C.1. ACUTE TOXICITY FOR FISH

1. METHOD

1.1. INTRODUCTION

The purpose of this test is to determine the acute lethal toxicity of a substance to fish 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 to help in the selection of the most appropriate test method (static, semi-static or flow-through) for ensuring satisfactorily constant concentrations of the test substance over the period of the test.

Additional information (for instance structural formula, degree of purity, nature and percentage of significant impurities, presence and amounts 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

Acute toxicity is the discernible adverse effect induced in an organism within a short time (days) of exposure to a substance. In the present test, acute toxicity is expressed as the median lethal concentration (LC_{50}), that is the concentration in water which kills 50% of a test batch of fish within a continuous period of exposure which must be stated.

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 response of tested species have not changed significantly.

No reference substances are specified for this test.

1.4. PRINCIPLE OF THE TEST METHOD

A limit test may be performed at 100 mg per litre in order to demonstrate that the LC_{50} is greater than this concentration.

The fish are exposed to the test substance added to water at a range of concentrations for a period of 96 hours. Mortalities are recorded at least at 24-hour intervals, and the concentrations killing 50% of the fish (LCso) at each observation time are calculated where possible.

1.5. QUALITY CRITERIA

The quality criteria shall apply to the limit test as well as the full test method.

The mortality in the controls must not exceed 10% (or one fish if less than ten are used) by the end of the t est.

The dissolved oxygen concentration must have been more than 60% of the air-saturation value throughout.

The concentrations of the test substance shall be maintained to within 80% of the initial concentrations 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 fish.

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 THE TEST METHOD

Three types of procedure can be used:

Static test:

Toxicity test in which no flow of test solution occurs. (Solutions remain unchanged throughout the duration of the test.)

Semi-static test:

Test without flow of test solution, but with regular batchwise renewal of test solutions after prolonged periods (e.g. 24 hours).

Flow-through test:

Toxicity test in which the water is renewed constantly in the test chambers, the chemical under test being transported with the water used to renew the test medium.

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 substance, and additional control fish 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 dilution water unless there are specific reasons not to do so. HCl 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, t his should be reported.

1.6.1.2. Holding and dilution water

Orinking-water supply (uncontaminated by potentially harmful concentrations of chlorine, heavy metals or other substances), good-quality natural water or reconstituted water (See Appendix I) may be used. Waters with a total hardness of between 10 and 250 mg per litre (as $CaCO_3$) and with a pH from 6,0 to 8,5 are preferred.

1.6.2. Apparatus

All apparatus must be made of chemically inert material.

-automatic dilution system (for flow-through test),

-oxygen meter ,

-equipment for determination of hardness of water ,

-adequate apparatus for temperature control,

-pH meter.

1.6.3. Test fish

The fish should be in good health and free from any apparent malformation.

The species used should be selected on the basis of practical criteria, such as their ready availability throughout the year, ease of maintenance, convenience for testing, relative sensitivity to chemicals, and any economic, biological or ecological factors which have any bearing. The need for comparability of the data obtained and existing international harmonization (reference 1) should also be borne in mind when selecting the fish species.

A list of fish species which are recommended for the performance of this test is given in Appendix 2; Zebra fish and rainbow trout are the preferred species.

1.6.3.1. Holding

Test fish should preferably come from a single stock of similar length and age. The fish must be held for at least 12 days, in the following conditions:

loading:

appropriate to the system (recirculation or flow-through) and the fish species,

water:

see 1.6.1.2,

light:

12 to 16 hours illumination daily,

dissolved oxygen concentration:

at least 80 % of air-saturation value,

feeding:

three times per week or daily, ceasing 24 hours before the startof the test.

1.6.3.2. Mortality

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

-greater than 10 % of population in seven days:

rejection of entire batch,

-between 5 and 10% of population:

holding period continued for seven additional days. If no further mortalities occur, the batch is acceptable, otherwise it must be rejected,

-less than 5 % of population:

acceptance of the batch.

1.6.4. Adaptation

All fish must be exposed to water of the quality and the temperature to be used in the test for at least seven days before they are used.

1.6.5. Test Procedure

A range-finding test can precede a 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.

Depending on the physical and chemical properties of the test compound, a static, semi-static, or a flow-through test should be selected as appropriate, to fulfil the quality criteria.

Fish are exposed to the substance as described below:

- duration: 96 hours

-number of animals: at least 7 per concentration,

-tanks: of suitable capacity in relation to the recommended loading,

-loading: maximum loading of 1 g per litre for static and semi-static tests is recommended; for flow-through systems, higher loading is acceptable,

-test concentration: At least five concentrations differing by a constant factor not exceeding 2,2 and as far as possible spanning the range of 0 to 100 % mortality,

-water: see 1.6.1.2,

-light: 12 to 16 hours illumination daily,

-temperature: appropriate to the species (Appendix 2) but within ± 1 °C within any particular test,

-dissolved oxygen concentration: not less than 60 % of the air-saturation value at the selected temperature,

-feeding: none.

The fish are inspected after the first 2 to 4 hours and at least at 24-hour intervals. Fish are considered dead if touching of the caudal peduncle produces no reaction, and no breathing movements are visible. Dead fish are removed when observed and mortalities are recorded. Records are kept of visible abnormalities (e.g. loss of equilibrium, changes in swimming behaviour, respiratory function, pigmentation, etc.).

Measurements of pH, dissolved oxygen and temperature must be carried out daily.

Limit test

Using the procedures described in this test method, a limit test may be performed at 100 mg per litre in order to demonstrate that the LC_{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 7 to 10 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 LC_{50} is greater than the concentration used in the limit test. With 7, 8 or 9 fish, the absence of mortality provides at least 99 % confidence that the LC_{50} is greater than the concentration used.)

If mortalities occur, a full study must be carried out. If sublethal effects are observed, these should be recorded.

2. DATA AND EVALUATION

For each period where observations were recorded (24, 48, 72 and 96 hours), plot percentage mortality for each recommended exposure period against concentration on logarithmic-probability paper .

When possible and for each observation time, the LC_{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 LC_{50}, a graphical estimate of this value is sufficient.

When two consecutive concentrations, at a ratio of 2,2 give only 0 and 100% mortality, these two values are sufficient to indicate the range within which the LC_{50} falls.

If it is observed that the stability or homogeneity of the test substance cannot be maintained, this should be reported and care should be taken in the interpretation of the results.

3. REPORTING

The test report shall, if possible, include the following information:

-information about test fish (scientific name, strain, supplier, any pretreatment, size and number used in each test concentration);

-dilution-water source and major chemical characteristics (pH, hardness, temperature);

-in the case of a substance of low aqueous solubility, the method of preparation of stock and test solutions;

-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 solution;

-if chemical analyses are performed, methods used and results obtained;

-results of the limit test if conducted;

-reasons for the choice and details of the test procedure used (e.g. static, semi-static, dosing rate, flow-through rate, whether aerated, fish loading, etc.);

-description of test equipment;

-lighting regime;

-dissolved oxygen concentrations, pH values and temperatures of the test solutions every 24 hours;

-evidence that the quality criteria have been fulfilled;

-a table showing the cumulative mortality at eaeh concentration and the control (and control with the auxiliary substance if required) at each of the recommended observation times;

-graph of the concentration/ percentage response curve at the end of the test;

-if possible, the LC_{50} values at each of the recommended observation times (with 95 % confidence limits);

-statistical procedures used for determining the LC_{50} values;

-if a reference substance is used, the results obtained,

-highest test concentration causing no mortality within the period of the test;

-lowest test concentration causing 100% mortality wirhin the period of the test.

4. **REFERENCES**

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Appendix 1

Reconstituted water

Example of a suitable dilution water

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}.

Apparatus for distillation of water must not contain any parts made of copper.

Stock solutions

$CaCI_2$. 2H2O (calcium chloride dihydrate): 11,76 g
Dissolve in, and make up to 1 litre with water .

$MgSO_4$. 7H2O (magnesium sulphate heptahydrate): 4,93 g
Dissolve in, and make up to 1litre 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 11itre with water.

Reconstituted dilution water

Mix 25 ml of each of the four stock solutions and make up to 1 litre with water.
Aerate until the dissolved oxygen concentration 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 must 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

Fish species recommended for testing

Recommended species	Recommended range of test temperature (°C)	Recommended total length of test animal (cm)
Brachydanio rerio (Teleostei, Cyprinidae) (Hamilton-Buchanan) Zebrab-fish	20 to 24	3,0 ± 0,5
Pimephales promelas (Teleostei, Cyprinidae) (Rafinesque) Fathead minnow	20 to 24	5,0 ± 2,5
Cyprinus carpio (Teleostei, Cyprinidae) (Linneaus 1758) Common carp	20 to 24	6,0 ± 2,0
Oryzias latipes (Teleostei, Poeciliidae) Cyprinodontidae (Tomminck and Schlege 1850) Red killifish	20 to 24	3,0 ± 1,0
Poecilia reticulata (Teleostei, Poeciliidae) (Peters 1859) Guppy	20 to 24	3,0 ± 1,0
Lepomis macrochirus (Teleostei, Centrarchidae) (Rafinesque Linneaus 1758) Bluegill	20 to 24	5,0 ± 2,0
Onchorhynchus mykiss (Teleostei, Salmonidae) (Walbaum 1988) Rainbow trout	12 to 17	6,0 ± 2,0
Leuciscus idus (Teleostei, Cyprinidae) (Linneaus 1758) Golden Orfe	20 to 24	6,0 ± 2,0

Collection

The fish listed above are easy to rear and/or are widely available throughout the year. They are capable of being 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.

Appendix 3

Example of concentration: percentage mortality

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