INTERNATIONAL STANDARD

ISO 7346-1

Second edition
1996-06-15

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

Part 1:
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 1: Méthode statique

Reference number
ISO 7346-1:1996(E)

Foreword

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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 7346-1 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 7346-1:1984), 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 this part of ISO 7346, 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 ISO 7346-2, 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.

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

Part 1:
Static method

1 Scope

This part of ISO 7346 specifies a 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 $\pm$ 5 mm, which, in principle, corresponds to a mass of 0,3 g $\pm$ 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 $\pm$ 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 $\pm$ 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. 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.

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.

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 then, if possible, measure the concentrations of the test substance in the test vessels at least at the beginning and end of the test.

Measure the dissolved oxygen concentration, the pH and temperature in each vessel at least once daily and at the beginning and end of the test. The temperature and pH of the test solutions should not vary by more than ± 1 °C or 0,2 pH units, respectively.

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 ($K_2Cr_2O_7$)] if available, 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).

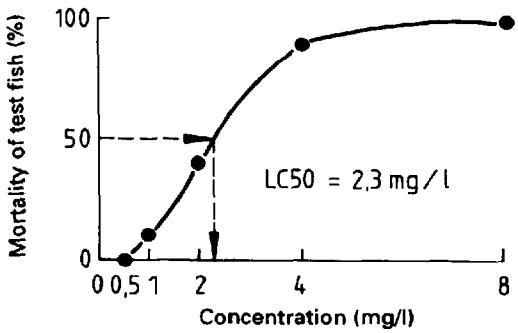

Figure 1 — Graphical interpolation of LC50 (linear scales)

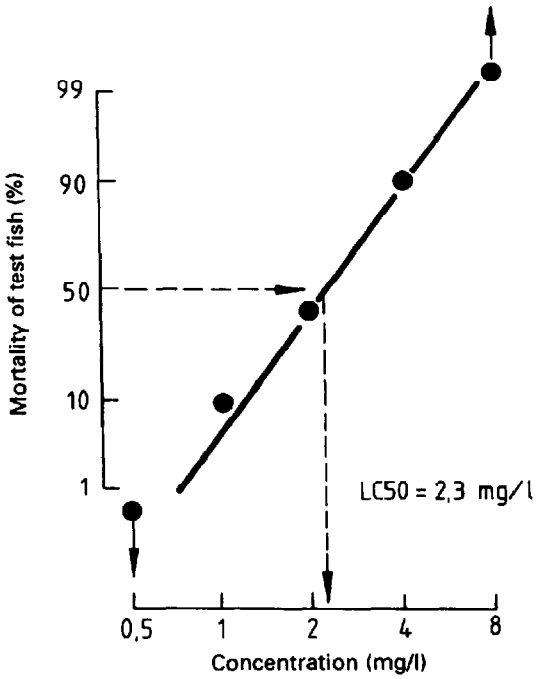

Figure 2 — Graphical interpolation of LC50 (logarithmic and probability scales)

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.

8 Test report

The test report shall include the following information:

a) a reference to this part of ISO 7346;

b) the chemical identity and any additional available information about the test substance (e.g. water solubility, volatility, octanol/water partition coefficient, degradation rate);

c) the method of preparing the dilution water, stock solutions and test solutions;

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

e) the data taken into account when assessing the validity of the test:

   1) concentration of dissolved oxygen;

   2) mortality observed among control fish;

   3) proportion of control fish showing abnormal behaviour;

   4) LC50 of the reference substance;

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

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

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

i) 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.

Annex A
(informative)

Environmental parameters for maintenance and breeding of zebra fish (*Brachydanio rerio* Hamilton-Buchanan)

A.1 General

The species originates from the Coromandel coast of India where it inhabits fast flowing streams. It is a common aquarium fish, so that information about procedures for its care and culture can be found in standard reference books on tropical fish culture. Its biology has recently been reviewed by Laale [5].

The fish rarely exceeds 45 mm in length. The body is cylindrical with 7 to 9 dark blue horizontal stripes on silver. These stripes run into the caudal and anal fins. The back is olive green. Males are slimmer than females and possess a golden sheen. Females are more silvery and the abdomen is distended, particularly prior to spawning.

A.2 Environmental parameters

The fish are capable of withstanding wide ranges of temperature, pH and water hardness. Axelrod [4] states a temperature range of 15,5 °C to 43,3 °C and a pH of 6,6 to 7,2. Fish may be bred, reared and maintained in tap water with a total hardness as high as 300 mg/l (as calcium carbonate) and a pH of 7,7 to 8,2. The temperature is maintained at 26 °C ± 1 °C and raised to 27 °C ± 1 °C to induce spawning.

A.3 Materials and methods

The fish may readily be spawned in glass tanks of capacity about 70 litres. The fry are later transferred to a tank of capacity 200 litres.

Since the adult fish are avid egg eaters, a method of protecting newly laid eggs and young fish is necessary. One method, used successfully, is to confine the adult fish in mesh cages in the water so that, as the female lays her eggs, these fall through the mesh to the bottom of the tank out of reach of the adults.

The mesh cages are made of plastics netting with 3 mm mesh, of dimensions approximately 250 mm × 250 mm × 80 mm. They are clipped to the lips of the tanks so that the whole of the upper edge of the cage is above water with the mesh dropping 60 mm into the water. An undergravel filter system should not be used to cleanse the water because it is likely to damage the eggs. The tanks should be illuminated for 8 h per day.

A.4 Conditioning

This period lasts for approximately 2 weeks. Males and females are separated and fed on live food. This consists of white worms (*enchytraeids*), *Daphnia* and brine shrimps (*Artemia*). The density of stocking during conditioning is kept below 30 fish in tanks of capacity 70 litres.

At the end of 2 weeks, the males possess a deep golden sheen and the females are greatly distended with ova.

A.5 Breeding stage

The spawning tank can be set up as follows.

Fill an empty tank with fresh tap water aged at 27 °C for 48 h and place a plastic cage inside the tank under the lip, allowing the fish a swimming space of volume about 1 litre. Place six females in the basket in the morning and feed with freeze-dried brine shrimps.

Add nine males to the basket in the evening and feed the fish once more with freeze-dried brine shrimps before the lights are switched off.

Spawning is induced by the morning light and is completed after the lights have been switched on for approximately 4 h. The eggs, which are non-adhesive, fall through the mesh, out of reach of the adults.

When the females are exhausted of eggs, remove the adults and leave the eggs to hatch.

A.6 Development of fry

The eggs hatch in 4 d to 5 d, and the fry or alevins adhere to the side of the tank and remain motionless for 24 h to 48 h. When the fry become free-

swimming, feed them on suitable proprietary fish food of small particle size. At 3 weeks, the fry can be fed newly hatched brine shrimps and growth then becomes more rapid. After 1 month, they can be transferred to a 200 litre tank and fed on a mixture of live and proprietary foods. The fish are sexually mature at 3 months and attain a length of 3,5 cm. It should be noted that spontaneous abnormalities in the developing larvae have been observed in certain strains ([9] in annex C).

Further studies ([7] in annex C) indicate that a dietary factor is responsible for the deformities and that the zebra fish is especially susceptible to this factor (other species breed normally when fed the same proprietary fish food).

Annex B
(informative)

Suggested form for recording data

Laboratory	**Operator**
Sample No.	**Date of start of test**
Substance	
Purity	
Impurities	
If a formulation is being tested, the identity of the components	
Method of preparing the stock solution	Stock solution concentration (mg/l) Maximum concentration of solvent in test vessels (ml/l)
Method of chemical analysis	

Control vessels
1 Dilution water only

Determinands	Time from start of test (h)							
	0							
Dissolved oxygen concentration (% ASV[1]) pH Temperature (°C) Number of dead fish								

2 Dilution water and _____ ml/l solvent

Determinands	Time from start of test (h)							
	0							
Dissolved oxygen concentration (% ASV[1]) pH Temperature (°C) Number of dead fish								

Test vessel No.

Initial (measured or calculated) concentration of test substance _____ mg/l

Determinands	Time from start of test (h)							
	0							
Test substance concentration [mg/l (by analysis)] Dissolved oxygen concentration (% ASV[1]) pH Temperature (°C) Number of dead fish								

1) Air saturation value.

Annex C
(informative)

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