International Standard ISO 7346/3

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Water quality — Determination of the acute lethal toxicity of substances to a freshwater fish [*Brachydanio rerio* Hamilton-Buchanan (Teleostei, Cyprinidae)] — Part 3: Flow-through method

Qualité de l'eau — Détermination de la toxicité aiguë létale de substances vis-à-vis d'un poisson d'eau douce [Brachydanio rerio Hamilton Buchanan (Teleostei, Cyprinidae)] — *Partie 3: Méthode avec renouvellement continu*

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Foreword

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Draft International Standards adopted by the technical committees are circulated to the member bodies for approval before their acceptance as International Standards by the ISO Council. They are approved in accordance with ISO procedures requiring at least 75 % approval by the member bodies voting.

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Water quality — Determination of the acute lethal toxicity of substances to a freshwater fish [*Brachydanio rerio* Hamilton-Buchanan (Teleostei, Cyprinidae)] — Part 3: Flow-through method

0 Introduction

The three parts of ISO 7346 describe methods of determining the acute lethal toxicity of substances to the zebra fish (*Brachydanio rerio* Hamilton-Buchanan), but it must be emphasized that the recommended use of the zebra fish does not preclude the use of other species. The methodologies presented here may also be used for other species of freshwater, marine of brackish water fish, with appropriate modifications of, for example, dilution water quality and the temperature conditions of the test.

Within the three parts of ISO 7346, a choice can be made between static, semi-static and flow-through methods. The static test, described in ISO 7346/1, in which the solution is not renewed, has the advantage of requiring simple apparatus although the substance in the test vessel may become depleted during the course of the test and the general quality of the water may deteriorate. The flow-through method, described in ISO 7346/3, in which the test solution is replaced almost continuously, overcomes such problems but requires the use of more complex apparatus. In the semi-static procedure, described in ISO 7346/2, the test solutions are renewed daily, this method being a compromise between the other two.

The flow-through method can be used for most types of substances, including those unstable in water, but the concentrations of the test substance are determined wherever possible. The static method is limited to the study of substances whose tested concentrations remain relatively constant during the test period. The semi-static method can be used for testing those substances whose concentrations can be maintained satisfactorily throughout the test by renewal of the solutions every 24 h.

To assist in the preparation and maintenance of concentrations of substances which may be lethal at concentrations close to that of their aqueous solubility, a small volume of solvent may be used , as specified in the methods.

1 Scope and field of application

This part of ISO 7346 specifies a flow-through method for the determination of the acute lethal toxicity of substances soluble in water under specified conditions to a species of freshwater fish [*Brachydanio rerio* Hamilton-Buchanan (Teleostei, Cyprinidae) — common name, zebra fish] in water of a specified quality.

The method is applicable for assigning, for each test substance, broad categories of acute lethal toxicity to *Brachydanio rerio* under the test conditions.

The results are insufficient by themselves to define water quality standards for environmental protection.

The method is also applicable when using certain other species of freshwater fish as the test organism.[1)]

The method may be adapted for use with other freshwater fish and marine and brackish water fish with appropriate modification of the test conditions, particularly with respect to the quantity and quality of the dilution water and temperature.

1) The following species of freshwater fish can be used, in addition to *Brachydanio rerio*, without modification to this part of ISO 7346:

- *Cichlasoma nigrofasciatum* (Teleostei, Cichlidae)
- *Lepomis macrochirus* (Teleostei, Centrachidae)
- *Oryzias latipes* (Teleostei, Poeciliidae)
- *Pimephales promelas* (Teleostei, Cyprinidae)
- *Poecilia reticulata* (Teleostei, Poeciliidae)

The results obtained from a test with one species cannot, however, be extrapolated to other species.

2 Principle

The method determines, under specified conditions, the concentrations at which a substance is lethal to 50 % of a test population of *Brachydanio rerio* after exposure periods of 24; 48; 72; and 96 h to that substance in the ambient water. These median lethal concentrations are designated the 24 h LC50; 48 h LC50; 72 h LC50 and 96 h LC50.

The test is carried out in two stages:

a) a preliminary test which gives an approximate indication of the acute median lethal concentrations and serves to determine the range of concentrations for the final test;

b) a final test, the results of which alone are recorded.

Where evidence is available to show that test concentrations remain relatively constant (i.e. within about 20 % of the nominal values) throughout the test then either measured or nominal concentrations may be used in the estimation of the LC50. Where such analyses show that the concentrations present remain relatively constant but are less than about 80 % of the nominal values, then the analytical values shall be used in estimating the LC50. Where evidence is not available to show that the test concentrations remained at an acceptable level throughout the test period or where it is known (or suspected) that the concentrations of the test chemical have declined significantly at any stage during the test then irrespective of whether or not chemical analytical data are available the LC50 cannot be defined, using this test method. In these cases the test is not necessarily invalidated but it can only be stated that the LC50 of the substance is $\leqslant x$ mg/l, the value, x, given being estimated from the nominal concentrations used.

3 Test organism and reagents

The reagents shall be of recognized analytical grade. The water used for the preparation of solutions shall be glass-distilled water or deionized water of at least equivalent purity.

3.1 Test organism

The test species shall be *Brachydanio rerio* Hamilton-Buchanan (Teleostei, Cyprinidae), commonly known as the zebra fish. Each test fish shall have a total length of 30 ± 5 mm and a mass of 0,3 ± 0,1 g. They shall be selected from a population of a single stock. This stock should have been acclimated and, in any case, maintained for at least 2 weeks prior to the test in dilution water, continuously aerated (using bubbled air) (see 3.2), under conditions of water quality and illumination similar to those used in the test. They shall be fed as normal up to the 24 h period immediately preceding the test.

Test fish shall be free of overt disease or visible malformation. They shall not receive treatment for disease during the test or in the 2 weeks preceding the test.

Environmental conditions for the maintenance and breeding of zebra fish are given in annex A.

3.2 Standard dilution water

The freshly prepared standard dilution water shall have a pH of 7,8 ± 0,2, and calcium hardness of approximately 250 mg/l, expressed as calcium carbonate, and shall be prepared as follows.

Prepare the following solutions using distilled or deionized water:

a) Calcium chloride solution

Dissolve 11,76 g of calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$) in water and dilute to 1 litre.

b) Magnesium sulfate solution

Dissolve 4,93 g of magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$) in water and dilute to 1 litre.

c) Sodium hydrogen carbonate solution

Dissolve 2,59 g of sodium hydrogen carbonate ($NaHCO_3$) in water and dilute to 1 litre.

d) Potassium chloride solution

Dissolve 0,23 g of potassium chloride (KCl) in water and dilute to 1 litre.

Mix 25 ml of each of these four solutions and dilute to 1 litre with water.

Aerate the dilution water until the concentration of dissolved oxygen reaches its air saturation value (ASV) and the pH value is constant at 7,8 ± 0,2. If necessary, adjust the pH of the solution by adding sodium hydroxide solution or hydrochloric acid. The dilution water thus prepared shall receive no further forced aeration before use in the tests.

3.3 Stock solutions of test substances

A stock solution of the test substance should be prepared by dissolving a known amount of test substance in a defined volume of dilution water, deionized water or glass-distilled water. The stock solution should be prepared daily except where it is known that the material is stable in solution, in which case sufficient solution for use over 2 days may be prepared. To enable stock solutions to be prepared and to assist in their transfer to the test vessels, substances of low aqueous solubility may be dissolved or dispersed by suitable means, including ultrasonic devices and using organic solvents of low toxicity to fish. If any such organic solvent is used, its concentration in the test solution shall not exceed 0,1 ml/l, and two sets of controls, one containing solvent at the maximum concentration used in any test vessel and one without solvent or test substance, shall be included.

3.4 Test solutions

Test solutions are prepared by adding appropriate amounts of the stock solution of the test substance to the dilution water to give the required concentrations. It is recommended that when a stock solution is prepared in distilled or deionized water, no more than 100 ml of stock solution should be added per 10 l of dilution water.

4 Apparatus

All materials which may come into contact with any liquid into which the fish are to be placed, or with which they may come into contact, shall be inert and should not absorb the test substance significantly.

Usual laboratory equipment [including a dip-net, made of nylon or of another chemically inert material, for the control vessels and another for all the test vessels (4.1)], and

4.1 Test vessels

Test vessels shall be either multiple angle neck, round-bottom glass flasks, of capacity 1 or 2 l, having ground-glass joints (see figure 1) or similar glass vessels. When 1 litre flasks are used, only five fish can be used per flask and two flasks shall be used for each test solution concentration. With the multiple-neck flask, one of the necks should have a standard inlet tube and the second, that is used for the outlet, should preferably be fitted with a screen.

Before use, new test vessels shall be carefully washed and then rinsed successively with water and the dilution water. At the end of the test, the flasks shall be emptied, cleaned by appropriate means, rinsed with water to remove all traces of the test substance and cleaning aid, and dried.

Test flasks shall be rinsed with dilution water just before use.

4.2 Temperature control equipment

The temperature of the test solutions and the water in the stock tanks shall be regulated to 23 ± 1 °C by a suitable method.

4.3 Solution replacement equipment

The dosing and mixing apparatus shall be capable of maintaining the required concentrations of the stock solution in the test flasks to within 10 %, and the apparatus shall be set to renew the test solutions in the flasks at a rate which is sufficient to prevent the concentration of dissolved oxygen in the vessels from falling below 60 % ASV.

5 Test environment

The preparation and storage of solutions, the holding of fish, and all the manipulations and tests shall be carried out in premises with an atmosphere free from harmful concentrations of airborne contaminants.

Take care to avoid any unwanted disturbance that may change the behaviour of the fish. All tests should be carried out under normal laboratory illumination with a daily photoperiod of 12 to 16 h.

6 Procedure

6.1 Condition of the fish

Whenever there is a change of stock population, a toxicity test using the method specified in this part of ISO 7346 should be carried out using a suitable reference substance. The results of such tests shall be in reasonable agreement with results obtained previously in the same laboratory.

6.2 Preliminary test

6.2.1 If possible, the flow-through method should be used for the preliminary test, adopting the same range of concentrations, number of fish per test solution concentration and method of observation of the fish as described in 6.2.2 for the alternative, preliminary static test.

6.2.2 Add at least 2,5 l, preferably 5 l, of standard dilution water (3.2) to each of six vessels and aerate if necessary to restore the concentration of dissolved oxygen to its air saturation value. Prepare test solutions by adding appropriate amounts of stock solution of the test substance (3.3) to five of the vessels in order to obtain an adequate range of concentrations, for example 1 000; 100; 10; 1; and 0,1 mg/l. Nothing is added to the sixth vessel, which serves as a control. The solutions should be adjusted to and maintained at 23 ± 1 °C.

Place five fish in each vessel.

At least twice a day for a suitable period, note the numbers of dead fish and the dissolved oxygen concentration in each vessel. Remove dead fish.

If there are insufficient data for establishing the range of concentrations required for the final test, repeat this preliminary test with alternative ranges of concentrations.

6.3 Final test

Select at least five concentrations, forming an approximately geometric series, for example 8; 4; 2; 1; and 0,5 mg/l, between, but including, the lowest concentration killing all the fish in the preliminary test, and the highest non-lethal concentration in 48 h. This selected series of concentrations should provide the possibility of obtaining mortalities of between 20 and 80 % in at least three consecutive concentrations of the geometric series used, for estimation of the LC 50.

In some instances, a narrower range of concentrations may be required to provide the necessary data and for others a wider range may be needed.

Assemble at least six 2 l flasks (or 12 × 1 litre flasks) and ancillary equipment and fill all but one or two of the flasks with one of the series of test solutions to give the particular range of concentrations of test substance which has been selected for

testing. Fill one of the remaining flasks[1] with standard dilution water only to act as a control. If an organic solvent has been used to dissolve or disperse a substance, prepare a second "control" with the standard dilution water containing sufficient of the organic solvent to give the maximum concentration at which this solvent is present in any of the test solutions. When the test solution has been adjusted to 23 ± 1 °C, place 10 fish in each 2 l flask (or five fish in each 1 litre flask; see 4.1), as follows.

Select the fish at random from the stock and distribute them at random into the test flasks, without delay, using a small mesh dip-net of soft inert material. Discard any fish dropped or otherwise mishandled during the transfer. In a given test, all fish should be added within a period of 30 min.

Set the apparatus to replace the test solution at a rate of at least 25 l/day, either continuously or by additions at short intervals. The rate of replacement can be reduced to as low as 12 l/day provided that the dissolved oxygen concentration of the outgoing solution remains greater than 60 % ASV.

The test solution to be used for replacement should be adjusted to, and maintained at, a temperature close to 23 °C before being added to the test flasks.

Record the number of dead fish in each flask at least twice daily over the period of the test. Remove each dead fish from the flask as soon as possible. Observations can be made more frequently, for example to enable median periods of survival to be calculated for each concentration. Note any abnormal behaviour of the fish.

If possible, the concentrations of the test substance in the stock solutions and in the outgoing solutions from the test flasks shall be measured at least at the beginning and end of the test.

Measure the dissolved oxygen concentration, the pH and the temperature of the outgoing solution from each of the test flasks at least once daily and at the beginning and end of the test.

A suggested form suitable for recording the data is given in annex B.

7 Expression of results

7.1 Validity

The results shall be considered valid if the following requirements were fulfilled:

a) the dissolved oxygen concentration in the test solutions during the test was at least 60 % ASV;

b) the concentrations of the test substance were not known (or suspected) of having declined significantly throughout the test (but see clause 2);

c) the mortality of the control fish did not exceed 10 %;

d) the proportion of control fish showing abnormal behaviour did not exceed 10 %;

e) the 24 h LC50 of the reference chemical for the stock of fish was in reasonable agreement with results obtained previously in the same laboratory.

7.2 Estimation of LC50

Where a simple graphical estimation of the LC50 is considered adequate this can be obtained by plotting mortality (expressed as a percentage of test fish in each test vessel) against concentration of test substance. Using axes with linear scales this will produce a sigmoid relationship from which the LC50 can be derived by interpolating the concentration expected to cause 50 % mortality (see figure 2).

It is more appropriate to plot the data on graph paper having axes with probability and logarithmic scales. Data plotted in this way should produce a linear relationship from which the LC50 can be interpolated as above (see figure 3).

Where estimation of slope and 95 % confidence limits of this and the LC50 are required, the data can be analysed graphically.[2]

Where computing facilities are available probit analysis can be applied.[1]

If insufficient data are available to estimate the LC50 at 24; 48; 72; and 96 h, record the minimum concentration at which 100 % mortality occurred and the maximum concentration giving 0 % mortality at 24; 48; 72; and 96 h. These concentrations will indicate the limits within which the LC50 probably lies.

8 Test report

The test report shall include the following information:

a) the chemical identity and any additional available information about the test substance;

b) the method of preparing the dilution water, stock solutions and test solutions, including a detailed description of the solution-replacement equipment or a reference to it;

c) all biological, chemical and physical data pertaining to the test not otherwise specified in this part of ISO 7346, including details of the acclimation conditions of the test fish, and the mass of fish in grams per litre;

d) the data taken into account when assessing the validity of the test

1) concentration of dissolved oxygen,

2) mortality observed among control fish,

1) If 1 litre flasks are used, two control flasks with dilution water only and, if appropriate, two with solvent should be used.

3) proportion of control fish showing abnormal behaviour,

4) LC50 of the reference substance;

e) a tabulated list showing the nominal concentrations tested (with chemical analytical values, where available), and the total percentage mortalities in each, 24; 48; 72; and 96 h, after the start of the test;

f) the LC50 values and confidence limits if available at 24; 48; 72; and 96 h, of the substance tested; reference should be given to the method of calculation, and the method of chemical analysis, where applicable;

g) the slope of the concentration-response curve (and its 95 % confidence limit if available);

h) a graphical illustration of the concentration-response relationship;

j) any unusual reactions by the fish under the test conditions and any visible external effects produced by the test substance;

k) any deviation from the procedure specified in this part of ISO 7346, and the reason for it;

m) a reference to this part of ISO 7346.

9 Bibliography

[1] FINNEY, D.J. *Statistical Methods in Biological Assay.* Wycombe, United Kingdom, Griffin, 1978.

[2] LITCHFIELD, J.T. and WILCOXON, F. A simplified method of evaluating dose-effect experiments. *J. Pharmacol. Exp. Ther.* **96** 1949: 99-113.

[3] STEPHAN, C.E. *Measurements for calculating an LC50. Aquatic Toxicology and Hazard Evaluation.* ASTM (1977), ST, p. 634.

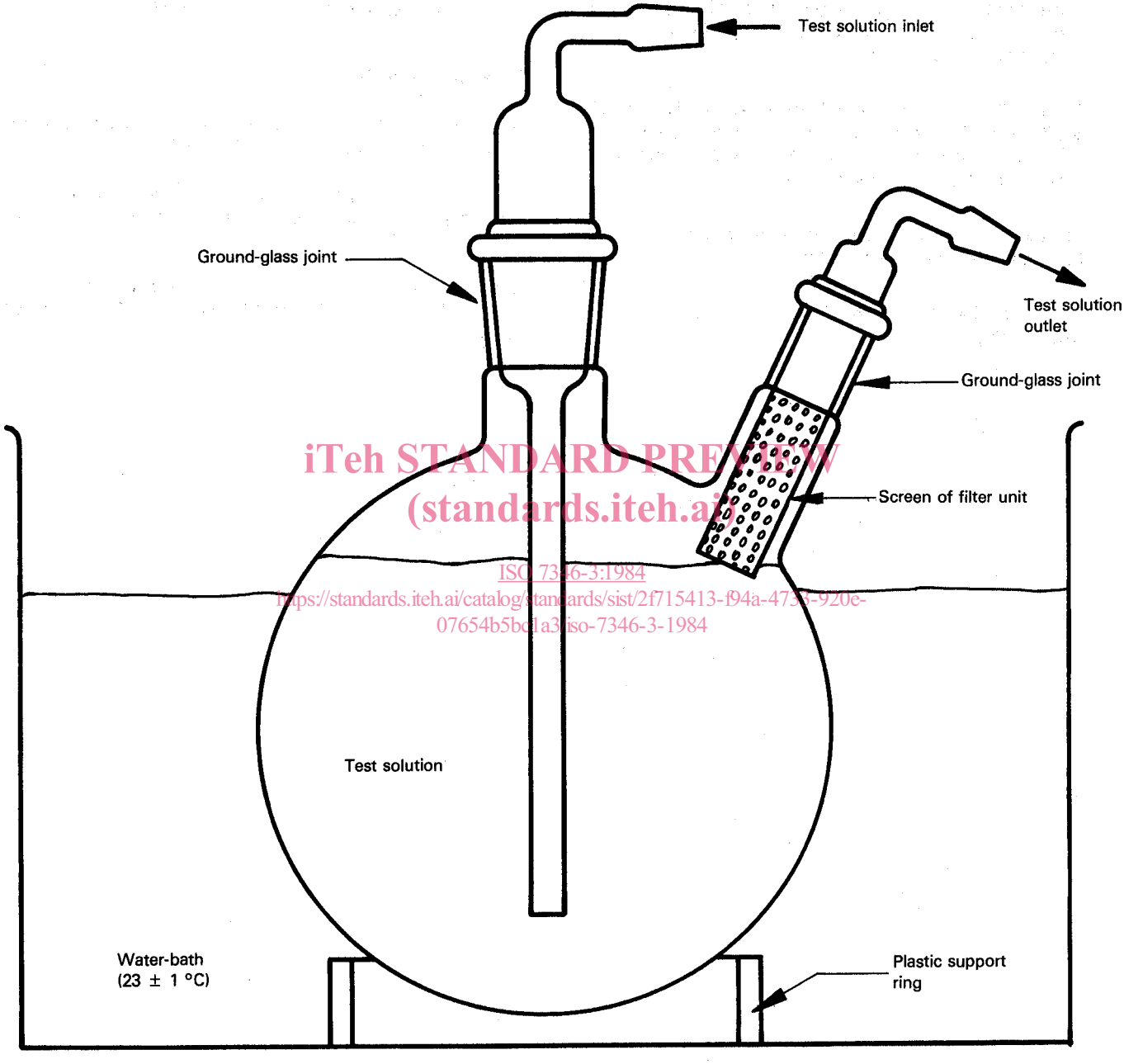

Figure 1 — Example of apparatus for flow-through method

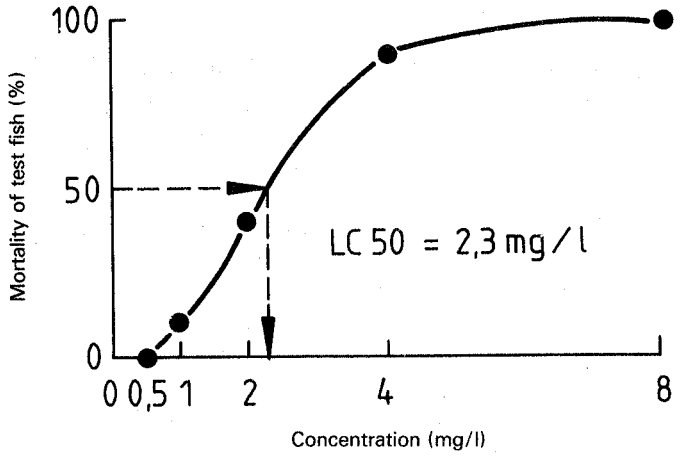

Figure 2 — Graphical interpolation of LC50 (linear scales)

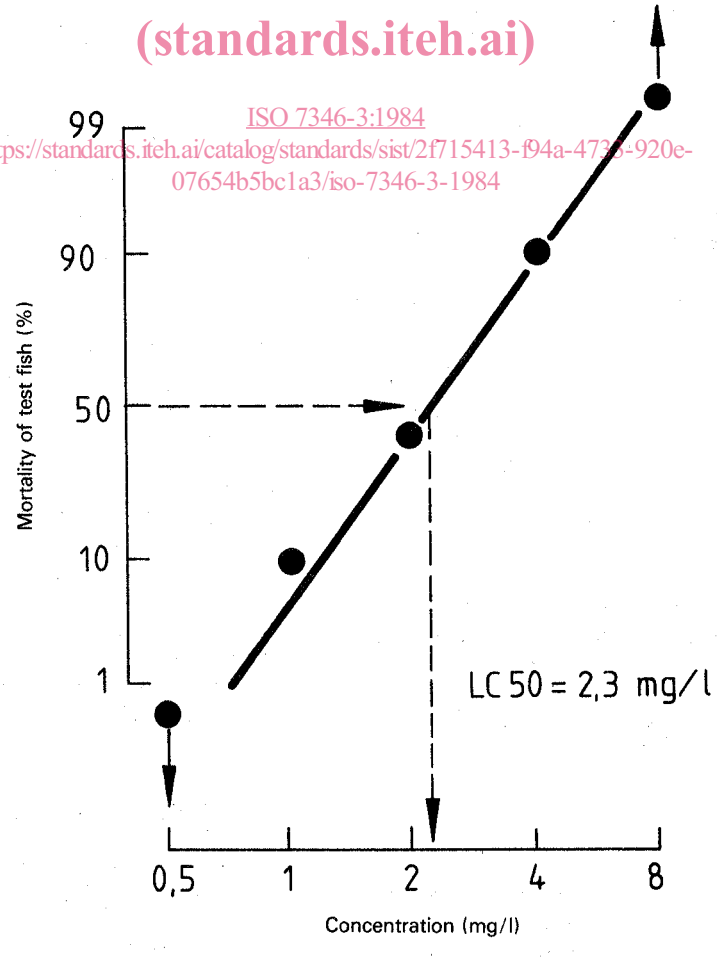

Figure 3 — Graphical interpolation of LC50 (logarithmic and probability scales)