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
  - $pK_a$
  - n-Octanol/water partition coefficient
  - Results of a biodegradability test (see Test Guidelines 301 A-E)

- **Qualifying statements**
  - For chemicals of limited solubility under the test conditions it may not be possible to determine the LC 50.

- **Standard documents**
  See references (1) to (11), Section 4, Literature.

2. **METHOD**

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

- **Definitions**

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

Users of this Test Guideline should consult the Preface, in particular paragraphs 3, 4, 7 and 8.
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 water is renewed continuously in the test chambers, the test substance being transported with the water used to renew the test medium.

LC 50 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).

- Reference substances

No reference substances are recommended for this test. However, if a reference substance has been tested, the results should be given.

- Principle of the test method

The fish are exposed to various concentrations of 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 (LC 50) are determined where possible.

The maximum concentration tested producing no mortality and the minimum concentration tested producing total mortality should be recorded.

- Conditions for the validity 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.

- The mortality in the controls should not exceed 10 per cent at the end of the test.

- The dissolved oxygen concentration (throughout the test) must have been at least 60 per cent of air saturation value.
There must be evidence that the concentration of the substance being tested has been satisfactorily maintained (it should be at least 80 per cent of the nominal concentration) over the test period. The results should be based on the measured concentration if the deviation from the nominal concentration is greater than 20 per cent.

B. DESCRIPTION OF THE TEST PROCEDURE

Preparations

Equipment

Normal laboratory equipment and especially the following is necessary:

- Oxygen meter
- Equipment for determination of hardness of water
- Adequate apparatus for temperature control
- Test tanks made of chemically inert material and of a suitable capacity

Solutions of the test substance

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, if necessary, by use of vehicles, such as organic solvents, emulsifiers or dispersants of low toxicity to fish. 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 preferably not exceed 100 mg/l.

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 advised 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 physical precipitation of the test substance is caused. HCl or NaOH are preferred.

**Experimental animals**

**Selection of species**

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, their ease of maintenance, their convenience for testing and any relevant economic, biological or ecological factors. The fish should be in good health and free from any apparent malformation.

Examples of fish recommended for testing are given in Table 1. The fish mentioned in Table 1 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 animal 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.
Table 1: FISH SPECIES RECOMMENDED FOR TESTING

<table>
<thead>
<tr>
<th>Recommended species</th>
<th>Recommended test temperature (range in °C)</th>
<th>Recommended total length of test fish in cm *</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Brachydanio rerio</em> (Teleostei, Cyprinidae) (Hamilton-Buchanan) Zebra-fish</td>
<td>21 - 25</td>
<td>2.0 ± 1.0</td>
</tr>
<tr>
<td><em>Pimephales promelas</em> (Teleostei, Cyprinidae) Fathead minnow</td>
<td>21 - 25</td>
<td>2.0 ± 1.0</td>
</tr>
<tr>
<td><em>Cyprinus carpio</em> (Teleostei, Cyprinidae) (Linné 1758) Common carp</td>
<td>20 - 24</td>
<td>3.0 ± 1.0</td>
</tr>
<tr>
<td><em>Oryzias latipes</em> (Teleostei, Poeciliidae) (Schlegel 1850) Red killifish</td>
<td>21 - 25</td>
<td>2.0 ± 1.0</td>
</tr>
<tr>
<td><em>Poecilia reticulata</em> (Teleostei, Poeciliidae) (Peters 1859) Guppy</td>
<td>21 - 25</td>
<td>2.0 ± 1.0</td>
</tr>
<tr>
<td><em>Lepomis macrochirus</em> (Teleostei, Centrarchidae) (Linné 1758) Bluegill</td>
<td>21 - 25</td>
<td>2.0 ± 1.0</td>
</tr>
<tr>
<td><em>Salmo gairdneri</em> (Teleostei, Salmonidae) (Richardson 1836) Rainbow trout</td>
<td>13 - 17</td>
<td>5.0 ± 1.0</td>
</tr>
</tbody>
</table>

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

Acclimatisation: At least 12 days. All fish must be exposed to water of the quality to be used in the test for at least seven days before they are used.

Water: Drinking water supply (dechlorinated if necessary), good quality natural water or reconstituted water (see Annex). Waters with a total hardness of between 50 and 250 mg CaCO₃ per liter, and with a pH 6.0 to 8.5 are preferable. The reagents used for the preparation of the dilution water should be of analytical grade and the deionised or distilled water should be of conductivity equal to or less than 10 uS cm⁻¹.

Light: 12 to 16 hours photoperiod daily

Temperature: Appropriate to the species (see Table 1)

Oxygen concentration: At least 80 per cent of air saturation value

Feeding: Three times per week or daily, at least until 24 hours before the test is started

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

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

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

One blank and, if relevant, one control containing the vehicle are run in addition to the test series. Mortality in the controls and blanks should not exceed 10 per cent.

Disturbances that may change the behaviour of the fish should be avoided.

Conditions of exposure

Duration: Preferably 96 hours

Tanks: Of suitable capacity in relation to the recommended loading

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 acceptable. At least 10 fish must be used at each test concentration and in the controls.

Test concentrations At least five concentrations in a geometric series with a factor preferably not exceeding 2

Water: Drinking water supply (dechlorinated if necessary), good quality natural water or reconstituted water (see Annex). Waters with a total hardness of between 50 and 250 mg of CaCO₃ per liter, and with a pH 6.0 to 8.5 are preferable. The reagents used for the preparation of the dilution water should be of analytical grade and the deionised or distilled water should be of conductivity equal to or less than 10 µS/cm⁻¹

Light: 12 to 16 hours photoperiod daily
Temperature: Appropriate to the species (see Table 1) constant within ± 1°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

Observations

The fish are inspected at least after 24, 48, 72 and 96 hours. Fish are considered dead if touching of the caudal peduncle produces no reaction. Dead fish are removed when observed and mortalities are recorded. Observations at three hours 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.). Measurements of pH, dissolved oxygen and temperature should be carried out at least daily.

3. DATA AND REPORTING

- Treatment of results

The cumulative percentage mortality for each recommended 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 (see references, Section 4, Literature). Confidence limits (p = 0.95) for the calculated LC50 values can be determined using the standard procedures quoted.

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).
Interpretation of results

If it is observed that the stability or homogeneity of the test substance cannot be maintained, care should be taken in the interpretation of the results and note made that these may not be reproducible.

Test report

The test report should include the following information:

Test substance: chemical identification data

Test animals: 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
- any available 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 according to the recommended observation time

- LC 50 values at each of the recommended observation times (with 95 per cent confidence limits, if possible)

- graph of the concentration-mortality curve at the end of the test

- statistical procedures used for determining the LC 50 values

- mortality in the controls

- incidents in the course of the test which might have influenced the results

- abnormal responses of the fish

4. LITERATURE

- Standard procedures

(1) Draft International Standard ISO/DIS 7346/1, 2, 3: Water Quality-Determination of the Acute Lethal Toxicity of Substances to Freshwater Fish (25 Nov. 1982).


(3) DIN Draft 38 412, L 1 und L 15: Testverfahren mit Wasserorganismen.

(4) AFNOR T 90 303: Détermination de la toxicité aiguë d'une substance vis à vis de Brachydano rio (French standard, 1978).


(11) Commission of the European Communities, Study D.8368: Inter-laboratory Test Programme concerning the Study of the Ecotoxicity of a Chemical Substance with respect to the Fish (22 March 1979).

- Other


5. **ANNEX**

An example of a suitable dilution water is given below (ISO 6341-1982):

(a) Calcium chloride solution  
Dissolve 11.76 g CaCl$_2$.2H$_2$O in deionised water; make up to 1 litre with deionised water

(b) Magnesium sulphate solution  
Dissolve 4.93 g MgSO$_4$.7H$_2$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 $\mu$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 solution is 2.5 mmol/l. The proportion of Ca:Mg ions is 4:1 and of Na:K ions 10:1. The acid capacity K$_5$H$_4$.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.