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Water quality — Determination of fresh water sediment toxicity to *Heterocypris incongruens* (*Crustacea*, *Ostracoda*)

Qualité de l'eau — Détermination de la toxicité des sédiments d'eau douce envers Heterocypris incongruens *(*Crustacea*,* Ostracoda*)*

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Foreword

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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 14371 was prepared by Technical Committee ISO/TC 147, *Water quality*, Subcommittee SC 5, *Biological methods*.

Introduction

The evaluation of harmful effects on water quality has for several years involved the performance of biological tests. Historically, toxicity tests have mainly focused on the impact of pollutants present in the water column of aquatic ecosystems, without considering the hazard of toxicants present and accumulating in the sediments.

“Direct contact” tests in which the test organisms are exposed to the whole sediment have been gradually developed with endobenthic species, such as chironomid larvae [*Chironomus riparius* or *Chironomus dilutus* (formerly *C. tentans*)] or epibenthic amphipod crustaceans (*Hyalella azteca*).

The test specified in this International Standard is a direct contact test for determination of the percentage mortality and/or growth inhibition on the fresh water ostracod *Heterocypris incongruens* (Ramdohr, 1808) after 6 d exposure to a whole sediment (see References [1], [2]).

H. incongruens is a cosmopolitan ostracod species, which has to date already been used extensively for toxicity testing not only of whole sediments, but also by extension on sludges and soils (see References [3]–[21]).

The direct contact test with *H. incongruens* has a sensitivity which is quite similar to that of the amphipod crustacean *Hyalella azteca* and the midge larva *Chironomus riparius* (see References [22]–[25]).

The assays are performed with neonates hatched from dormant eggs (cysts), which bypasses the need for culturing or maintaining live stock cultures of test organisms.

H. incongruens neonates (150 µm to 200 µm) are substantially smaller than *Hyalella azteca* and *Chironomus riparius*, and the assays can be performed in much smaller test containers, hence require much less bench space and incubator space.

The effects are evaluated after a shorter exposure time (6 d) than in the assays with the amphipod crustacean (10 d to 28 d) and midge larvae species (10 d to 28 d).

Water quality — Determination of fresh water sediment toxicity to *Heterocypris incongruens* (*Crustacea*, *Ostracoda*)

WARNING — Persons using this International Standard should be familiar with normal laboratory practice. This 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 in accordance with this International Standard be carried out by suitably qualified staff.

1 Scope

This International Standard specifies a method for the determination of lethal as well as sublethal effects of contaminated sediments on the ostracod crustacean *Heterocypris incongruens* after 6 d exposure.

The method is applicable not only to fresh water sediments, but also by extension to solid wastes and soils after addition of (uncontaminated) water.

The method can also be applied to chemicals or preparations which are spiked into a reference sediment.

This International Standard is not applicable to the testing of sediments from the estuarine or marine environment.

2 Normative references

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

ISO 5667-15:2009, *Water quality — Sampling — Part 15: Guidance on the preservation and handling of sludge and sediment samples*

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

3 Terms and definitions

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

3.1
neonate
newly hatched individual

3.2
pre-feeding
feeding of the neonates (3.1) with a small amount of dried algae (*Spirulina*) prior to the test

4 Principle

Freshly hatched *Heterocypris incongruens* larvae are exposed to the sediment sample under analysis and the percentage mortality of the test organisms is determined after 6 d exposure.

If the percentage mortality is low (e.g. <30 %), the growth inhibition of the organisms in the sediment sample is determined in comparison to the control sediment, as a sublethal effect criterion.

5 Test environment

The test shall be carried out in the dark, in a temperature-controlled room or incubator at (25 ± 1) °C in the test containers.

Maintain the atmosphere free from toxic dusts or vapours. The use of control solutions is a double check that the test is performed in an atmosphere free from toxic dusts and vapours.

6 Reagents, test organisms and media

Use only reagents of recognized analytical grade, unless otherwise specified.

6.1 Test organisms. The test organisms are neonates of the fresh water ostracod *Heterocypris incongruens* which are hatched from dormant eggs (cysts) of this crustacean.

Cysts of *H. incongruens* are obtained from laboratory cultures, as described in Annex A, or can be purchased from a specialized company[1)].

6.2 Pure water, having a conductivity below 10 µS/cm.

6.3 Test medium, prepared by dissolving the following mineral substances in 1 l of pure water (6.2):

$NaHCO_3$	96 mg
$CaSO_4 \bullet 2H_2O$	60 mg
$MgSO_4$	60 mg
KCl	4 mg

This test medium corresponds to a synthetic water of moderate hardness, i.e. containing $CaCO_3$ at concentrations of 80 mg/l to 100 mg/l (see Reference [26]). Thus prepared, the medium has a pH of 7,6 ± 0,3.

The test medium can be used for two weeks when stored in a refrigerator (4 °C to 7 °C) in the dark.

Aerate the test medium until the dissolved oxygen concentration has reached the air saturation value and until the pH has stabilized. If necessary, adjust the pH to 7,6 ± 0,3 using a sodium hydroxide or hydrochloric acid solution. The concentration of the acid or base required shall be selected so that the volume to be admixed is as small as possible. Bring the temperature of the test medium up to (25 ± 1) °C prior to use.

6.4 Algal food.

6.4.1 *Spirulina* suspension. A suspension of powder of the blue-green alga *Spirulina platensis* prepared by mixing 150 mg dry mass *Spirulina* in 10 ml pure water (6.2). *Spirulina* powder is available commercially in health stores.

6.4.2 Green algae. A 25 ml suspension of green algae (e.g. *Scenedesmus* spp.) at a concentration of around $1{,}5 \times 10^7$ cells/ml, prepared with test medium (6.3).

The algae can also be encapsulated in algal beads, which can be stored for long periods of time in a refrigerator, and "de-immobilized" at the time of use (see ISO 8692:2012[31], Annexes B and C).

1) MicroBioTests Inc., Mariakerke (Gent), Belgium, is an example of a supplier able to provide suitable *Heterocypris incongruens* cysts. This information is given for the convenience of the users of this document and does not constitute an endorsement by ISO of this supplier.

6.5 Lugol solution, to immobilize and fix the test organisms at the end of the test.

Lugol solution is prepared by mixing 5 g iodine (I_2) and 10 g potassium iodide (KI) with 85 ml pure water (6.2).

6.6 Control sediment. Commercial (not toxic and not calcareous) river sand or marine sand, which is water washed and sieved to eliminate dirt and debris, and air-dried is a suitable control sediment[2)] provided it is of the category "medium size" sand, with a granulometry 0/2 (i.e. a particle size range between 0 mm and 2 mm).

At the time of testing, the control sediment shall first be "water saturated" as follows.

Fill a small glass beaker with control sediment and pour test medium (6.3) on it until the sediment is completely wet. During this operation, the sediment shall be stirred with a spatula to ensure complete water saturation. Supernatant test medium shall be decanted.

6.7 Reference substance. Copper sulfate pentahydrate ($CuSO_4 \bullet 5H_2O$) is the recommended reference chemical.

7 Apparatus and material

Usual laboratory apparatus and, in particular, the following.

7.1 Temperature-controlled room or **chamber**.

7.2 Petri dishes, diameter 5 cm, glass or inert plastic material for hatching of the cysts and transfers of the test organisms.

7.3 Test containers. Disposable microplates made from chemically inert material, with wells of a capacity of at least 10 ml. Six (2 × 3) well microplates with a well diameter of approximately 35 mm are suitable.

7.4 Micropipette, in glass for sampling the test organisms, with an internal diameter of ~1 mm at the tip, for capturing the animals while allowing sampling of only a small volume of medium.

Micropipettes provided with a bulb at the end are very suitable for the operations.

7.5 "Large mouth" micropipettes, in an inert plastic material and with a wide opening, for transfer of sediment suspensions.

7.6 Spatula scoop, stainless steel or inert plastic material, with a capacity of 500 µl or 1 000 µl.

7.7 Flat spatula, stainless steel or inert plastic material.

7.8 Microsieve, with a 100 µm mesh.

7.9 Stereomicroscope with incident (bottom) illumination, with a magnification of at least 8 times and, if possible, a continuous magnification.

7.10 Calibrated eyepiece for the stereomicroscope, for length measurements of the test organisms.

7.11 Light source, providing a range of light intensity in the hatching Petri dish (7.2) of 3 000 lx to 4 000 lx.

7.12 Sample-collecting containers, in accordance with ISO 5667-15:2009, Clause 7.

2) River sand or marine sand can be obtained in every country from aquarium shops.

8 Special precautions for sampling, transportation, storage and treatment of the sediment samples

Perform sampling, transportation, and storage of the samples in accordance with the general procedures specified in ISO 5667-16.

The containers with the sediment samples shall be stored at (4 ± 2) °C in darkness with no head space above the sediment, and the assays shall be carried out as soon as possible after collection. The toxicity tests should be performed within two weeks of sampling, and preferably within one week. In any case, the assays shall start no later than six weeks after sample collection.

NOTE Scientific studies report that the toxicity of sediments stored at 4 °C did not change after several months of storage, but in other cases toxicity changes were noted within days to weeks.

Prior to the test performance, the samples shall be homogenized by stirring with a spoon or spatula in a container of stainless steel or inert plastic material. Large debris or large indigenous macro-organisms should be removed manually.

9 Procedure

9.1 Hatching of the cysts

Heterocypris incongruens cysts shall be hatched as follows.

Put 8 ml test medium (6.3) into a small Petri dish (7.2) and add a sufficient amount of ostracod cysts (6.1) to perform a complete test[3)].

Cover the Petri dish with its lid and incubate for 48 h at (25 ± 1) °C under continuous illumination (7.11).

9.2 Pre-feeding of the ostracod neonates

The neonates shall be provided with food for a few hours immediately after they have hatched (i.e. after 48 h incubation of the cysts).

Take the container with the *Spirulina* suspension (6.4.1) and homogenize the contents by hand shaking. Add 1 ml *Spirulina* suspension to the hatching Petri dish and gently shake the Petri dish to distribute the food suspension evenly.

Put the hatching Petri dish back in the incubator for 4 h.

9.3 Length measurement of the neonates

Pick up 10 ostracods from the hatching Petri dish with a glass micropipette, taking care to aspirate as little test medium (6.3) as possible, and drop the organisms in the centre of a glass slide.

Place the slide on the stage of the stereomicroscope and focus on the field with the ostracods. Put one drop of Lugol solution (6.5) on the organisms and wait for a few minutes until the neonates are completely immobile. Measure the length of the ostracods with the aid of the calibrated eyepiece and score the results on the data report template 1 (see Table 1). Calculate and score the mean length, L_{start}, of the neonates on the template.

NOTE The size of ostracod neonates ranges from 150 µm to 250 µm.

3) The amount depends on the hatchability of the cysts and should be sufficient to provide enough nauplii to perform a complete toxicity test (i.e. > 120 nauplii). 10 mg dry ostracod cysts is normally sufficient.

Table 1 — Data report template 1: Length of ostracod neonates

Ostracod neonate	Length µm
1	
2	
3	
4	
5	
6	
7	
8	
9	
10	
Mean length, L_{start}	

9.4 Addition of sediment and algal food to the test container

Perform the direct contact test on the whole sediment in six replicates, in parallel to a control sediment (6.6) also in six replicates. When using six-well microplates (7.3), the assay requires two such plates.

Inoculate three wells in each microplate with whole sediment and the three other wells with control sediment. Alternatively, the six wells of the first microplate can be filled with whole sediment, and those of the second plate with control sediment.

Put 2 ml test medium (6.3) into all the cups of the two microplates.

Fill the cup of the spatula scoop (7.6) with test sediment and strike off the excess of sediment with the flat spatula (7.7) to keep a volume of sediment of either 500 µl or 1 000 µl sediment in the scoop (depending of the type of spatula).

Transfer the sediment into the six wells of the two microplates using the tip of the flat spatula to empty the cup of the spatula scoop completely. Each well shall receive 1 000 µl sediment (i.e. two scoops of 500 µl when using a spatula scoop of 500 µl capacity).

Proceed similarly with the control sediment (6.6), filling the remaining six wells of the microplates with 1 000 µl control sediment.

Keeping the microplates horizontal, gently shake them to distribute the sediment evenly over the bottom surface of the wells.

Take the container with the algal food suspension (6.4.2), shake it gently to homogenize the algal suspension, and add 2 ml algal suspension to each well of the two microplates.

NOTE Algal food is provided to the organisms at the start of the test to avoid starvation which may lead to mortality or decreased growth of the test organisms.

9.5 Transfer of the ostracod neonates into the test container

Put the hatching Petri dish with the neonates on the stage of the stereomicroscope.

Fill a small Petri dish (7.2) with 10 ml test medium (6.3) and transfer with the glass micropipette about 65 to 70 ostracods into the Petri dish.

Put the Petri dish with the ostracods under the stereomicroscope and transfer 10 neonates into each well of the first microplate.