INTERNATIONAL STANDARD

ISO 7346-2

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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 2: Semi-static 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 (Téléostei, Cyprinidae)*] —

Partie 2: Méthode semi-statique

Reference number
ISO 7346-2:1996(E)

Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

International Standard ISO 7346-2 was prepared by Technical Committee ISO/TC 147, *Water quality*, Subcommittee SC 5, *Biological methods*.

This second edition cancels and replaces the first edition (ISO 7346-2:1984), which has been technically revised.

ISO 7346 consists of the following parts, under the general title *Water quality — Determination of the acute lethal toxicity of substances to a freshwater fish* [Brachydanio rerio *Hamilton-Buchanan (Teleostei, Cyprinidae)*]:

— *Part 1: Static method*

— *Part 2: Semi-static method*

— *Part 3: Flow-through method*

Annexes A, B and C of this part of ISO 7346 are for information only.

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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 or 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 substances 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 replenished continuously, overcomes such problems but requires the use of more complex apparatus. In the semi-static procedure, described in this part of ISO 7346, the test solutions are renewed every 24 h or 48 h, 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 or 48 h. Special arrangements may be necessary for substances which are highly volatile.

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.

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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 2:
Semi-static method

1 Scope

This part of ISO 7346 specifies a semi-static method for the determination of the acute lethal toxicity of stable, non-volatile, single substances, soluble in water under specified conditions, to a 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 the temperature.

2 Principle

Determination, under specified conditions, of the concentrations at which a substance is lethal to 50 % of a test population of *Brachydanio rerio* after exposure periods of 24 h, 48 h, 72 h 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 reported.

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

— *Lepomis macrochirus* (Teleostei, Centrarchidae)

— *Oryzias latipes* (Teleostei, Poeciliidae)

— *Pimephales promelas* (Teleostei, Cyprinidae)

— *Poecilia reticulata* (Teleostei, Poeciliidae)

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 are used in the estimation of the LC50. Where such analyses show that the concentrations present remain relatively constant but are less than about 80 %, or greater than 120 %, of the nominal values, then the analytical values are 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, 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 mm ± 5 mm, which, in principle, corresponds to a mass of 0,3 g ± 0,1 g. They shall be selected from a population of a single stock. This stock should have been acclimatized and, in any case, maintained for at least 7 d 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. Subsequent to the test, fish remaining alive should be suitably disposed of.

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 a calcium hardness of approximately 250 mg/l, expressed as calcium carbonate, and shall contain the following concentrations of salts dissolved in distilled or deionized water:

294,0 mg/l $CaCl_2 \cdot 2H_2O$

123,3 mg/l $MgSO_4 \cdot 7H_2O$

63,0 mg/l $NaHCO_3$

5,5 mg/l KCl

Aerate the dilution water until the concentration of dissolved oxygen reaches at least 90 % of its air saturation value (ASV) and the pH 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 at a frequency appropriate to the stability of the test substance. 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 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, or the volume containing 0,1 g/l, whichever is the greater. Where a solvent is used, 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 litres 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 and the following.

4.1 Test vessels, of sufficient capacity (which may need to be greater than 10 litres), with a large area of interface between the air and the test medium (of about 800 cm^2 for 10 litres of medium) and equipped with a securely fixed and close-fitting cover. The volume of the test vessels should be sufficient that a loading rate of 1 g of fish per litre of water should not be exceeded at any time during the test.

Before use, the test vessels shall be cleaned thoroughly, for example with a non-ionic detergent (followed by acid and solvent washes for substances expected to adsorb strongly to the vessel).

4.2 Temperature control equipment, to regulate the temperature of the test solutions and the water in the stock tanks to 23 °C ± 1 °C by a suitable method.

4.3 Dip-net, made of nylon or of another chemically inert material, for the control vessels and another for all the test vessels (4.1).

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. Carry out all tests under normal laboratory illumination with a daily photoperiod of 12 h to 16 h.

6 Procedure

6.1 Condition of the fish

Whenever there is a change of stock population, carry out a toxicity test using the method specified in this part of ISO 7346 using a suitable reference chemical [e.g. potassium dichromate ($K_2Cr_2O_7$)]. The results of such tests shall be in reasonable agreement with results obtained previously in the same laboratory.

Test fish shall not have been used for any previous testing procedure.

Maintain the temperature of the water in the stock tanks at 23 °C ± 1 °C (4.2).

6.2 Limit test

Using the procedures described in this part of ISO 7346, a limit test may be performed at the limit of aqueous solubility under the conditions of the test or at 100 mg/l, whichever is the lower, in order to demonstrate that the 96 h - LC50 is greater than this concentration. If no fish die in the limit test, no further testing is required.

Perform the limit test using 10 fish, with the same number in the control(s).

NOTE 1 Binominal theory dictates that, when 10 fish are used, with zero mortality there is a 99,9 % confidence that the 96 h - LC50 is greater than the limit-test concentration. If mortalities occur, a complete study (see 6.3 and 6.4) may need to be considered. If sub-lethal effects are observed, these should be recorded.

6.3 Preliminary test

Add at least 2,5 litres, preferably 5 litres, of standard dilution water (3.2) to each of six test vessels (4.1) and aerate if necessary to restore the concentration of dissolved oxygen to at least 90 % of 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 geometric range of concentrations, for example 1 000 mg/l; 100 mg/l; 10 mg/l; 1 mg/l and 0,1 mg/l. Nothing is added to the sixth vessel, which serves as a control. The solutions shall be adjusted to and maintained at 23 °C ± 1 °C (4.2) and shall not be forcibly aerated during the test.

Place three fish in each vessel.

After 24 h or 48 h, prepare new test solutions in new test vessels and transfer the live fish to them without delay. Repeat every 24 h or 48 h throughout the preliminary test.

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

6.4 Final test

Select at least five concentrations, forming an approximately geometric series, for example 8 mg/l; 4 mg/l; 2 mg/l; 1 mg/l 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 96 h. This selected series of concentrations shall provide the possibility of obtaining mortalities of between 0 % and 100 % in at least two consecutive concentrations of the geometric series used, which is necessary for an estimation of the LC50 using the probit method.

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

Take at least six test vessels (4.1) and into each pour, for example, 10 litres of standard dilution water (3.2). Nothing is added to one of these (the control) but, to the remainder, add the different amounts of stock solution (3.3) required to give the particular range of concentrations of test substance which has been selected for testing. If an organic solvent has been used to dissolve 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 (3.4) has been adjusted to 23 °C ± 1 °C (4.2), place at least seven fish in each of the vessels, as follows.

Select the fish at random from the stock and place them at random in the test vessels, without delay, using a small mesh dip-net of soft inert material (4.3). Discard any fish dropped or otherwise mishandled during the transfer. In a given test, add all the fish within a period of 30 min.

After 24 h or 48 h, prepare new test solutions in new test vessels and transfer the live fish to them without delay. The renewal of test solutions and transfer of fish shall be repeated every 24 h or 48 h during the test. In order to avoid significant transfer of test substances between test vessels via the dip-net (4.3), the transfer of fish should begin with the lowest concentration and proceed towards the highest concentration.

The solutions shall not be forcibly aerated. Record the number of dead fish in each vessel at least daily over the period of the test. Remove each dead fish from the vessel 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 the substance is shown to be stable over the period of exposure, (i.e. losses of less than 20 % of the initial measured concentration are expected or have been demonstrated) then, if possible, measure the concentrations of the test substance in the test vessels at least at the beginning and end of the first and final renewal periods. If the test substance is shown to be unstable over the period of exposure, then if possible measure the concentration of the test substance in the test vessels at the beginning and end of each renewal period throughout the duration of the test.

Measure the dissolved oxygen concentration, the pH and temperature in each vessel at least at the beginning of the test and immediately before and after the renewal of the test solution.

A suggested form which is 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 are 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) to have declined significantly throughout the test (but see clause 2);

c) the mortality of the control fish did not exceed 10 % or one per tank;

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

e) the 24 h - LC50 of the reference chemical [e.g. potassium dichromate ($K_2Cr_2O_7$)] 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 1).

It is more appropriate to plot the data on graph paper having axes with logarithmic and probability scales. Data plotted in this way should produce a linear relationship from which the LC50 can be interpolated as above (see figure 2).

Where estimation of slope and 95 % confidence limits of this and the LC50 are required, and it is recommended that these statistics are frequently valuable in expressing results, the data can be analysed graphically ([2] in annex C).

Where computing facilities are available, probit analysis can be applied ([1] in annex C).

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

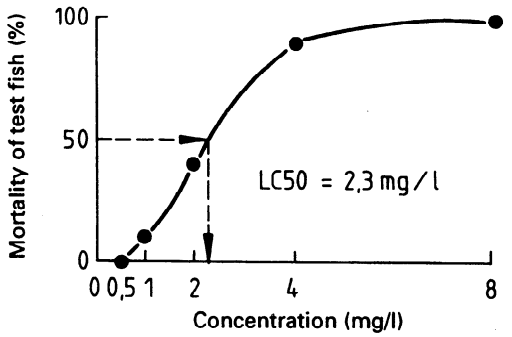

Figure 1 — Graphical interpolation of LC50 (linear scales)

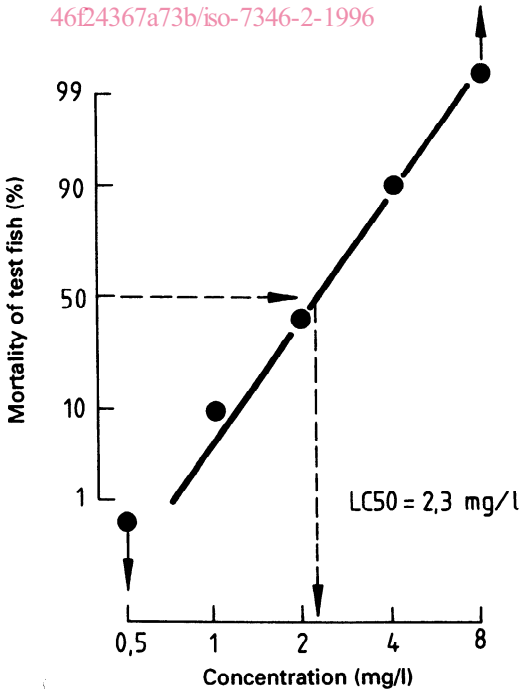

Figure 2 — Graphical interpolation of LC50 (logarithmic and probability scales)