

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
<u>Brachydanio rerio</u> (Teleostei, Cyprinidae) (Hamilton-Buchanan) Zebra-fish	21 - 25	2.0 ± 1.0
<u>Pimephales promelas</u> (Teleostei, Cyprinidae) (Rafinesque) Fathead Minnow	21 - 25	2.0 ± 1.0
<u>Cyprinus carpio</u> (Teleostei, Cyprinidae) (Linnaeus) Common carp	20 - 24	3.0 ± 1.0
<u>Oryzias latipes</u> (Teleostei, Cyprinodontidae) (Temminck and Schlegel) Ricefish	21 - 25	2.0 ± 1.0
<u>Poecilia reticulata</u> (Teleostei, Poeciliidae) (Peters) Guppy	21 - 25	2.0 ± 1.0
<u>Lepomis macrochirus</u> (Teleostei, Centrarchidae) (Rafinesque) Bluegill	21 - 25	2.0 ± 1.0
<u>Oncorhynchus mykiss</u> (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 1DEFINITIONS

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 2EXAMPLE OF A SUITABLE RECONSTITUTED WATER (ISO 6341-1982)

- (a) Calcium chloride solution
Dissolve 11.76 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ in deionised water; make up to 1 litre with deionised water
- (b) Magnesium sulphate solution
Dissolve 4.93 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ in deionised water; make up to 1 litre with deionised water
- (c) Sodium bicarbonate solution
Dissolve 2.59 g NaHCO_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 $10 \mu\text{Scm}^{-1}$.

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 $K_{\text{S4.3}}$ 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.

