INTERNATIONAL STANDARD

ISO 12890

First edition
1999-12-15

Water quality — Determination of toxicity to embryos and larvae of freshwater fish — Semi-static method

Quallité de l'eau — Détermination de la toxicité vis-à-vis des embryons et larves de poissons d'eau douce — Méthode semi-statique

Reference number
ISO 12890:1999(E)

PDF disclaimer

This PDF file may contain embedded typefaces. In accordance with Adobe's licensing policy, this file may be printed or viewed but shall not be edited unless the typefaces which are embedded are licensed to and installed on the computer performing the editing. In downloading this file, parties accept therein the responsibility of not infringing Adobe's licensing policy. The ISO Central Secretariat accepts no liability in this area.

Adobe is a trademark of Adobe Systems Incorporated.

Details of the software products used to create this PDF file can be found in the General Info relative to the file; the PDF-creation parameters were optimized for printing. Every care has been taken to ensure that the file is suitable for use by ISO member bodies. In the unlikely event that a problem relating to it is found, please inform the Central Secretariat at the address given below.

ISO copyright office
Case postale 56 • CH-1211 Geneva 20
Tel. + 41 22 749 01 11
Fax + 41 22 734 10 79
E-mail copyright@iso.ch
Web www.iso.ch

Printed in Switzerland

Contents

Page

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.

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 3.

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 12890 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.

Introduction

Fish are particularly susceptible to the influence of substances, for example chemicals, during the reproductive stage (gametogenesis) and early developmental stages (embryo and larval stages). Determination of the toxicity to fish in early developmental stages is thus a more sensitive index of tolerance than that obtained by determination of acute toxicity to adult fish.

Only tests incorporating all stages of the life cycle of fish are expected to give an accurate estimate of the chronic toxicity of chemicals to fish. A reduced exposure with respect to life stages may reduce the sensitivity and, thus, underestimate the chronic toxicity. Therefore, the present method using embryos and larvae is expected to be less sensitive than a full life cycle test, and it may also be less sensitive than an early life-stage test incorporating the growth of the larvae for several weeks. The difference in sensitivity between these types of test will depend upon several factors, including the chemicals exerting toxicity. Therefore, it is not possible to generalize about the relationship between the sensitivity of full life cycle, embryo-larval (including growth) and embryo-larval (excluding growth) tests. However, experience has shown that for many chemicals the sensitivity obtained in embryo-larval tests correlates with that obtained in full life cycle tests.

Most experience with embryo-larval tests in Europe has been obtained with the freshwater fish *Danio rerio* (Hamilton-Buchanan) *Teleostei, Cyprinidae*, commonly called zebrafish. The systematic name of this species was recently changed from *Brachydanio rerio* to *Danio rerio*. More detailed guidance on the maintenance of stock fish and egg production for testing with this species is given in annex A. References to previous studies on these matters and embryo-larval testing are given in annex B. Reference to the zebrafish does not preclude the use of other species of freshwater fish for which experience is available.

Water quality — Determination of toxicity to embryos and larvae of freshwater fish — Semi-static method

1 Scope

This International Standard specifies a semi-static method for determination of toxicity of chemicals, waters and wastewaters to embryos and early larval developmental stages of a species of freshwater fish, *Danio rerio* (Hamilton-Buchanan), *Teleostei, Cyprinidae* (common name zebrafish). Where necessary this determination may include an acute toxicity test using *Danio rerio* to determine the 96-h LC_{50} for zebrafish in accordance with ISO 7346-1, ISO 7346-2 or ISO 7346-3.

The method is also applicable to freshwater fish other than zebrafish if appropriate modifications of the test conditions are made, particularly with regard to the temperature and volume per fish biomass.

2 Normative references

The following normative documents contain provisions which, through reference in this text, constitute provisions of this International Standard. For dated references, subsequent amendments to, or revisions of, any of these publications do not apply. However, parties to agreements based on this International Standard are encouraged to investigate the possibility of applying the most recent editions of the normative documents indicated below. For undated references, the latest edition of the normative document referred to applies. Members of ISO and IEC maintain registers of currently valid International Standards.

ISO 5667-16, *Water quality — Sampling — Part 16: Guidance on biotesting of samples*.

ISO 6341, *Water quality — Determination of the inhibition of the mobility of Daphnia magna Straus (Cladocera, Crustacea) — Acute toxicity test*.

ISO 7346-1, *Water quality — Determination of the acute lethal toxicity of substances to a freshwater fish [Brachydanio rerio Hamilton-Buchanan (Teleostei, Cyprinidae)] — Part 1: Static method*.

ISO 7346-2, *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, *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 Terms and definitions

For the purposes of this International Standard, the following terms and definitions apply.

3.1
effect concentration
EC_X
⟨for *X* % effect⟩ the experimentally derived concentration that is calculated to affect *X* % of the test organisms

NOTE The EC_{50} for survival is equivalent to the LC_{50}, the concentration lethal to 50 % of the test organisms.

3.2
lowest observed effect concentration
LOEC
lowest tested concentration of the test sample with a significant effect (at $p \leqslant 0,05$) when compared with the control

NOTE All test concentrations above the LOEC have a harmful effect equal to or greater than those observed at the NOEC.

3.3
no effect concentration
NEC
zero equivalent point
ZEP
estimate of the test concentration at which the response is equal to the response of the untreated control

NOTE NEC is especially useful when stimulation (hormesis) is observed at low concentrations.

3.4
no observed effect concentration
NOEC
test concentration immediately below the LOEC.

4 Principle

Newly fertilized eggs are exposed to a series of concentrations of the sample to be tested, including controls (concentration 0). For practical reasons, for the purposes of this International Standard eggs are regarded as embryos. The number of surviving and hatched eggs or larvae is recorded daily in all test solutions, which are renewed daily (semi-static method). No food is provided. The standard period of exposure for this test is 10 days, but the test may be prolonged up to 14 days in order to increase its sensitivity if desired.

The entire procedure, including preparation and calculations, takes about 4 weeks. The data obtained are used for calculations of the median times for hatching and survival in the tested concentrations and in the controls. No effect concentrations (NECs) are determined from the concentration-effect relationships for hatching and survival.

Effect concentrations with desired percentage effects (EC_Xs) can also be determined, and the results can also be evaluated with regard to the highest concentration without significant effect (NOEC) and the lowest concentration with significant effect in relation to the controls (LOEC).

Prior to the determination it may be useful to determine the acute toxicity of the sample (the 96-h LC_{50}) to zebrafish in accordance with ISO 7346-1, ISO 7346-2 or ISO 7346-3.

5 Test environment

Carry out the storage of the solutions and the exposure of test organisms as described in this International Standard under normal laboratory illumination with a 12 h/12 h, a 14 h/10 h or a 16 h/8 h light/dark exposure period. Stock solutions may be stored refrigerated and/in darkness when appropriate. Maintain the testing atmosphere at 26 °C ± 2 °C and free from vapours or dusts toxic to the test organism. Stock storage of fish and all handling and testing shall take place in rooms where air is free from hazardous concentrations of dust and toxic vapour.

6 Test fish

Carry out the test on *Danio rerio*, zebrafish, of specified source. Make the test on newly fertilized eggs (embryos) obtained from parental fish that have been acclimatized to specified environmental conditions (clause 5) for at least two weeks before the test. Water for acclimatization and spawning of parental fish shall be of recognized fish-breeding quality and with characteristics (pH, hardness and dissolved oxygen) similar to the dilution water (7.1).

Collect eggs produced in the morning within an hour after the light is turned on. Start the test (time 0) after a further 2 h to 3 h. The eggs (embryos) used to start the test (time 0) then have an age of 2 to 4 h. This corresponds to the stages when the blastula develops.

NOTE The conditions for production of eggs are given in annex A.

7 Reagents

7.1 Dilution water

All chemicals used for preparing the dilution water shall be of analytical grade and the diluent water shall be of high purity. Distilled or deionized water for the production of dilution water should be free from chemical substances and have a conductivity value of >18 MΩ/cm. Then the salts should be added to prepare the dilution (test) water.

Prepare the dilution water 1 day to 7 days before use and store in thoroughly cleaned vessels of chemically inert material. The dilution water shall have a pH of $7,5 \pm 0,2$ and a hardness corresponding to (100 ± 10) mg $CaCO_3$ per litre. It shall be prepared as follows:

a) Calcium chloride solution

   Dissolve 11,76 g $CaCl_2 \cdot 2H_20$ in water and make up to 1 litre with water.

b) Magnesium sulfate solution

   Dissolve 4,93 g $MgSO_4 \cdot 7H_2O$ in water and make up to 1 litre with water.

c) Hydrogen carbonate solution

   Dissolve 2,59 g $NaHCO_3$ in water and make up to 1 litre with water.

d) Potassium chloride solution

   Dissolve 0,23 g KCl in water and make up to 1 litre with water.

Add 100 ml of each of these four solutions a) to d) to approximately 5 l diluent water and dilute to a total volume of 10 l. This dilution water is made from the same stock solutions as used in the *Daphnia* acute toxicity test (ISO 6341) but is used here in a more dilute form.

Aerate the dilution water through a glass tube until the concentration of dissolved oxygen reaches 90 % to 100 % of the air saturation value at 26 °C. At the same time this stabilizes the pH value. If necessary, adjust the pH using additions of appropriate dilutions of hydrochloric acid or sodium hydroxide solution. The dilution water thus prepared shall receive no further forced aeration before use.

NOTE If the test is performed for purposes necessitating the use of a dilution water with characteristics differing from those described above, reference should be made in the test report, giving the main characteristics of the dilution water used.

7.2 Stock solutions

Prepare a stock solution of the substance to be tested by weighing in a known amount and dissolving it in a known volume of dilution water (7.1), deionized water or distilled water.

Prepare the stock solution daily except when it is known that the substance is stable in solution. In such situations a sufficient amount for the entire test may be prepared.

Solubilize stock solutions of substances which are poorly soluble in water directly in the test medium (7.1) by suitable means, e.g. using ultrasonic devices or solvents of low toxicity to fish. Subsequently, use dilution water

(7.1) to make up the final volume. When acetone or another suitable solvent is used, the concentration of solvent in the final test solution shall not exceed 0,1 ml/l, and two control solutions, one with no solvent and one with the maximum concentration of solvent, shall be included in the test (see ISO 5667-16)

When wastewater is to be tested, the original sample shall constitute the stock solution. If a wastewater cannot be tested immediately, divide it into smaller volumes in sampling vessels of inert plastics and store it frozen (– 20 °C) so that the required amounts of test solution at start and renewals can be thawed and, if necessary, adjusted to the appropriate pH during the preparation of test solutions in accordance with ISO 5667-16.

If the pH of the stock solution is outside 7,5 ± 0,2, it may be necessary to adjust the pH of the test solution within 7,5 ± 0,2. Any adjustment needed shall be made with dilute hydrochloric acid or sodium hydroxide solution.

7.3 Test solutions

Prepare test solutions daily within 1 h of use using the test fish or eggs by mixing the stock solution (7.2) and dilution water (7.1) in suitable proportions. Prepare and store stock and test solutions only in vessels of chemically inert material.

8 Apparatus

All material that may come into contact with the water or the solution, and which in turn may come into contact with parental fish during the conditioning period or with eggs and larvae being tested, shall be made of a chemically inert material [e.g. glass, stainless steel, nylon (polyamide), polyethene or polypropylene, silicone, teflon], i.e. materials that do not leach into water to any extent that may influence the results. All materials used shall be thoroughly cleaned before use and rinsed with the dilution water (7.1).

8.1 Shallow test vessels, ca. 100 ml capacity, of the Petri dish type, with an inner diameter of ca. 100 mm and equipped with a cover.

8.2 Temperature control, e.g. cupboards, water-baths or a thermostat-controlled room, with the capacity to keep the temperature of the test solutions at (26 ± 2) °C shall be used for the test and control incubations.

NOTE A thermostatically temperature-controlled room maintained at (26 ± 2) °C is preferable since it allows faster equilibration of test solutions and minimizes temperature variations during transfer of eggs and larvae to new solutions. Newly fertilized eggs are especially sensitive to temperature variations.

Record the temperature in the test solutions, even if the thermostat operates on the air temperature. Air temperature shows larger variability than the water temperature.

8.3 Nets and pipettes

Handle parental fish with dip-nets of nylon (polyamide) or other soft materials. Handle eggs and larvae by pipettes (e.g. Pasteur pipettes) with a polished opening of ca. 2 mm.

8.4 Measurement equipment

Oxygen electrode, pH-meter, thermometer.

9 Procedure

9.1 Condition of the fish

The quality of zebrafish eggs can vary between individual pairs and also from one spawning to another. Viable eggs are transparent. Poor egg quality becomes apparent several hours after the start of the tests when dead eggs become white. After 24 h all dead eggs have become white. The percentage proportion of dead eggs in the control vessels after 24 h shall not normally exceed 30 %.

The median time for hatching in the controls should be between 2 days and 4 days. The median time for survival of the embryo-larvae in the controls is usually between 12 days and 16 days. This reduces the exposure period to a standard exposure period of 10 days, and a maximum period of about 14 days. Longer exposure periods may increase the sensitivity, due to a combined effect of starvation and prolonged accumulation of toxic chemicals in the test solutions. If the proportion of dead eggs in the controls and solutions of the tested substance exceeds 30 % the precision of the test will be decreased and if possible the test shall be terminated and repeated with a new batch of eggs.

9.2 Test procedure

The embryo-larval toxicity test is usually preceded by determination of the acute toxicity in accordance with ISO 7346-1, ISO 7346-2 or ISO 7346-3. The embryo-larval toxicity test shall be comprised of a control and at least six different concentrations of the sample, which shall be selected so that at least the two highest concentrations give a significant effect on hatching or on survival in relation to the controls and that at least the lowest concentration produces no significant effect. The concentrations shall be selected so that they constitute a geometric series, e.g. 2, 1, 1/2, 1/4, 1/8, and 1/16 $\times$ 96-h LC_{50} for the test in accordance with ISO 7346-1, ISO 7346-2 or ISO 7346-3.

If it is suspected that the tested sample contains substances for which the toxic effect is delayed, increase the number of concentrations by several lower concentrations (1/32,1/64,1/128,1/256,1/512 $\times$ 96-h LC_{50}). Test lower concentrations if the lowest concentration produces any kind of effect relative to the controls.

NOTE In this type of test, it is not uncommon that low concentrations result in a higher survival than the controls.

Use at least two Petri dishes containing at least 25 ml test solution for each concentration (duplicated exposure concentrations) and at least four Petri dishes with at least 25 ml dilution water (7.1) for the controls (quadruplicated control).

At the start of the test (day 0) measure the oxygen concentration, pH and temperature in the sample and the control solutions. The levels obtained shall be within the limits stated in 10.1

Within 1 h of the preparation of the test solutions, transfer 15 eggs (2 h to 4 h after spawning) to all dishes (day 0). Select the eggs at random from a population originating from one or several females, and distribute them at random into the test and control dishes. Handle eggs and larvae using pipettes (8.3); they should not come into contact with air. Keep to a minimum the amount of liquid accompanying the eggs upon transfer. Cover the test and control vessels and incubate at (26 $\pm$ 2) °C under normal laboratory light intensity for 12 h light and 12 h darkness, 14 h light and 10 h darkness or 16 h light and 8 h darkness per day.

After 24 h, a variable number of the 15 embryos will have died and the eggs will have become white. Record the number of dead eggs in each dish and reduce the number of viable eggs per dish to a maximum of 10 when transferring to new solutions.

Determination of median times for hatching and survival shall be based only on these remaining 10 individuals which are now 1 day old.

Prepare new test vessels with at least 25 ml new solution (<1 h old) at the corresponding time on subsequent days. Transfer viable eggs (transparent) and live larvae (exhibit mobility spontaneously or after stimulation by tapping on the edge of the dish or by a flush of water from the pipette) into the new dishes. Record the number of dead and living eggs and larvae. Measure the oxygen content, pH and temperature in both the new and the old solutions. Check the controls and the samples with highest and lowest concentrations first. If the difference in values is large between these solutions, then all test solutions shall be measured.

Hatching normally occurs after 2 days to 4 days. In order to decide whether the time of hatching has been affected, make observations of the number of eggs and hatched larvae every morning and afternoon (record the exact time) on the second, third and fourth days. These results can then be used to calculate the median hatching time. Terminate the test after 10 days or, to increase the sensitivity of the test, when at least 90 % of the larvae in all test solutions have died.

10 Results

10.1 Validity

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

— the concentration of dissolved oxygen in the controls has been maintained between 70 % and 110 % of the air-saturation value for dilution water at 26 °C;

— the pH in all fresh solutions has been 7,5 ± 0.2;

— the temperature in the test solutions has been maintained at (26 ± 2) °C;

— more than 70 % of the embryos (eggs) in the controls were alive after 24 h;

— the median time for hatching was 2 days to 4 days in the controls;

— the proportion of surviving larvae in the controls after 10 days was > 90 %;

— if the test was prolonged, the median time for survival in the controls is 12 day to 16 days.

10.2 Determination of effect levels

The determination of effect levels (concentrations with prescribed effect or no effect) can be made by regression of dose response relationships (NEC and EC_X approach) or hypothesis testing (LOEC-NOEC approach). These two approaches often produce similar effect levels. However, sometimes they produce dissimilar results. Therefore, it is recommended to use both methods in order to establish the uncertainty associated with the interpretation of the data in estimating the toxicity of the tested sample.

Determine the median times for hatching and survival either graphically on log-probit paper or by probit analysis or a similar method for each concentration and for the controls. The method of determination used shall be given in the report. Methods that give a 95 % confidence interval shall be used preferentially.

In a graph with logarithmic scales, plot the values determined for median times of hatching and survival against the corresponding concentrations. Check the validity of the results (according to 10.1) and that suitable concentrations have been used (9.2). Fit the lines according to Figure B.1 of annex B and determine from the diagram the No Effect Concentration (NEC) for hatching and survival. Recorded data on hatched larvae and larval survival/mortality after various times can also be used for determination of EC/LC_X. The 10-day EC_{50} for effects on hatch and survival is shown in Figure B.2. EC_X values for different percentage effects can be calculated with 95 % two-sided confidence limits after various times of exposure. Confidence limits tend to be wider for lower percentage effect levels compared to the 50 % effect level.

Determine the LOEC and NOEC (according to clause 3) by a suitable statistical procedure like analysis of variance (ANOVA) followed by multiple comparisons for the different test concentrations with the control using e.g. Dunnett's or William's test. This is exemplified in the data in Table B.1 (the same data as used in Figures B.1 and B.2).

11 Test report

The test report shall include the following information:

a) a reference to this International Standard (ISO 12890);

b) name of person responsible for the test and the address of laboratory;

c) date of the test (day 0);

d) test substance(s);

e) identification and origin of the sample;

   1) for substances details whether it is a pure substance (purity) or a formulated product (active and other ingredients), and if the concentrations refer to a pure substance or a formulated product,

   2) for wastewater, unstable substances and mixtures, details shall be given of sampling times, source, storage vessels and temperatures and pretreatments, together with the name(s) of the person(s) responsible for the sampling;

f) method for preparation of stock solution;

g) test fish:

   1) scientific name of the tested species (*Danio rerio* or other),

   2) origin of the parental fish and their handling and conditions for maintenance and spawning (temperature, light exposure period, water quality characteristics), and

   3) method of collection of the fertilized eggs and their subsequent handling;

h) test conditions taken into account when assessing the validity of the test, such as measured ranges for oxygen concentration, pH and temperature;

i) the nominal concentration of the substance tested in the test solutions used and, if available, analysed concentrations of the test substance;

j) results:

   1) initial mortality of embryos (24 h),

   2) median times of hatching and survival (number of days $\pm$ 0,1) in the controls and the different test solutions. State the method used to determine median times and, if possible, the 95 % confidence interval for median times;

k) determined effect levels:

   1) the No Effect Concentration (NEC) determined by regression of the dose response relationships for effects on median hatching and survival times,

   2) the Lowest Observed Effect Concentration (LOEC) and No Observed Effect Concentration (NOEC) for hatching and survival;

      Justify estimated values in doubtful cases;

l) conditions or events which might have influenced the results, and any divergences from this International Standard.

Annex A
(informative)

Conditions for the production of eggs of zebrafish

A.1 Introduction

The zebrafish (*Danio rerio*, formerly called *Brachydanio rerio* [1], [15]) originates from the Coromandel coast of India, where it inhabits fast-flowing streams. It is a common aquarium fish, and information about procedures for its care and culture can be found in standard reference books on tropical fish. Laale (see [10]) has reviewed its biology and use in fishery research.

The fish rarely exceeds 45 mm in length. The body is cylindrical, with seven to nine 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.

Adult fish are able to tolerate large fluctuations in temperature, pH and water hardness. However, in order to obtain healthy fish which produce eggs of good quality, optimal conditions should be provided.

During spawning the male pursues and butts the female, and as the eggs are expelled they are fertilized. The eggs, which are transparent and non-adhesive, fall to the bottom where they risk being eaten. About 100 to 400 eggs are produced during each spawning, which will last for about half an hour. Spawning is influenced by light. If the morning light is adequate, the fish will most likely spawn in the early hours following daybreak.

A.2 Conditioning for spawning and production of eggs

Select a suitable number of healthy fish. Keep them in good quality water similar in characteristics (dissolved oxygen, pH, hardness) to the dilution water (7.1) for at least two weeks prior to the intended spawning, and separate males and females 5 days to 10 days before spawning. The density of fish during this period should not exceed 30 fish per 70 litres. Regular changes of water, completely or partially, allows higher stocking densities to be used, e.g. 30 fish/20 litres if 3/4 of the water is replaced every second day.

It is important during this period that the fish receive a varied diet, which may consist of, for example, live newly hatched *Artemia*, chironomids, *Daphnia*, white worms (Enchytraeids) and finely chopped ox liver. If suitable live food is not available, deep-frozen food of corresponding types may be used together with, for example, fine chopped frozen liver pâté, but particular care should be taken to remove any food residues. After the male and female fish have been separated for 5 days to 10 days, the abdomens of the females will be distended and their genital papillae are visible. Male fishes lack genital papillae.

The spawning tank should be equipped with a false mesh bottom, enabling the eggs to be protected from being eaten by the parent fish. Fill the spawning tank with new dilution water (7.1) so that the level of water above the net is ca. 5 cm deep. Adjust the temperature to 27 °C to initiate spawning. Transfer the male and female fish in a ratio of one female per two males in the evening. Turn out the light. Occasionally the fish will spawn in darkness. Such eggs should be removed in the morning when the light is turned on and spawning normally starts. Only those eggs that are spawned within the first hour are to be used, as these eggs will have a defined age when the test is started 2 h to 3 h after the completion of spawning.

Thus, the age of the eggs (embryos) should be 2 h to 4 h at the start (time 0). Only visibly healthy, transparent eggs should be used. The number of eggs and their quality may vary considerably among different fish and from one spawning to another.

After 2 h to 4 h the embryos will be in the blastula stage, developmental stages 8-12 (see [7]), which can be checked in a microscope or using a magnifier. Earlier papers on spawning and/or development in the zebrafish are listed in the Bibliography ([5], [6], [7], [8], [12] and [18]).

Induction of the spawning of the parental fishes at appropriate times may require some experience, and spawning should not be expected from all females. Therefore, it is recommended to use at least 10 females and 20 males, either individually in smaller, separate tanks (ca. 5 litres) or in a larger aquarium common to all the fish. A funnel-shaped spawning tank for this purpose has been described [9].

The same parental fish may be used repeatedly for several consecutive spawnings. Good spawning results have been obtained when fish are allowed or induced to spawn repeatedly at weekly intervals.

Another egg production method, which eliminates the need for keeping males and females separate, is as follows. In an aquarium containing 75 l of water (height 15 cm) with a 20 l/h water renewal and aeration, keep approximately 60 fish (~ 20 females and ~40 males) at a temperature of (26 ± 2) °C and a 14 h/10 h light exposure cycle with progressive lighting. On the evening preceding the day of spawning (fixed day of the week), a container covered with dark-coloured 5 mm mesh netting is introduced and the water level is lowered to ~1 cm above the lid. A spawning rate of 2 000 to 5 000 eggs per week for over a year (if and only if the spawning is conducted once a week throughout this whole period) using this procedure has been obtained by one investigator.

The eggs and larvae not used in the tests may be reared, so that a stock of parental fish of known history can be established in the laboratory. This may also limit problems with diseases induced by transportation and adjustment to new environments.

It is not permitted to use any chemical treatment of the eggs prior to or during testing, since this may affect the response to the tested substance(s).

A.3 Problems

If, despite having received adequate diet, the parental fish are still unwilling to spawn, attempts may be made to stimulate the actual spawning. If light is directed from one side (ca. 30 ° towards the surface of the water) and increased gradually during about 30 min in the morning, this implies a simulation of dawn, the time when the fish frequently spawn in nature. In addition, a spawning substrate consisting of green threads (ca. 1 mm in diameter) can be placed in the spawning tank.

In some cases, the initial mortality among the eggs may be abnormally high. The reasons for this are not fully understood. Possible causes which should be considered are poor water quality resulting from overfeeding, inadequate nutrition, endogenous parasites or the start of senescence in parental fish. Installation of a mechanical filter in the holding tanks; more careful feeding and renewal of old and obviously defective fish are measures which may cure the problem. Check and ensure that the temperature in the holding and spawning tanks is correctly maintained and that water quality is acceptable. Careless handling of the eggs, particularly during the first 24 h, may also contribute to high mortality.

Annex B
(informative)

Example of data on initial mortality, hatching and survival times

Table B.1 — Example of data on initial mortality (after 24 h) and median times for hatch and survival in a test with a wastewater (mean value with replicate values in parenthesis)

Test concentration volume fraction (%)	**Initial mortality** %	**Median time for hatch** days	**Median time for survival** days
0 (control)	13 (7; 13; 13; 20)	2,9 (2,5; 2,9; 2,2; 4,0)	14,7 (14,3; 14,9; 13,3; 16,3)
1,56	13 (7; 20)	2,6 (2,3; 2,9)	14,1 (12,7; 16,2)
3,13	7 (7, 7)	2,9 (2,7; 3,2)	13,7 (12,4; 15,2)
6,25	7 (7, 7)	2,6 (2,4; 2,8)	13,1 (12,6; 13,6)
12,5	3 (7, 0)	2,6 (2,4; 2,9)	12,6 (11,7; 13,7)
25	3 (7; 0)	3,6 (2,2; 5,0)	11,8 (10,8; 12,8) [b]
50	3 (7; 0)	>16 (>16; >16) [a]	2,7 (2,2; 3,1) [a]
100	23 (27; 20)	>16 (>16; >16) [a]	3,7 (3,4; 4,1) [a]

[a] Different from control by Dunnett´s and William´s test at $p < 0,05$.

[b] Different from control by William´s but not by Dunnett´s test at $p < 0,05$.

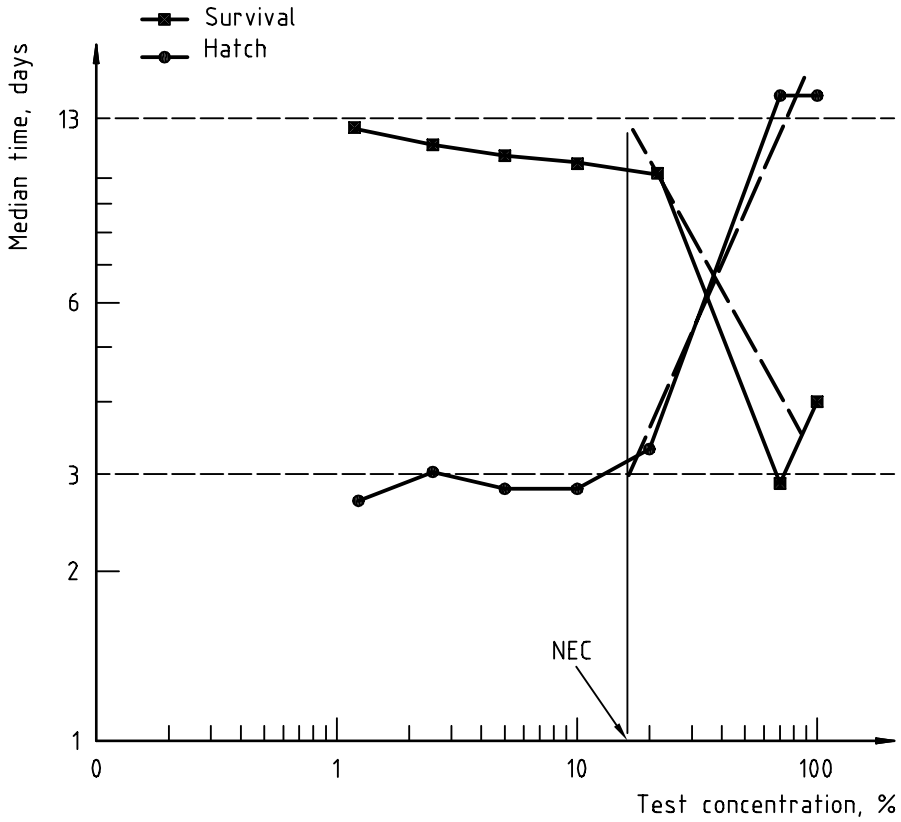
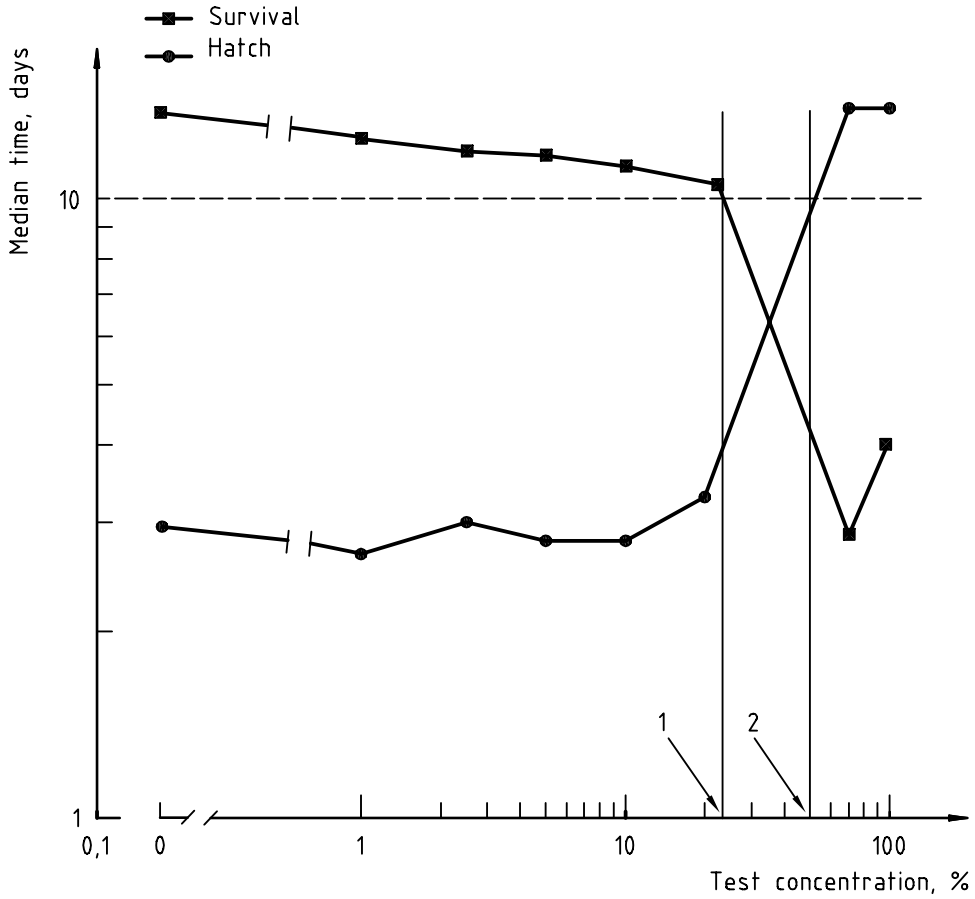


Figure B.1 — Mean values for median times for hatch and survival in controls at different test concentrations (values from Table B.1) demonstrating the graphical determination of NEC for hatch and survival (In this case the NECs for hatch and survival were identical)



1 10-d EC_{50} (survival)

2 10-d EC_{50} (hatch)

NOTE EC_{50} as well as EC_X for lower percentages of effect with 95 % confidence limits may be determined from the original data set after various times of exposure.

Figure B.2 — Mean values for median times for hatch and survival in controls different test concentrations (values from Table 1) demonstrating the relative positions of the EC_{50} for hatch and survival

Bibliography

[1] BARMAN, R.P. A taxonomic revision of the Indo-Burmese species of *Danio* Hamilton-Buchanan (Pisces: Cyprinidae). *Rec. Zool. Surv. India* occ. Paper, (1991) **137**:1-91.

[2] DAVE, G. Replicability, repeatability, and reproducibility of embryo-larval toxicity tests with fish, (1993) In: *Progress in Standardization of Aquatic Toxicity Tests*, A.M.V.M. Soares and P. Calow, Eds., Lewis Publ., pp. 129-157.

[3] DAVE, G. and XIU, R.: Toxicity of mercury, nickel, lead and cobalt to embryos and larvae of zebrafish, *Brachydanio rerio. Arch. Environm. Cont. Toxicol.*, (1991) **21**:126-134.

[4] DAVE, G., DAMGAARD, B., GRANDE, M., MARTELIN, J.-E., ROSANDER, B. and VIKTOR, T. Ring test of an embryo-larval toxicity test with zebrafish *(Brachydanio rerio)* using chromium and zinc as toxicants. *Environm. Toxicol. Chem.*, (1987) **6**:61-71.

[5] EATON, R.C. and FARLEY, R.D. Spawning cycle and egg production of zebrafish, *Brachydanio rerio*, in the laboratory. *Copeia*, (1974) **1**:195-204.

[6] EATON, R.C. and FARLEY, R.D. Growth and the reduction of depensation of zebrafish, *Brachydanio rerio*, reared in the laboratory. *Copeia*, (1974) **1**:204-209.

[7] HISAOKA, K.K. and BATTLE, H.I. The normal developmental stages of the zebrafish, *Brachydanio rerio* (Hamilton-Buchanan). *J. Morphol.*, (1958) **102**:311-328.

[8] HISAOKA, K.K. and FIRLIT, C.F. Ovarian cycle and egg production in the zebrafish, *Brachydanio rerio*. *Copeia*, (1962) **4**:788-792.

[9] KIHLSTROM, J.E., LUNDBERG, C. and HULT, L.: Number of eggs and young produced by zebrafishes, *Brachydanio rerio* (Hamilton-Buchanan) spawning in water containing small amounts of phenylmercuric acetate. *Environ. Res.*, (1971) **4**:355-359.

[10] KRISTENSEN, P. *Evaluation of the Sensitivity of Short Term Fish Early Life Stage Tests in relation to other FELS test methods*. Final report to the Commission of the European Communities. (1990) 60 pp.

[11] LAALE, H.W. The biology and use of the zebrafish, *Brachydanio rerio* in fisheries research. A literature review. *J. Fish Biol.*, (1977) **10**:121-173.

[12] LEGAULT, R. A technique for controlling the time of daily spawning and collecting of eggs of the zebrafish, *Brachydanio rerio* (Hamilton-Buchanan). *Copeia*, (1958) **4**:328-330.

[13] MACEK, K.J. and SLEIGHT III, B.H. Utility of toxicity test with embryos and fry of fish in evaluating hazards associated with the chronic toxicity of chemicals to fishes. In: *Aquatic Toxicology and Hazard Evaluation*. ASTM STP 634, Mayer F.L. and Hamelink J.L., eds., American Society for Testing and Materials, (1977) pp. 137-146.

[14] McKIM, J.M. Evaluation of tests with early life stages of fish for predicting long-term toxicity. *J. Fish Res. Board Can.*, (1977) **34**(8):1148-1154.

[15] MEYER, A., BIERMAN, C.H. and ORTI, G. The phylogenetic position of the zebrafish *(Danio rerio)*, a model system in developmental biology: an invitation to the comparative method. *Proc. R. Soc. Lond. B.*, (1993) **252**:231-236.

[16] NAGEL, R., BRESCH, H., CASPERS, N., HANSEN, P.D, MARKET, M., MUNK, R., SCHOLZ, N. and HOFTE, B.B. Effect of 3,4-dichloroaniline on the early life stages of the zebrafish *(Brachydanio rerio)*: results of a comparative laboratory study. *Ecotox. Environ. Safety*, (1991) **21**:157-164.

[17] NEWSOME, C.S. and PIRON, R.D.: Aetiology of skeletal deformities in the zebra danio fish, *Brachydanio rerio*, (Hamilton-Buchanan). *J. Fish Biol.*, (1982) **21**:231-237.

[18] NIIMI, A.J., LAHAM, Q.N.: Influence of breeding time interval on egg number mortality, and hatching of the zebrafish, *Brachydanio rerio*. *Can. J. Zool.*, (1974) **52**:515-517.

[19] OECD: Fish, toxicity test on egg and sac-fry stages. *OECD Guideline for Testing of Chemicals*. Proposal for a new Guideline, revised draft document (December 1996). 20 pp.

ICS 13.060.70

Price based on 14 pages