OECD GUIDELINE FOR TESTING OF CHEMICALS

Adopted by the Council on 17th July 1992

Fish, Acute Toxicity Test

INTRODUCTION

1. This new version of the guideline, originally adopted in 1981 and first updated in 1984, is based on a proposal from the United Kingdom to reduce the numbers of fish in tests of acute aquatic toxicity. The proposal was discussed at a meeting of OECD experts convened at Medmenham (United Kingdom) in November 1988.

2. The main differences in comparison with the earlier versions are the reduction in group-size allowing the use of seven fish per group, the extension of the concentration range by allowing a spacing factor of 2.2 instead of 2 and the introduction of a limit test at 100 mg/l of test substance.

PRINCIPLE OF THE TEST

3. The fish are exposed to the test substance preferably for a period of 96 hours. Mortalities are recorded at 24, 48, 72 and 96 hours and the concentrations which kill 50 per cent of the fish (LC50) are determined where possible.

INFORMATION ON THE TEST SUBSTANCE

4. It is necessary to know the water solubility of the substance under the conditions of the test. A reliable analytical method for the quantification of the substance in the test solutions must also be available.

5. Useful information includes the structural formula, purity of the substance, stability in water and light, pK_a, P_{ow}, vapour pressure and results of a test for ready biodegradability (see Guideline 301). Solubility and vapour pressure can be used to calculate Henry's constant which will indicate if losses of the test substance may occur.

VALIDITY OF THE TEST

6. For a test to be valid the following conditions should be fulfilled:

- the mortality in the control(s) should not exceed 10 per cent (or one fish if less than ten are used) at the end of the test;

- constant conditions should be maintained as far as possible throughout the test and, if necessary, semi-static or flow-through procedures should be used (see Annex 1 for definitions);

- the dissolved oxygen concentration must have been at least 60 per cent of the air saturation value throughout the test;

- there must be evidence that the concentration of the substance being tested has been satisfactorily maintained, and preferably it should be at least 80 per cent of the nominal concentration throughout the test. If the deviation from the nominal concentration is greater than 20 per cent, results should be based on the measured concentration.

DESCRIPTION OF THE METHOD

Apparatus

7. Normal laboratory equipment and especially the following is necessary :

(a) oxygen meter;
(b) equipment for determination of hardness of water;
(c) adequate apparatus for temperature control;
(d) tanks made of chemically inert material and of a suitable capacity in relation to the recommended loading.

Selection of species

8. One or more species may be used, the choice being at the discretion of the testing laboratory. It is suggested that the species used be selected on the basis of such important practical criteria as, for example, their ready availability throughout the year, ease of maintenance, convenience for testing and any relevant economic, biological or ecological factors. The fish should be in good health and free from any apparent malformation.

9. Examples of fish recommended for testing are given in the Table. The fish mentioned in the Table are easy to rear and/or widely available throughout the year. They can be bred and cultivated either in fish farms or in the laboratory, under disease- and parasite-controlled conditions, so that the test fish will be healthy and of known parentage. These fish are available in many parts of the world. If other species fulfilling the above criteria are used, the test method should be adapted in such a way as to provide suitable test conditions.

Holding of fish

10. All fish must be obtained and held in the laboratory for at least 12 days before they are used for testing. They must be held in water of the quality to be used in the test for at least seven days immediately before testing and under the following conditions:

Light: 12 to 16 hours photoperiod daily;

Temperature: appropriate to the species (see Table);

Oxygen concentration: at least 80 per cent of air saturation value;

Feeding: three times per week or daily until 24 hours before the test is started.

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

- mortalities of greater than 10 per cent of population in seven days: rejection of entire batch;
- mortalities of between 5 and 10 per cent of population: acclimatisation continued for seven additional days;
- mortalities of less than 5 per cent of population: acceptance of batch.

Water

12. Good quality natural water or reconstituted water (see Annex 2) is preferred, although drinking water (dechlorinated if necessary) may also be used. Waters with total hardness of between 10 and 250 mg $CaC0_3$ per liter, and with a pH 6.0 to 8.5 are preferable. The reagents used for the preparation of reconstituted water should be of analytical grade and the deionised or distilled water should be of conductivity equal to or less than 10 μScm^{-1}.

Test solutions

13. Test solutions of the chosen concentrations are prepared by dilution of a stock solution. Stock solutions of substances of low water solubility may be prepared by ultrasonic dispersion or other suitable physical means. If necessary, vehicles such as organic solvents, emulsifiers or dispersants of low toxicity to fish may be used. When such vehicles are used an additional control should be exposed to the same concentration of the vehicle as that used in the most concentrated solution of the test substance. The concentration of organic solvents, emulsifiers or dispersants should not exceed 100 mg/l.

14. The test should be carried out without adjustment of pH. If there is evidence of marked change in the pH of the tank water after addition of the test substance, it is advisable that the test be repeated, adjusting the pH of the stock solution to that of the tank water before addition of the test substance. This pH adjustment should be made in such a way that the stock solution concentration is not changed to any significant extent and that no chemical reaction or precipitation of the test substance is caused. HC1 and Na0H are preferred.

PROCEDURE

Conditions of exposure

15. Duration: preferably 96 hours.

Loading: maximum loading of 1.0 g fish/litre for static and semi-static tests is recommended; for flow-through systems higher loading can be accepted.

Light: 12 to 16 hours photoperiod daily.

Temperature: appropriate to the species (see Table) and constant within a range of 2°C.

Oxygen concentration: not less than 60 per cent of the air saturation value. Aeration can be used provided that it does not lead to a significant loss of test substance.

Feeding: none.

Disturbance: disturbances that may change the behaviour of the fish should be avoided.

Number of fish

16. At least 7 fish must be used at each test concentration and in the controls.

Test concentrations

17. At least five concentrations in a geometric series with a factor preferably not exceeding 2.2. A range-finding test properly conducted before the definitive test enables the choice of the appropriate concentration range.

Controls

18. One blank and, if relevant, one control containing the solubilising agent are run in addition to the test series.

Observations

19. The fish are inspected at least after 24, 48, 72 and 96 hours. Fish are considered dead if there is no visible movement (e.g. gill movements) and if touching of the caudal peduncle produces no reaction. Dead fish are removed when observed and mortalities are recorded. Observations at three and six hours after the start of the test are desirable. Records are kept of visible abnormalities (e.g. loss of equilibrium, swimming behaviour, respiratory function, pigmentation, etc.). Measurement of pH, dissolved oxygen and temperature should be carried out at least daily.

LIMIT TEST

20. Using the procedures described in this Guideline, a limit test may be performed at 100 mg(active ingredient)/l in order to demonstrate that the LC50 is greater than this concentration. The limit test should be performed using a minimum of 7 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 LC50 is greater than 100 mg/l. With 7, 8 or 9 fish, the absence of mortality provides at least 99% confidence that the LC50 is greater than the concentration used in the limit test.) If any mortalities occur, a full study should be conducted. If sublethal effects are observed, these should be recorded.

DATA AND REPORTING

Treatment of results

21. The cumulative percentage mortality for each exposure period is plotted against concentration on logarithmic probability paper. Normal statistical procedures are then employed to calculate the LC50 for the appropriate exposure period. Confidence limits ($p = 0.95$) for the calculated LC50 values are determined using standard procedures (1)(2)(3)(4)(5).

22. Where the data obtained are inadequate for the use of standard methods of calculating the LC50, the highest concentration causing no mortality and the lowest concentration producing 100 per cent mortality should be used as an approximation for the LC50 (this being considered the geometric mean of these two concentrations).

Test report

23. The test report must include the following information:

Test substance:

- physical nature and, where relevant, physicochemical properties;
- identification data.

Test fish:

- scientific name, strain, size, supplier, any pretreatment, etc.

Test conditions:

- test procedure used (e.g. static, semi-static, flow-through; aeration; fish loading; etc.);
- water quality characteristics (pH, hardness, temperature);
- dissolved oxygen concentration, pH values and temperature of the test solutions at 24 hour intervals (in semi-static systems the pH should be measured prior to and after water renewal);
- methods of preparation of stock and test solutions;
- concentrations used;
- information on concentrations of the test substance in the test solutions;
- number of fish in each test solution.

Results:

- maximum concentration causing no mortality within the period of the test;
- minimum concentration causing 100 per cent mortality within the period of the test;
- cumulative mortality at each concentration at the recommended observation times;
- LC50 values, with 95 per cent confidence limits, at each of the recommended observation times, if possible;
- graph of the concentration-mortality curve at the end of the test;
- statistical procedures used for determining the LC50 values;
- mortality in the controls;
- incidents in the course of the test which might have influenced the results;
- abnormal responses of the fish.

Discussion of the results.

TABLE: FISH SPECIES RECOMMENDED FOR TESTING

Recommended species	Recommended test temperature range (°C)	Recommended total length of test fish (cm)[1]
Brachydanio rerio (Teleostei, Cyprinidae) (Hamilton-Buchanan) Zebra-fish	21 - 25	2.0 ± 1.0
Pimephales promelas (Teleostei, Cyprinidae) (Rafinesque) Fathead Minnow	21 - 25	2.0 ± 1.0
Cyprinus carpio (Teleostei, Cyprinidae) (Linnaeus) Common carp	20 - 24	3.0 ± 1.0
Oryzias latipes (Teleostei, Cyprinodontidae) (Temminck and Schlegel) Ricefish	21 - 25	2.0 ± 1.0
Poecilia reticulata (Teleostei, Poeciliidae) (Peters) Guppy	21 - 25	2.0 ± 1.0
Lepomis macrochirus (Teleostei, Centrarchidae) (Rafinesque) Bluegill	21 - 25	2.0 ± 1.0
Oncorhynchus mykiss (Teleostei, Salmonidae) (Walbaum) Rainbow trout	13 - 17	5.0 ± 1.0

[1] If fish of sizes other than those recommended are used, this should be reported together with the rationale.

LITERATURE

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

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

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

(4) Stephan C.E. (1977). Methods for calculating an LC50. In Aquatic Toxicology and Hazard Evaluation (edited by Mayer F.I. and Hamelink J.L.). ASTM STP 634, pp 65-84, American Society for Testing and Materials.

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

ANNEX 1

DEFINITIONS

Static test is a test with aquatic organisms in which no flow of test solution occurs. Solutions remain unchanged throughout the duration of the test.

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

Flow-through test is a test in which solutions are automatically and continually renewed in the test chambers, the displaced solutions running to waste.

LC50 in this Test Guideline is the median lethal concentration, i.e. that concentration of the test substance in water which kills 50 per cent of a test batch of fish within a particular period of exposure (which must be stated).

ANNEX 2

EXAMPLE OF A SUITABLE RECONSTITUTED WATER (ISO 6341-1982)

(a) Calcium chloride solution
Dissolve 11.76 g $CaCl_2.2H_20$ in deionised water; make up to 1 litre with deionised water

(b) Magnesium sulphate solution
Dissolve 4.93 g $MgS0_4.7H_20$ in deionised water; make up to 1 litre with deionised water

(c) Sodium bicarbonate solution
Dissolve 2.59 g $NaHC0_3$ in deionised water; make up to 1 litre with deionised water

(d) Potassium chloride solution
Dissolve 0.23 g KCl in deionised water; make up to 1 litre with deionised water

All chemicals must be of analytical grade.

The conductivity of the distilled or deionised water should not exceed 10 μScm^{-1}.

25 ml each of solutions (a) to (d) are mixed and the total volume made up to 1 litre with deionised water. The sum of the calcium and magnesium ions in this solutions is 2.5 mmol/l. The proportion Ca:Mg ions is 4:1 and Na:K ions 10:1. The acid capacity $K_{S4.3}$ of this solution is 0.8 mmol/l.

Aerate the dilution water until oxygen saturation is achieved, then store it for about two days without further aeration before use.