration tested producing no immobile Daphnia

– The lowest tested concentration producing 100 per cent immobile Daphnia

For the reproduction test, also report:

- The EC 50 (immobilisation) and EC 50 (reproduction) values as far as possible at 24 hours, 48 hours, 96 hours, 7 days, 14 days and at the end of the test, preferably with 95 per cent confidence limits, obtained either by computation or graphically and the method applied (for the determination a probit method should be used.)

- The length of time for the appearance of the first brood for each concentration

- The number of young alive in each test vessel at any given day at which counts were made (the minimum requirement is for counts thrice weekly)

- The number of dead young on each day of counting

- Source, kind and amount of food, feeding frequency

For each of the above a statistical analysis of the homogeneity of replicate results for each concentration should be made. If homogeneity is found, it should be determined, through an appropriate statistical analysis, whether a significant difference exists between the control and the test concentrations.

Then report:

- The highest concentration tested at which no significant difference is found versus the controls with respect to mortality, reproduction and other observed effects

- The lowest concentration tested with significant difference versus the controls

4. LITERATURE

• Standard procedures

1. ISO 6341: Water Quality - Determination of the Inhibition of the Mobility of *Daphnia magna* Straus (Cladocera, Crustacea) (15 March 1982).