BRITISH STANDARD

BS EN ISO
15088:2008

# Water quality — Determination of the acute toxicity of waste water to zebrafish eggs (Danio rerio) (ISO 15088:2007)

ICS 13.060.70

# National foreword

This British Standard is the UK implementation of EN ISO 15088:2008. It is identical to ISO 15088:2007.

The UK participation in its preparation was entrusted to Technical Committee EH/3/5, Biological Methods.

A list of organizations represented on this committee can be obtained on request to its secretary.

This publication does not purport to include all the necessary provisions of a contract. Users are responsible for its correct application.

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EUROPEAN STANDARD

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# EN ISO 15088

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ICS 13.060.70

English Version

# Water quality - Determination of the acute toxicity of waste water to zebrafish eggs (Danio rerio) (ISO 15088:2007)

Qualité de l'eau - Détermination de la toxicité aiguë des eaux résiduaires vis-à-vis des oeufs de poisson-zèbre (Danio rerio) (ISO 15088:2007)

Wasserbeschaffenheit - Bestimmung der akuten Toxizität von Abwasser auf Zebrafisch-Eier (kursiv(Danio rerio)) (ISO 15088:2007)

This European Standard was approved by CEN on 29 October 2008.

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Ref. No. EN ISO 15088:2008: E

# Foreword

The text of ISO 15088:2007 has been prepared by Technical Committee ISO/TC 147 “Water quality” of the International Organization for Standardization (ISO) and has been taken over as EN ISO 15088:2008 by Technical Committee CEN/TC 230 “Water analysis” the secretariat of which is held by DIN.

This European Standard shall be given the status of a national standard, either by publication of an identical text or by endorsement, at the latest by May 2009, and conflicting national standards shall be withdrawn at the latest by May 2009.

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**Endorsement notice**

The text of ISO 15088:2007 has been approved by CEN as a EN ISO 15088:2008 without any modification.

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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.

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.

**INTERNATIONAL STANDARD** **ISO 15088:2007(E)**



# 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

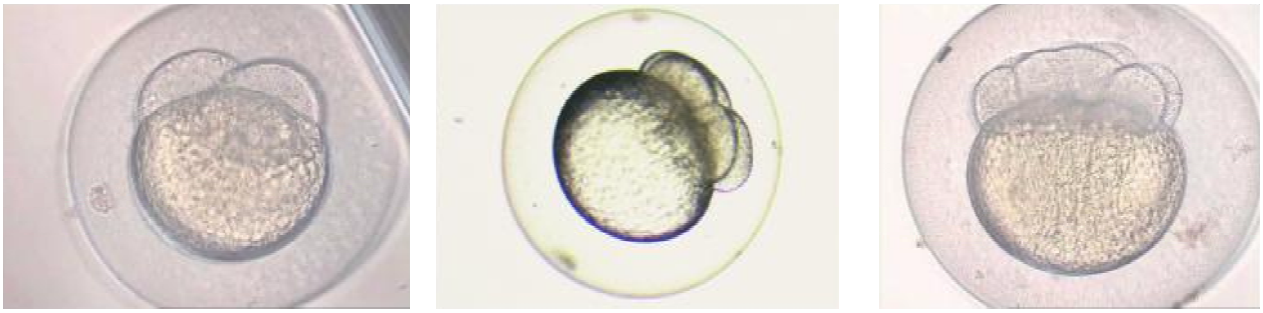
To differentiate the eggs, use a microscope or a binocular. Freshly spawned eggs show the following structures: the perivitelline space containing the yolk is surrounded by the egg membrane; the germinal disc is formed at the animalic pole (upper side of the yolk). After fertilization of the eggs, the 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.



**a) 2-cell stage** **b) 4-cell stage** **c) 8-cell stage**

**Figure 1 — Relevant development stages for the differentiation of eggs**

## 8.3 Test procedure

Consider the general recommendations on biotesting and handling of samples as specified in ISO 5667-16.

Determine the pH and oxygen concentration of the original sample.

Adjust the pH of the waste water to 7,0 ± 0,2 by the addition of hydrochloric acid (6.2) or sodium hydroxide solution (6.3) respectively, using beakers.

Make sure that the volume of the acid or base required for neutralisation is as small as possible.

Avoid surpassing the neutral point.

Neutralization should be omitted if the effect of the pH is to be reflected in the test result or if physical modification or chemicals reactions (e.g. precipitation) are observed due to pH adjustment. Following Table 1, prepare a set of dilutions from 1 volume of waste water and the appropriate volume of dilution water.

**Table 1 — Dilution levels, $D$**

| **Multiple of dilution water** $X$ | **Dilution level** $D$ | **Portion of sample in** $D$ % |
|---|---|---|
| 0 | 1 | 100,00 |
| 1 | 2 | 50,00 |
| 2 | 3 | 33,00 |
| 3 | 4 | 25,00 |
| 5 | 6 | 16,67 |
| 7 | 8 | 12,50 |
| 11 | 12 | 8,33 |
| 15 | 16 | 6,25 |
| 23 | 24 | 4,17 |
| 31 | 32 | 3,13 |

Ensure that $O_2$ concentration does not fall below 4 mg/l $O_2$ (about 50 % saturation) in each dilution. If aeration is necessary, the $O_2$ concentration should not exceed the corresponding saturation value.

To start the exposure as soon as possible, immediately transfer about 40 eggs into the prepared dilution (preferably in glass crystallization dishes of 100 ml). Subsequently differentiate eggs into fertilized and unfertilized. Transfer only fertilized eggs separately into each well of the microplate, such that each well contains one egg in a constant volume of test concentration (i.e. 2 ml in a 24-well microplate).

For each dilution, incubate at least 10 fertilized eggs. If incubation is carried out with 24-well microplates, it is possible to arrange two dilution levels on one microplate.

Use the remaining four wells on this microplate for microplate control, placing one fertilized egg per well and filling with dilution water.

In the same way, prepare a negative dilution water control consisting of at least 10 fertilized eggs (see Annex A).

With each dilution series, measure a positive control with 3,7 mg/l of reference substance 3,4-dichloroaniline, also consisting of at least 10 fertilized eggs.

Cover the microplates with self-adhesive foil and incubate at 26 °C ± 1 °C for 48 h.

After incubation, examine the eggs/embryos and record the results.

## 9 Determination of toxicological endpoints

### 9.1 General

After 48 h of incubation, investigate, determine and record the following toxicological endpoints.

### 9.2 Number of coagulated eggs

Even without magnification, coagulated eggs can be clearly identified. They are opaque and seem to be dark when observed through the microscope.

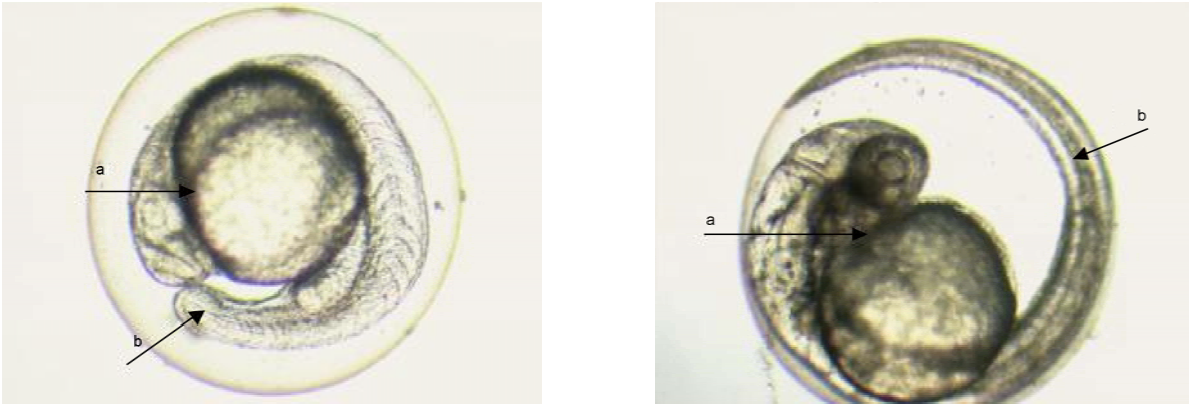
### 9.3 Tail detachment

It should be verified if the embryo's tail has been detached from the yolk sac due to its growth in length. The detachment should have developed at least as shown in Figure 2. If not, record as "no detachment".

### 9.4 Heartbeat

In Figure 2, “a” shows the location of the heart.

Record if a heartbeat is detected or not.




24 h 48 h

**Key**

a heart

b detached tail

**Figure 2 — Embryos of zebrafish after 24 h and 48 h**

Acute toxicity can be assumed if detachment of embryonic tail has not proceeded, and heartbeat cannot be detected; also, coagulated eggs indicate lethality. Supplementary investigations have shown that embryos do not hatch if one of the described effects has been detected.

After 48 h, an embryo is defined as dead, if

— it is coagulated, or

— it does not show tail detachment (if, after 48 h, embryonic tail is not more detached than after 24 h, it is defined as “not detached”, see Figure 2), or

— a heartbeat is not detectable.

## 10 Validity criteria

The test is valid if

— at least 90 % of the embryos in the negative dilution water control survive after the 48 h incubation time, and

— the results of the positive control are within the defined range (3,7 mg/l of reference substance should cause an effect > 10 %, see 8.3).

As a positive control, a defined concentration of reference substance (3,7 mg/l of 3,4-dichloroaniline) should be tested with 10 fertilized eggs (8.3) in parallel when the test is carried out.

## 11 Determination of $LID_{egg}$

Summarize the number of dead eggs for each test batch after 48 h (9.2). If one of the criteria described in 9.4 is fulfilled, the egg is considered dead.

The test result is the lowest tested value for $D$ (LID) in which at least 90 % of the eggs have survived. This dilution level is specified as the result.

If more than one egg dies in a microplate control, the respective microplate should be excluded from calculation.

Mortality in microplate controls has not been considered within validity criteria. They give some indication of unsuitable microplates. Normally, mortality in microplate controls does not occur (< 0,1 %). If so, plausibility has to be checked. In case of doubt, the microplate is excluded from the evaluation of the test result.

## 12 Expression of results

Values for LID are reported as integral numbers.

EXAMPLE $LID_{egg} = 2$

It is generally possible to calculate $EC_{50}$ values from dilution series of different volume fractions of waste water. In this case, ISO/TS 20281 should be taken into account.

## 13 Test report

This clause specifies which information shall be included in the test report. It is required that information is given on at least the following aspects of the test:

a) a reference to this International Standard (ISO 15088);

b) identification of the method;

c) identity of waste water;

d) pH and oxygen concentration in milligrams per litre (or percentage saturation) of the original sample and the dilutions, if necessary, at the beginning of test;

e) report of test results according to Clause 12;

f) any deviation from the test protocol and report of all marginal conditions of relevance according to the test result.

## 14 Reproducibility

An interlaboratory trial based on the method described in this International Standard was carried out in 2000. Results with the reference substance 3,4-dichloroaniline and the percentage of mortality at a given concentration (3,7 mg/l of 3,4-dichloroaniline) are given in Table 2. Additional information referring to the test procedure and comparisons between the acute fish test and the fish egg test are given in a validation document, which is available under http://www.gdch.de/strukturen/fg/wasser/publikat/vali/vdok_t6.pdf [1)].

1) At the time of publication, this document is available in German only. An English translation is in progress.

**Table 2 — Interlaboratory test results for $EC_{50}$ of 3,4-dichloroaniline and mortality at 3,7 mg/l of 3,4-dichloroaniline**

| Parameter | $l$ | $n$ | $o$ % | $\bar{\bar{x}}$ | $s_R$ | $CV_R$ % | $s_r$ | $CV_r$ % |
|---|---|---|---|---|---|---|---|---|
| A | 15 | 43 | 8,5 | 3,31 mg/l | 0,455 mg/l | 13,8 | 0,289 mg/l | 8,7 |
| B | 16 | 46 | 0,0 | 68,0 % | 24,0 % | 35,0 | 16,9 % | 25,0 |

where

$l$ is the number of laboratories;

$n$ is the number of valid measured values;

$o$ is the percentage of outliers;

$\bar{\bar{x}}$ is the overall mean;

$s_R$ is the reproducibility standard deviation;

$CV_R$ is the reproducibility variation coefficient;

$s_r$ is the repeatability standard deviation;

$CV_r$ is the repeatability variation coefficient.

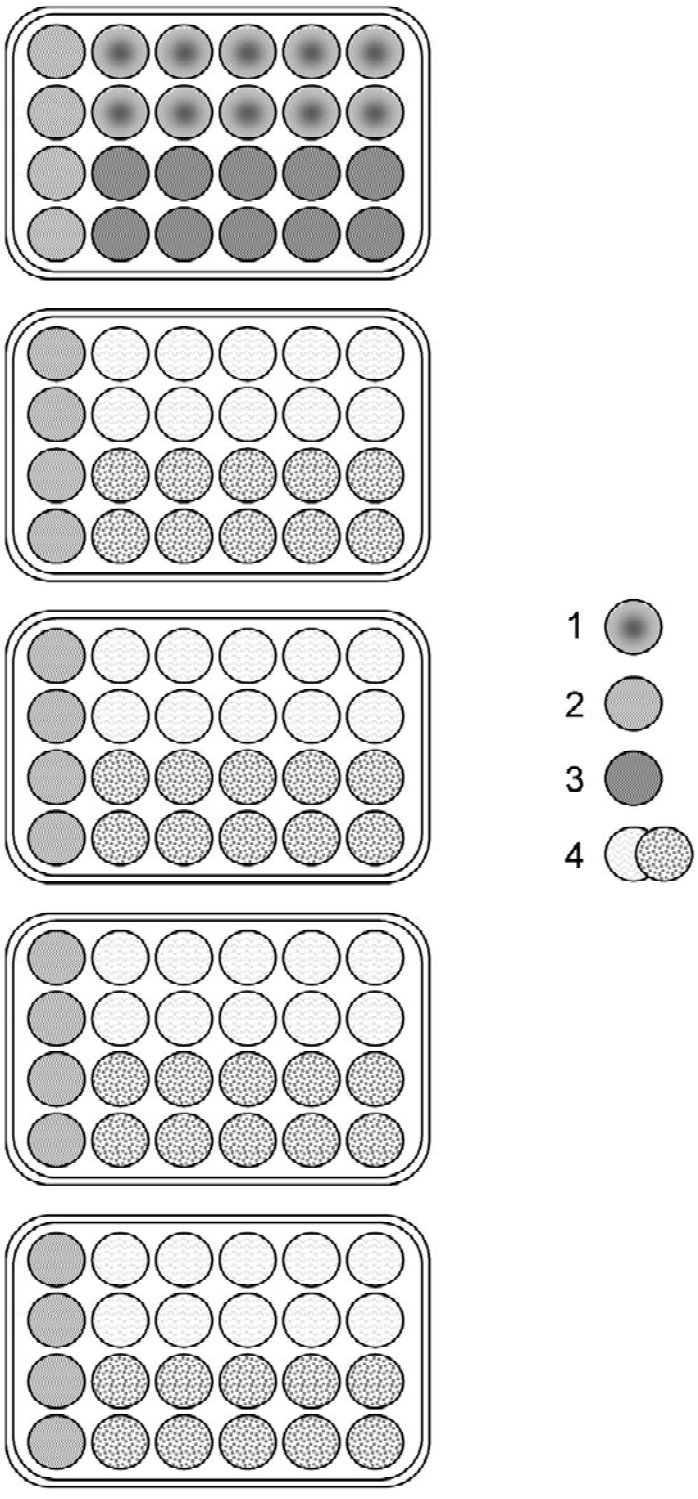
Parameter:

A $EC_{50}$ of 3,4-DCA (mg/l).

B Mortality at 3,7 mg/l 3,4-DCA (%).

# Annex A
(informative)

## Scheme for layout and distribution of eggs in microplates




**Key**

1 positive control (3,7 mg/l 3,4-DCA)
2 microplate control
3 negative dilution water control
4 dilution level

**Figure A.1 — Scheme for layout and distribution of eggs in microplates**

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