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Water quality — Determination of the prolonged toxicity of substances to freshwater fish — Method for evaluating the effects of substances on the growth rate of rainbow trout [*Oncorhynchus mykiss* Walbaum (Teleostei, Salmonidae)]

Qualité de l'eau — Détermination de la toxicité prolongée de substances vis-à-vis d'un poisson d'eau douce — Méthode d'évaluation des effets de substances sur le taux de croissance de la truite arc-en-ciel [Oncorhynchus mykiss *Walbaum (Teleostei, Salmonidae)*]

Reference number
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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 10229 was prepared by Technical Committee ISO/TC 147, *Water quality*, Subcommittee SC 5, *Biological methods*.

Annexes A and B of this International Standard are for information only.

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Introduction

This International Standard describes methods of determining the long-term toxicity of substances to freshwater fish. The methods include the determination of lethal and sublethal responses. In all cases, the specified use of one species does not preclude the use of other species. It is possible for the methods presented here to be used for other species of freshwater fish, provided that appropriate modifications to, for example, quality of water used for dilution, temperature conditions and feeding rates are made and the relationships between growth rates, feeding rates and conversion efficiencies are established.

Within this International Standard, a choice may be made between semi-static and flow-through methods. These have already been described for the determination of the acute lethal toxicity of substances to a freshwater fish in ISO 7346-2 and ISO 7346-3 respectively. The choice of the method of presentation of the test solution depends on two factors: the nature of the test substance and the form of the test method.

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 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 invoke the chosen responses in the fish at concentrations close to that of their aqueous solubility, a small volume of solvent is sometimes used, as specified in the methods.

Water quality — Determination of the prolonged toxicity of substances to freshwater fish — Method for evaluating the effects of substances on the growth rate of rainbow trout [*Oncorhynchus mykiss* Walbaum (Teleostei, Salmonidae)]

1 Scope

This International Standard specifies a method for the determination of the long-term sublethal toxicity of substances soluble in water under specified conditions to a species of anadromous fish [*Oncorhynchus mykiss* Walbaum (Teleostei, Salmonidae) — common name, rainbow trout] in fresh water.

NOTE 1 "Substances" is considered to include pure chemicals tested singly or in mixtures, waste waters, process waters or other waters whose sublethal toxicity to fish it is necessary to determine. A range of concentrations of waste and process waters should be tested (see 8.2).

The response measured is the change in rate of growth of fish exposed to the test substance over periods of 14 d and 28 d (see [1] in annex B).

NOTE 2 The mass of each fish at the start of the test, the period of exposure, the feeding regime and temperature range have been chosen to ensure that the control fish will be growing exponentially throughout the test.

The method is applicable for assigning, for each test substance, a category of prolonged sublethal toxicity to *Oncorhynchus mykiss* under the test conditions.

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

It is possible for the method to 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 temperature, the quantity and quality of the dilution water, the food and the fish-marking technique.

2 Normative references

The following standards contain provisions which, through reference in this text, constitute provisions of this International Standard. At the time of publication, the editions indicated were valid. All standards are subject to revision, and parties to agreements based on this International Standard are encouraged to investigate the possibility of applying the most recent editions of the standards indicated below. Members of IEC and ISO maintain registers of currently valid International Standards.

ISO 7346-2:1984, *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.*

ISO 7346-3:1984, *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.*

3 Principle

Determination, under specified conditions, of the concentrations at which a substance statistically and significantly reduces the rate of growth of a test population of *Oncorhynchus mykiss* after exposure periods of 14 d and 28 d to that substance in the ambient water.

The test is carried out in two stages:

a) a preliminary test, carried out using rainbow trout as the test species but in accordance with ISO 7346-2 or ISO 7346-3 (although the rainbow trout is not specifically named in these parts of ISO 7346), which determines the concentrations at which the substance is lethal to 50 % of a test population after exposure to the substance in the ambient water for 96 h: this median lethal concentration is designated as 96 h LC50 and serves to determine the range of concentrations for the final test (see note 11 to 8.1).

b) a final test, using 16 fish per concentration, in which the results are recorded as both the lowest observed effect concentration (LOEC) and the no observed effect concentration (NOEC). They are prefixed by an indication of the exposure period concerned, for example "28 d LOEC". The data may also be fitted to a regression model in order to calculate the IC10 (inhibition concentration for a 10 % effect), or a similar statistic.

Where analytical data show that median test concentrations remain relatively constant (i.e. within 20 % of the nominal values) throughout the test, the method allows the use of either measured or nominal concentrations in the statements on LOEC and NOEC. Where such analyses show that the concentrations present remain relatively constant (i.e. ± 20 % of the median) but are less than 80 % or greater than 120 % of the nominal values, then the analytical values derived from this method are used in presenting the LOEC and NOEC. Where no evidence is available to show that the test concentrations remained at an acceptable level throughout the test period or where it is known that the concentrations of the test chemical have changed significantly (i.e. by greater than 20 % of the median) at any stage during the test, then irrespective of whether or not chemical analytical data are available the LOEC and NOEC cannot be defined using this test method. In these cases, the test is not necessarily invalidated but it can only be stated that the LOEC of the substance is $\leqslant x$ mg/l, and that the NOEC of the substance is $\leqslant y$ mg/l, the values x and y given being estimated from the nominal concentrations used.

NOTE 3 Wherever possible, it is very important to determine the concentrations of the test substance to which the fish are exposed.

Fish can be individually recognized throughout the test if the freeze-branding technique or an equivalent method (e.g. tagging) is used for marking, but this is not mandatory.

4 Test organism

The recommended test species is *Oncorhynchus mykiss* Walbaum (Teleostei, Salmonidae), commonly known as the rainbow trout. Test fish shall be selected from a population of a single stock. This stock shall have been acclimatized and, in any case, maintained for at least 2 weeks prior to the test in dilution water, continuously aerated (using bubbled air), under conditions of water quality, feeding regime and illumination similar to those used in the test.

Except where otherwise stated (see clause 8), the fish shall be fed at a minimum rate of 1 % of their wet body mass per day during any pre-acclimation and acclimation period and 4 % of their mass per day during the final test (see 8.2).

NOTE 4 This latitude in feeding rate during the pretest period enables the fish to be grown to, or held at, a suitable size (see below) for the start of the test.

The food shall be a dry proprietary salmonid fry food and shall be divided into two equal portions and given to the fish in two feeds per day separated by at least 5 h. After exposure of 14 d to the test substance, when the fish are weighed again, the rations shall be recalculated for each test vessel.

At the start of the test, each fish shall have a mass of not less than 3 g and not more than 5 g (but see 8.2). For the whole batch of fish used in the test, the range in individual masses at the start of the test shall lie within ± 10 % of the arithmetic mean of the masses.

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.

Maintain the fish in environmental conditions suitable for rainbow trout.

NOTE 5 Fish cultivation manuals should be consulted, for example [2] in annex B.

5 Reagents

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

5.1 Dilution water

The dilution water shall be suitable for the long-term survival and growth of the test fish. The average pH

of the dilution water shall be within the range 6,7 to 8,5, but during a given test the pH of the dilution water shall not vary by more than ± 0,2 pH units from the mean value.

NOTE 6 Such a pH range can be expected to be associated with a bicarbonate alkalinity of 1 mg/l to 100 mg/l $CaCO_3$ and total hardness values of 20 mg/l to 300 mg/l $CaCO_3$, but these ranges will depend on the precise constitution of the dilution water and the time available for equilibration of the water. A suitable artificial water can be made with distilled water, as follows:

294,0 mg/l $CaCl_2 2H_2O$

123,3 mg/l $MgSO_4.7H_2O$

63,0 mg/l $NaHCO_3$

5,5 mg/l KCl

5.2 Stock solutions of test substances

Prepare a stock solution of the test substance by dissolving a known amount of test substance in a defined volume of dilution water, deionized water or glass-distilled water.

NOTE 7 In the case where the test "substance" is a waste water, process water or other water (see clause 1, note 1) and not one or more pure chemicals, these waters are to be taken as the stock solution.

Prepare or obtain the stock solution as frequently as necessary to maintain stable solutions. Although the use of solvents is not desirable, to enable stock solutions to be prepared and to assist in their transfer to the test vessels, it shall be permissible to dissolve or disperse substances of low aqueous solubility by suitable means, for example ultrasonic devices or the use of 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 preferably be below 0,01 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.

NOTE 8 It is preferable that the same concentration of solvent be used in all test vessels.

5.3 Test solutions

Prepare test solutions by adding appropriate amounts of the stock solution of the test substance (5.2) to the dilution water to give the required concentrations (8.2). 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. Wherever possible, it is very important to determine the concentrations of the test substance in each test vessel, with the exception of waste waters where this may not be possible.

6 Apparatus

6.1 General

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 shall 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 (6.2)] shall be used.

6.2 Test vessels, of capacity at least 45 litres. Their sides shall be covered with an opaque material to minimize disturbance of the fish.

Before use, carefully wash new test vessels and then rinse successively with water and the dilution water (5.1). At the end of the test, empty the vessels, clean them, for example with a non-ionic detergent (followed by acid and solvent washes for substances expected to adsorb strongly to the vessel), rinse with water to remove all traces of the test substance and cleaning aid, and dry.

Rinse test vessels with dilution water (5.1) just before use.

6.3 Temperature control, to maintain the temperature of the test solutions and the water in the fish stock tanks in the range 12,5 °C to 17,5 °C and to regulate it within ± 1 °C.

6.4 Solution-replacement equipment, comprising a dosing and mixing apparatus capable of maintaining the required concentrations of the stock solution in the test vessels to within 10 % of the mean. The apparatus shall be set to renew the test solutions in the vessels at a rate which is sufficient to prevent the concentration of dissolved oxygen in the vessels from falling below 70 % air saturation value (ASV).

6.5 Equipment for marking fish

If individual fish are to be marked, one of several techniques is permissible, for example freeze branding.

For freeze branding, the branding irons shall be made from stainless steel wire (e.g. 22 SWG 316) bent into the shape of an arabic numeral or letter and attached to a non-metallic handle. Figure 1 gives an example of suitable patterns for the brands. The brands shall be cooled in liquid nitrogen contained in a suitable insulated flask.

NOTES

9 Soon after branding, the mark is almost invisible but it gradually appears during the next 2 d and is then clearly visible for at least six weeks. Brands which include closed loops (as in 0,6,8,9) should be avoided, in case they encourage infection of the skin. The face of the brand should be smooth and flat, to avoid excessive pressure on any point of the area of skin being marked.

10 It is permissible to use methods of fish marking other than freeze-branding, provided that they do not interfere with the test.

7 Test environment

Carry out the preparation and storage of solutions, the holding of fish, and all the manipulations and tests in premises with an atmosphere free from concentrations of airborne contaminants that are harmful to fish.

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

8 Procedure

8.1 Preliminary tests

Carry out a preliminary test by performing a full 96 h LC50 test in accordance with ISO 7346-2 or ISO 7346-3 but using rainbow trout as the test species. The fish shall preferably be drawn from a single stock and be of approximately the same size as those to be used in the final test. The temperature, dilution water quality and illumination shall closely resemble the conditions to be adopted in the final test.

NOTE 11 It should be understood that, if the test substance has been tested in the past and relevant lethal toxicity data are available, there is no necessity to repeat the acute lethal toxicity test.

Report the results of the preliminary test as indicated in clause 8 of ISO 7346-2:1984 or ISO 7346-3:1984.

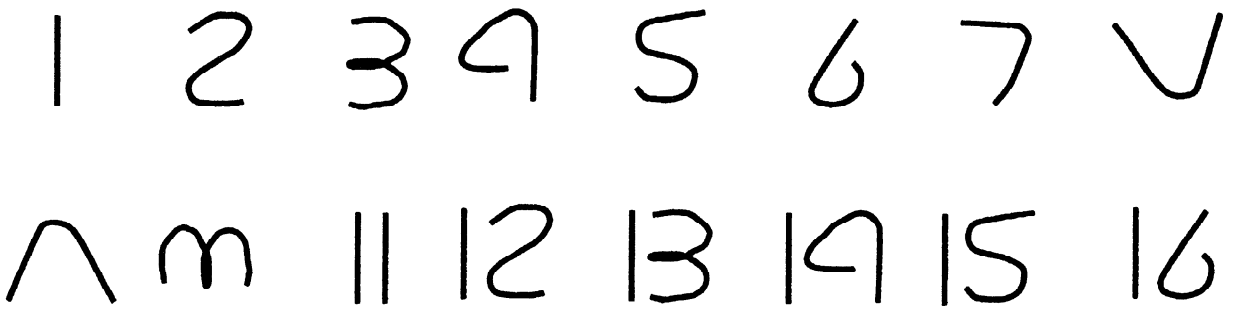

Figure 1 — Examples of marks used for freeze-branding fish

8.2 Final test

Normally, select at least five concentrations, forming an approximately geometric progression. The highest concentrations shall not usually be less than 10 % or greater than 32 % of the 96 h LC50 as determined in 8.1. If fewer than five concentrations are used, this procedure shall be justified.

NOTE 12 As a guide to the choice of the fraction of the 96 h LC50 to be used as the maximum concentration in the final test, if the toxicity curve determined in the preliminary test shows little evidence of flattening towards an asymptotic LC50, a fraction of approximately 10 % should be used. If an asymptotic LC50 has been defined within 96 h, or seems to be capable of definition shortly after 96 h, a fraction of approximately 32 % may be appropriate.

The factor between consecutive concentrations shall not exceed $\sqrt{10}$ (3,162).

NOTE 13 If it is necessary to repeat the test, a narrower or wider range of concentrations may have to be established.

Assemble at least six approximately 45 litre vessels (6.2) and ancillary equipment (6.3 to 6.5) and fill all but one or two of the vessels with 40 litres of the test solutions (5.3) to give the range of concentrations of test substance selected for testing. Fill one of the remaining vessels with dilution water (5.1) to act only as a control. If an organic solvent has been used to dissolve or disperse a substance, prepare a second "control" with the 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.

Adjust the temperature of the test solutions to the required level (see 6.3).

Measure the concentrations of the test substance in the solutions leaving the test vessels at least at the beginning, middle and end of the test.

NOTE 14 Preferably, the procedure for sampling and analysis of test water indicated below should be followed.

– 4	Start adding the test substance to the test vessels
– 3	Sample 1
– 2	Sample 2
0	If samples 1 and 2 are judged satisfactory, brand the fish if required and start the test
0 to 7	Sample 3
8 to 14	Sample 4
15 to 21	Sample 5
22 to 28	Sample 6

Set the apparatus (6.4) to replace the test solution in the test vessels at a rate of at least 200 l/day, either continuously or by additions at short intervals. If fish of an initial mass greater than 5 g are used, the test shall not be invalidated but the rate of replacement shall be increased so that, in all cases, the dissolved oxygen concentration of the outgoing solution remains greater than 70 % ASV and the conditions concerning concentrations of test substance (see 6.4) are met.

NOTE 15 When larger fish are used, the use of larger test vessels should be considered.

Adjust and maintain the temperature of the test solution to be used for replacement close to the test temperature prior to addition to the test vessels.

Perform the test either by

a) measuring individual growth rate, or

b) measuring the mean growth of each group of fish.

These methods may be equally sensitive, but this is probably only true if the range of masses or lengths at the start of the test is small, and conforms to the criteria stipulated in clause 4.

NOTE 16 Individual growth rate may be a more sensitive endpoint if initial masses and lengths are variable (see [3] in annex B).

a) Measuring individual growth rate

Withhold food from the test fish (clause 4) for 24 h before starting the test. Then choose the fish at random from among the stock fish, and anaesthetize them using an aqueous solution of 100 mg/l tricaine methanesulfonate or a similar substance (e.g. benzocaine). Weigh the fish to the nearest 100 mg and measure them (fork, total or standard length), to the nearest millimetre.

NOTE 17 Reference should be made to [4] in annex B for more information.

Place the anaesthetized fish one by one on a pad of moist tissue paper and remove excess moisture from the area on the fish to be branded. Then gently press a brand (see 6.5), cooled by immersion in liquid nitrogen, onto the fish below the dorsal fin for a period of 3 s. Allow them to recover from the anaesthetic in clean water. Place the fish, marked individually in batches of 16, in the test vessels.

WARNING — It should be noted that tricaine methanesulfonate may be carcinogenic.

NOTE 18 Fish should be transferred using a dip-net with a small mesh of soft inert material.

Discard any fish dropped or otherwise mishandled during the transfer.

In a given test, the first and last fish shall have been branded, weighed, measured and placed in exposure tanks within 4 h.

Fourteen days after the first exposure of the fish to the substance, withhold food again from the fish for 24 h. Repeat the anaesthesia and measurement of the fish and recalculate the ration (see clause 4). Return the fish to the test vessels for a further 14 d and then repeat the 24 h starvation, anaesthesia and measurement of the fish.

Examine the fish, preferably daily during the test, and note any abnormal behaviour. Record mortalities, if any, and remove each dead fish from the vessel as soon as possible. Replace dead fish with individuals of a similar mass from the stock population. (Do not include these replacement fish in the data analysis.)

NOTES

19 The replacement fish are intended to minimize any effects which might otherwise occur as the numbers of test fish decrease in a given test vessel.

20 If mortality in the highest test concentration exceeds 10 %, this may suggest that the range of test concentrations has been set too closely to the 96 h LC50.

Measure the dissolved oxygen concentration, the pH and the temperature of the solution from each of the test vessels at least once daily and at the beginning and end of the test. Clean faecal material and uneaten food from the test vessels each day.

b) Measuring mean specific growth rate

The procedure is the same as for the measurement of individual growth rate except that the fish are not individually identified and therefore are not anaesthetized and branded/tagged. The lengths and masses of each group of fish are recorded at the start of the test, after 14 d and after 28 d, but the mean increases in length or mass are used for calculating specific growth rates.

9 Precision of the test procedure

The results of a European ring test using two test substances, 3,4-dichloroaniline and linear alkylbenzenesulfonate, have yielded estimates for a coefficient of variation of 29 % and 31 % respectively. These estimates were based on the results of regression analysis of 11 datasets for the former substance and six datasets for the latter (see [3] in annex A).

10 Expression of results

10.1 Validity

Consider the results valid if the following requirements were fulfilled:

a) the dissolved oxygen concentration in the test solutions during the test was at least 70 % ASV;

b) the temperature was in the range 12,5 °C to 17,5 °C and did not vary by more than 2 °C;

c) the concentrations of the test substance were known to have remained within ± 20 % of the median value throughout the test (but see clause 3);

d) the mortality of the control fish did not exceed 10 %.

10.2 Data available

Tabulate the following data for each fish at 0 d, 14 d and, if required, 28 d:

a) live weight;

b) fork, total or standard length;

c) specific growth rate (r), calculated using the equation

$$r = \frac{\log_e m_{t_2} - \log_e m_{t_1}}{t_2 - t_1} \times 100$$

where

m_{t_1} is the mass, in grams, at time t_1;

m_{t_2} is the mass, in grams, at time t_2;

m_{t_1} and m_{t_2} may be either individual masses or the mean mass of the group.

NOTE 21 r can therefore be calculated for the following periods:

$t_1 = 0$, $t_2 = 14$ d

$t_1 = 14$, $t_2 = 28$ d

$t_1 = 0$, $t_2 = 28$ d

10.3 Estimation of LOEC and NOEC

For estimating the LOEC and NOEC directly, analyse the data for masses of individual fish, their lengths, and individual growth rates using a one-way analysis of variance. Compare the treatment means with those of the control fish using a suitable statistical method.

NOTE 22 Dunnett's test [5] or Williams' test [6] may be suitable, although neither should be used without true replicates.

If individual growth rates have not been measured, the mean specific growth rate data can be used to calculate the IC10, which can be taken as an approximation of the LOEC.

NOTE 23 Refer to 7.2 of ISO 7346-1:1984, *Water quality — Determination of the acute lethal toxicity of substances to a freshwater fish* [*Brachydanio rerio, Hamilton-Buchanan (Teleostei, Cyprinidae)*] — *Part 1: Static method* for guidance on the calculation of the IC10. The calculation of the EC10 (effective concentration for 10 % of the fish) is the same as that used for the IC10.

11 Test report

The test report shall include the following information:

a) a reference to this International Standard;

b) the chemical identity and any additional available information about the material tested;

c) 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;

d) all biological, chemical and physical data pertaining to the test and not otherwise specified in this International Standard, including details of the acclimatization conditions of the test fish and the method of marking the fish;

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

   1) concentrations of dissolved oxygen and test substance, and

   2) mortality observed among control fish;

f) a tabulated list showing the nominal concentrations of the test substance tested (with chemical analytical values where available), at least at the beginning, middle and end of the test and preferably according to the scheme given in 8.2, note 14;

g) a tabulated list showing the masses, lengths, values of r for every individual fish and/or mean masses and lengths;

h) results of the analysis of variance and comparison of treatment means, preferably in both tabular and graphical form (an example of the latter is shown in figure 2); together with a summary giving the LOEC and NOEC, or IC10, for each measurement (see 10.3);

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

j) any other facts that are relevant concerning the procedure followed.

NOTE 24 An example of a report form is given in annex A.