INTERNATIONAL STANDARD

ISO 15088

First edition
2007-10-01

Water quality — Determination of the acute toxicity of waste water to zebrafish eggs (*Danio rerio*)

*Qualité de l'eau — Détermination de la toxicité aiguë des eaux résiduaires vis-à-vis des œufs de poisson-zèbre (*Danio rerio*)*

Reference number
ISO 15088:2007(E)

ISO copyright office
Case postale 56 • CH-1211 Geneva 20
Tel. + 41 22 749 01 11
Fax + 41 22 749 09 47
E-mail copyright@iso.org
Web www.iso.org

Published 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 2.

The main task of technical committees is to prepare International Standards. 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.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

ISO 15088 was prepared by Technical Committee ISO/TC 147, *Water quality*, Subcommittee SC 5, *Biological methods*.

Introduction

Fish play a major role in the aquatic food web. They are high-order consumers (often final consumers) and have important functions of regulation in aquatic ecosystems. They are a confirmed part within test concepts regarding aquatic organisms from different trophic levels.

The eggs of the zebrafish (*Danio rerio* Hamilton-Buchanan) are used as test material. The zebrafish belongs to the family of Cyprinidae (carp-related fish) within the class of Osteichthyes (teleost fish). Zebrafish are easy to keep and produce transparent, nonadhesive eggs (diameter about 1 mm) throughout the whole year. Their embryonic development is well described. The zebrafish is one of the most important model fish in research on the developmental biology of vertebrates and is recommended as a test fish, i.e. in the OECD Guidelines 203, 204 and 210.

The development of fertilized fish eggs can be affected by water constituents and effluents. Death of embryos and certain defined disturbances of embryonic development, which finally lead to death, are considered effects.

Water quality — Determination of the acute toxicity of waste water to zebrafish eggs (*Danio rerio*)

WARNING — Persons using this International Standard shall be familiar with normal laboratory practice. This International Standard does not purport to address all of the safety problems, if any, associated with its use. It is the responsibility of the user to establish appropriate safety and health practices and to ensure compliance with any national regulatory conditions.

IMPORTANT — It is absolutely essential that tests conducted according to this International Standard be carried out by suitably qualified staff. When applying this International Standard, it is necessary in each case to determine if and to what extent additional conditions should be established.

1 Scope

This International Standard specifies a method for the determination of degrees of dilution or of concentrations as a measure of the acute toxic effect of waste water to fish eggs within 48 h. This International Standard is also applicable to treated municipal waste water and industrial effluents.

NOTE This International Standard has been elaborated as a substitute for the acute fish toxicity test. Applied to waste water, it gives the same or similar results as achieved from the acute fish toxicity test (e.g. ISO 7346-1 or ISO 7346-2). If used for single substances, different sensitivities from both test systems are possible.

2 Normative references

The following referenced documents are indispensable for the application of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

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

ISO 5814, *Water quality — Determination of dissolved oxygen — Electrochemical probe method*

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*

3 Terms and definitions

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

3.1
control
dilution water with fertilized fish eggs

3.2
dilution level
D
reciprocal value of the volume fraction of waste water in dilution water in which the test is conducted

EXAMPLE 250 ml of waste water in a total volume of 1 000 ml (volume fraction of 25 %) represents dilution level $D = 4$.

NOTE See ISO 5667-16.

3.3
EC_{50}
concentration at which there is an effect on 50 % of the organisms in line with the test criterion

NOTE In this International Standard, LC_{50} is the lethal concentration at which 50 % of the organisms are dead.

3.4
fish egg
generally used term for all development stages of an egg cell (inside the chorion) within this International Standard

NOTE If necessary, the following qualifiers for this term are used: “not fertilized”, immediately after spawning; “freshly fertilized”, between 4-cell stage and 128-cell stage; “embryo”, if the developing embryo is visible inside the egg before hatching.

3.5
lowest ineffective dilution
dilution factor
LID
lowest ineffective dilution tested, expressed as dilution level *D* (3.2), at which no inhibition, or only effects not exceeding the test-specific variability, are observed

3.6
LID_{egg}
lowest ineffective dilution within a test batch in which at least 90 % of the fish eggs do not show any effect according to this International Standard

NOTE See Clauses 11 and 12 and Figure 2.

3.7
test batch
defined dilutions with fertilized fish eggs

4 Interferences

The differentiation of eggs can be difficult in the case of heavily coloured and/or turbid waste water. In such cases, the following modified preparation order is used before starting the exposure:

— spawning;

— differentiation into fertilized and unfertilized eggs (8.2.2);

— transfer into dilution water (7.10, crystallization bowls);

— transfer into microplates without previous exposure.

While transferring into microplates, the error in dilution caused by adherent water should be kept as small as possible.

5 Principle

In a dilution series, waste water graduated to integral volume ratio is mixed with dilution water giving defined dilution levels, D_i. After exposure of fertilized fish eggs to the test batches for 48 h using microplates, the dilution limit in which no acute toxic effect occurs is determined (LID). At 26 °C, the embryos hatch after 72 h to 96 h. The test duration is 48 h. As a positive control, a solution of 3,7 mg/l of the reference substance, 3,4-dichloroaniline, is tested with 10 fertilized eggs (8.3).

This International Standard may also be used to calculate dose-response-based EC_{50} values as percentages of waste water without changing the test design.

6 Reagents

As far as available, use only reagent grade chemicals.

6.1 Water, deionized or of equivalent purity (conductivity < 10 µS/cm).

6.2 Hydrochloric acid, e.g. c(HCl) = 0,1 mol/l.

6.3 Sodium hydroxide solution, e.g. c(NaOH) = 0,1 mol/l.

6.4 Reference substance 3,4-dichloroaniline, stock solution, $\rho(C_6H_5Cl_2N)$ = 100 mg/l.

Stir 0,05 g of dichloroaniline in 500 ml of dilution water (6.5) for 24 h. Adjust the pH to 7,0.

Kept dark in a refrigerator, this stock solution may be stored for up to 6 months. A concentration of 3,7 mg/l is used as positive control, (8.3).

6.5 Dilution water

Use standard dilution water as specified in ISO 7346-1 and ISO 7346-2.

— 294,0 mg/l of calcium chloride dihydrate, $CaCl_2 \cdot 2\ H_2O$;

— 123,3 mg/l of magnesium sulfate heptahydrate, $MgSO_4 \cdot 7\ H_2O$;

— 63,0 mg/l of sodium hydrogen carbonate, $NaHCO_3$;

— 5,5 mg/l of potassium chloride, KCl.

Before adding the reference substance (6.4) or waste water samples to be tested, the dilution water shall be equilibrated with air to 100 % oxygen saturation at 26 °C ± 1 °C.

7 Apparatus

7.1 Inverse microscope and/or binocular, with a minimum magnification of 30×.

7.2 Exposure vessels, with a volume of 2,5 ml to 5 ml, plain ground, i.e. polystyrene one-way microplates (24 wells).

7.3 Self-adhesive foil, to cover the microplates.

7.4 Temperature-controlled incubator or climatization of the room to 26 °C ± 1 °C, controlled illumination during the keeping of parental fish and exposure of eggs.

7.5 pH-meter.

7.6 Oxygen probe, see ISO 5814.

7.7 Spawning dishes of inert plastic or glass to sample the eggs during spawning.

7.8 Artificial plants of glass or plastic.

7.9 Pipette, for transfer of the eggs.

7.10 Crystallization dishes or bowls.

7.11 Glassware to prepare the concentration levels and dilution water.

7.11.1 Volumetric flasks.

7.11.2 Graduated cylinders.

7.11.3 Graduated pipettes.

7.11.4 Petri dishes.

7.11.5 Beaker, e.g.150 ml.

7.12 Aquaria to keep the adult fish.

7.13 Laboratory thermometer.

8 Procedure

8.1 Keeping of fish and production of eggs

8.1.1 General

For the production of eggs, use only apparently healthy spawners free from externally visible diseases and aged between 6 months and 24 months.

Do not treat parental test fish with any pharmaceuticals (acute or prophylactic) during the 6 months immediately before spawning.

8.1.2 Aquaria

Keep spawners in aquaria in which sufficient space for swimming is available (i.e. 1 l per fish).

8.1.3 Water for fish keeping and breeding

Use standardized dilution water (6.5).

The oxygen saturation should be at least 80 %, the temperature 26 °C ± 1 °C. For keeping and breeding, suitable drinking water (e.g. free from disinfectants) may be used alternatively.

8.1.4 Food

Fish should be fed with commercially available dried food for aquarium fish.

Supplemental feeding with for example living food (*Artemia* spec.–*nauplia*, *Daphnia* in appropriate size) is recommended.

Overfeeding should be avoided.

Eggs of *Artemia* spec. can be purchased from the aquarist trade. Breeding conditions are generally given in the manufacturer's instructions. Before feeding the fish, rinse *Artemia-nauplia* thoroughly with water to remove salt.

8.1.5 Light conditions

The photoperiod is constantly adjusted throughout the year, i.e. 16/8 h (light/dark) or 12/12 h.

NOTE Any physiological tolerable light conditions and photoperiods are acceptable.

8.2 Providing eggs for the test

8.2.1 General

Mating and spawning usually take place within 30 min after starting the light period. A ratio for males to females of 2:1 is recommended and has been proved successful.

Adult zebrafish are known to feed upon their own spawned eggs. Therefore, spawning dishes covered with a grid of stainless steel are placed in the aquaria, thus allowing the eggs to be sampled without interference from the adults. As a supporting tactile stimulus to initiate spawning, artificial plants of plastic or glass (7.8) should be fixed to the grid covering the spawning dishes.

Insert spawning dishes (7.7) into the aquaria immediately before activation of light to ensure that the development stage of fertilized eggs is within the range of the 4-cell stage to 128-cell stage.

Other procedures to achieve fertilized eggs are permissable as long as criteria for the starting conditions (4-cell stage to 128-cell stage) are met.

Remove spawning dishes about 30 min after activation of light and before feeding.

The transparent eggs can easily be identified by putting the spawning dish on a black pad and using transverse light for investigation.

The fertilization rate should be more than 50 %. An approximation of the fertilization rate may be accepted (8.2.2).

8.2.2 Differentiation of eggs

To differentiate the eggs, use a microscope or a binocular. Freshly spawned eggs show the following structures: the perivitelline space containing the yolk is surrounded by the egg membrane; the germinal disc is formed at the animalic pole (upper side of the yolk). After fertilization of the eggs, the first cell division proceeds after about 15 min at 26 °C. Subsequently, the germinal disc is divided synchronously into 4, 8, 16 and 32 blastomeres (discoidal groove, see Figure 1). From the 4-cell stage onwards, fertilized eggs can be distinguished clearly and definitely from non-fertilized eggs.

Use only fertilized eggs from the 4-cell stage to the 128-cell stage for the test.

Differentiate eggs within the first hour after spawning.

Separate and reject eggs with conspicuous anomalies in cell division (asymmetries, vesicles) or damaged membranes.

NOTE Diminutive, completely white eggs are oocytes denatured in the females' bodies before spawning.