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

National foreword

This British Standard is the UK implementation of ISO 14371:2012.

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INTERNATIONAL STANDARD

ISO 14371

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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*)*

Reference number
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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

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).

INTERNATIONAL STANDARD ISO 14371:2012(E)

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.

Repeat this transfer operation for the second microplate.

On completion of the transfers, cover the two microplates with a sheet, e.g. of polyethylene, and the microplate cover.

9.6 Incubation of the test system

Incubate the two microplates at (25 ± 1) °C in darkness for 6 d.

9.7 Measurements

9.7.1 General

At the end of the exposure period, the ostracods have to be recovered from the test containers to determine the percentage mortality and to make the length measurements of the surviving ostracods.

The ostracods can be recovered “directly” from the wells containing control sediment since they are easily visible under a stereomicroscope. This is, however, mostly not the case for the test wells containing test sediment, for which a “sieving procedure” has to be applied to recover the ostracods.

9.7.2 Recovery of the living ostracods from the wells with control sediment

Place one multiwell on the stage of the stereomicroscope and switch on (preferably) both the top and the bottom illumination.

Centre one well containing control sediment and, with the aid of the glass micropipette, pick up the living ostracods one by one and transfer them into a small Petri dish (7.2) containing test medium (6.3)

Perform the same operation for all the wells containing control sediment.

9.7.3 Recovery of the ostracods from the wells with test sediment

Take one multiwell, and with a “large mouth” pipette (7.5) gently mix the sediment in the first well which contains test sediment, with the layer of test medium (6.3) on top of the sediment.

Aspirate part of the sediment suspension, transfer it into the microsieve (7.8) and gently rinse the contents of the microsieve with tap water from a wash bottle until all the fine sediment particles are washed out.

Proceed further with the stepwise transfer of the sediment suspension from the well to the microsieve, followed by rinsing with tap water, until most of the sediment has been transferred from the well to the microsieve.

Add a few millilitres of test medium (6.3) to the well, mix it with the remaining part of the sediment, and transfer it to the microsieve for rinsing. Repeat this operation several times, if necessary, to make sure that all the sediment and all the ostracods have been transferred.

Turn the microsieve upside down on top of a small Petri dish (7.2) and rinse the contents of the microsieve back into the Petri dish with test medium (6.3). Make sure that the full contents of the microsieve are transferred into the Petri dish.

These transfer and rinsing operations have, subsequently, to be performed for all the wells with test sediment of the two microplates.

NOTE The Petri dishes will contain the remaining (large) sediment particles and the living and dead ostracods which can now easily be seen under a stereomicroscope.

The user can, if desired, also apply other techniques to recover the ostracods.

9.7.4 Scoring of the mortality

Put, one by one, all the Petri dishes containing the ostracods on the stage of the stereomicroscope and count the number of living ostracods in each Petri dish.

Score the number, n_A, of living ostracods counted in each Petri dish on data report template 2 (Table 2) in the appropriate cells of the “Number of living ostracods” row.

Subtract each of the scored numbers, n_A, from 10 (i.e. the number of test organisms inoculated in the well at the start) to obtain the number of dead ostracods, $10 - n_A$, and score these numbers on data report template 2 in the cells of the “Number of dead ostracods” row.

NOTE The Petri dishes originating from the wells with test sediment can also contain “dead” ostracods. The total number of ostracods (i.e. dead plus living) can still be less than 10. The explanation for this is that, except for manipulation errors during the transfers and the sievings, dead ostracods decompose very rapidly, leaving no visible remains.

Proceed the same way with all the Petri dishes containing the test organisms from the other 11 wells and score the results on data report template 2.

9.7.5 Determination of the percentage mortality

With the data on mortality inserted into data report template 2 for each replicate, calculate the mean number of dead ostracods for the six replicates of the control sediment and the test sediment. Subsequently, calculate the mean percentage mortality in the control sediment and the test sediment. Record all these data in data report template 2.

Table 2 — Data report template 2: Mortality

Replicate	Control sediment						Test sediment					
	1	2	3	4	5	6	1	2	3	4	5	6
Number of living ostracods, n_A												
Number of dead ostracods, $10 - n_A$												
Mean number of dead ostracods for all replicates												
Mean percentage mortality												

9.7.6 Length measurement

Length measurements of the ostracods at the end of the 6 d exposure allow to evaluate the growth inhibition of the test organisms in the test sediment in comparison to the control sediment, as a “sublethal” effect criterion. The evaluation of this sublethal effect is clearly without purpose in case a substantial percentage of the organisms in the test sediment have died during the exposure.

Length measurements shall therefore only be performed in case the percentage mortality in the test sediment is below 30 %.

Pick up, one by one, the living ostracods from one Petri dish with the glass micropipette, and place them in the centre of a glass slide. Put the glass slide on the stage of the stereomicroscope and add one drop of Lugol solution (6.5) to immobilize the organisms.

Measure the length of the ostracods with the calibrated eyepiece of the stereomicroscope and score the length results on data report template 3 (Table 3) in the appropriate cells.

Calculate the mean length of the ostracods in the control sediment and the test sediment at the end of the assay, L_{end}.

Calculate the mean length increment, $L_{\text{increment}}$, of the ostracods in the control sediment and in the test sediment with Formula (1):

$$L_{\text{increment}} = L_{\text{end}} - L_{\text{start}} \quad (1)$$

Calculate the percentage growth inhibition of the ostracods in the test sediment with Formula (2):

$$100 - \frac{L_{\text{increment in test sediment}}}{L_{\text{increment in control}}} \times 100 \quad (2)$$

Table 3 — Data report template 3: Growth inhibition

Replicate	Control sediment						Test sediment					
	1	2	3	4	5	6	1	2	3	4	5	6
Test organism	Length of test organisms, µm											
1												
2												
3												
4												
5												
6												
7												
8												
9												
10												
Mean length of ostracods per replicate												
Mean length of ostracods for all the replicates, L_{end}												
Mean length increment for all the replicates, $L_{\text{end}} - L_{\text{start}}$	$L_{\text{increment in control}} =$						$L_{\text{increment in test sediment}} =$					
Mean percentage growth inhibition in the test sediment	—						$100 - \frac{L_{\text{increment in test sediment}}}{L_{\text{increment in control}}} \times 100 =$					

10 Reference test

Periodically perform a reference test with the reference chemical copper sulfate pentahydrate (6.7) in order to verify the sensitivity of the test organisms and the conformity to the test procedure.

The toxicant range to be used for the reference test is 1 mg/l to 10 mg/l of $CuSO_4 \bullet 5H_2O$.

Prepare solutions of the reference substance in test medium (6.3) at “double strength” (i.e. two times the concentrations to which the test organisms will be exposed during the incubation period).

After addition to each test cup of the 2 ml test medium containing the different toxicant solutions, also add 2 ml algal food suspension to each cup (9.4). This *de facto* decreases the toxicant concentration in the cups by half, which is why the solutions of the reference substance are originally prepared at double strength.

The following (log scale) dilution series of $CuSO_4 \bullet 5H_2O$ shall be prepared with test medium:

C1: 20 mg/l

C2: 11,2 mg/l

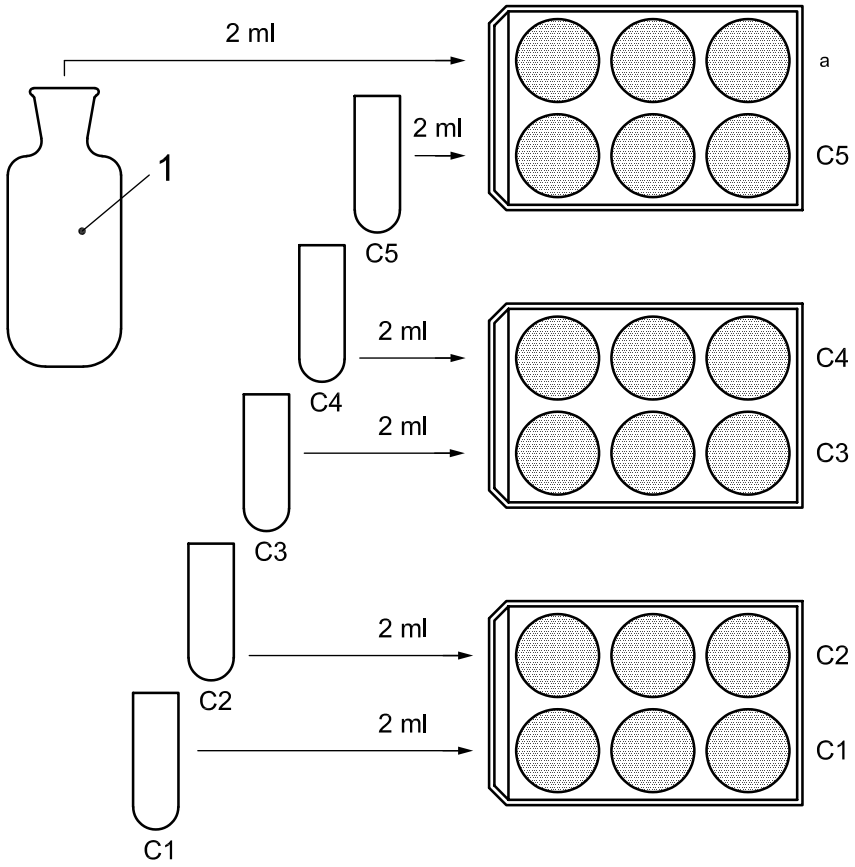
C3: 6,4 mg/l

C4: 3,6 mg/l

C5: 2 mg/l

Each toxicant concentration (plus the negative control) is tested in triplicate, in test cups each containing 1 000 µl reference sediment (and 10 ostracods per cup). The reference test therefore requires three six-well microplates (7.3).

Figure 1 shows the addition of the test medium (negative controls) and the toxicant solutions to the test wells in the three microplates.



Key

1	test medium
C1 ... C5	dilution series

[a] Control.

Figure 1 — Addition of test medium and toxicant solutions to the microplates

Similarly to the “direct” recovery of the test organisms from the control cups with reference sediment at the end of the assay (9.7.2), the surviving ostracods in the reference test can also be recovered “directly” from all the test cups without the need for a sieving step.

According to an international interlaboratory comparison (Annex B) with 26 participants, after having excluded outliers (Mandel’s h-statistic) the mean 6 d LC_{50} value is 5,79 mg/l $CuSO_4 \bullet 5H_2O$, with a repeatability standard deviation, s_r (within-laboratory variability), of 0,69 (giving a coefficient of variation of repeatability, $C_{V,r}$, expressed

as a percentage, of 11,91) and a reproducibility standard deviation, s_R (between-laboratory variability) of 1,79 (giving a coefficient of variation of reproducibility, $C_{V,R}$, expressed as a percentage, of 30,88).

Therefore, according to the data of this extensive international interlaboratory comparison, the results of a test with this reference chemical should be in the range 2,21 mg/l to 9,37 mg/l $CuSO_4 \bullet 5H_2O$ (calculated as the mean 6 d $LC_{50} + 2s_R$).

NOTE Since the percentage mortality in the highest test concentration is above 30 %, there is (according to 9.7.6) no need to perform the length measurements and calculate the growth inhibition EC_{50} for a reference test with copper sulfate performed with the toxicant range described above.

11 Tests on pure chemicals or preparations

Similarly to the reference test with copper sulfate (Clause 10), assays can be performed on pure chemicals or preparations by spiking a reference sediment with selected concentrations of a chemical or a preparation. By analogy to the reference test with copper sulfate, the solutions of the chemical or the preparation have to be prepared at “double strength” (see explanation in Clause 10, paragraph 3).

12 Validity criteria

The test is considered valid if the following conditions are met:

a) the percentage mortality in the control sediment is not higher than 20 %;

b) the mean length of the ostracods in the control sediment is at least 1,5 times higher than the mean length of the organisms at the start of the test.

13 Test report

The test report shall contain at least the following information:

a) the test method used, together with a reference to this International Standard (ISO 14371:2012);

b) all information required for the complete identification of the sediment under test;

c) the method of sampling and storage of the samples;

d) all biological, chemical, and physical information relative to the sediment;

e) all information relative to the test organism, and, if need be, the origin and number of the batch of *Heterocypris incongruens* cysts used;

f) all information relative to the test procedure;

g) the test results according to 9.7 and the method for calculation of the percentage mortality and/or growth inhibition;

h) data to prove that the validity criteria (Clause 12) have been met;

i) all operating details not specified in this International Standard, or regarded as optional, together with details of any incident that may have influenced the results;

j) name and address of the testing laboratory, the persons carrying out the test, and the person approving the report.

Annex A
(informative)

Culturing of *Heterocypris incongruens* for cyst production

A.1 Life cycle of *Heterocypris incongruens*

The ostracod *Heterocypris incongruens* is a cosmopolitic benthic microcrustacean living in temporary fresh water pools in temperate climates (see Reference [27]).

The strain of *H. incongruens* which is used for the ostracod microbiotest produces "simultaneously" two types of eggs: subitaneous eggs which develop into live offspring and "dormant eggs" (cysts) which resist dessication when the pools dry out. The ratio of production of subitaneous eggs versus dormant eggs is dependent on the environmental conditions in which the ostracod populations are living (see Reference [28]).

The cysts are deposited (cemented) in clusters on solid surfaces at the bottom of the pools and are resistant to dessication when the pools dry out.

When the pools are rehydrated (e.g. by rainfall), and provided the environmental conditions are suitable, the ostracod cysts hatch and the nauplii (150 µm to 250 µm) grow in several moults to the adult stage (about 1 500 µm) and start to reproduce.

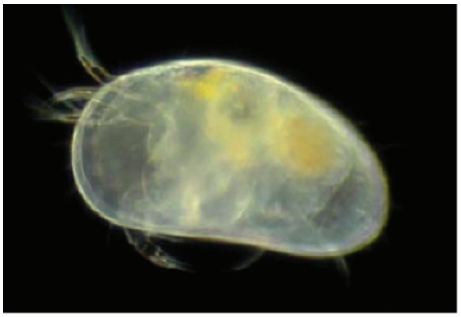


Figure A.1 — Freshly hatched larva (150 µm to 200 µm) of *Heterocypris incongruens*

A.2 Culturing of *Heterocypris incongruens* for cyst production

Start *Heterocypris incongruens* cultures with neonates hatched from cysts (see 9.1), in a loading preferably not exceeding 50 nauplii per litre.

The culturing medium can be natural water or reconstituted fresh water. Culturing can be done in small or large containers, dependent on the number of cysts which it is desired to produce. Culturing temperature is preferably 25 °C ± 1 °C, with a 12L:12D light cycle.

H. incongruens can be cultured on a variety of food sources (see Reference [29]), but preference should be given to micro-algae (e.g. *Scenedesmus* spp.), at concentrations of 50 000 cells/ml to 200 000 cells/ml.

Survival and growth of the organisms in laboratory cultures are totally a function of "good" culturing conditions, including control of a variety of abiotic and biotic factors, especially the amount and the frequency of feeding and the renewal of the culturing medium.

The cultures shall be aerated continuously (by gentle air bubbling) to guarantee a sufficient oxygen level.

After a few weeks, under appropriate conditions (which have to be determined by trial and error on an individual basis), the cultures reach the adult stage and start producing live offspring as well as cysts which are deposited in clusters on the walls and on the bottom of the culturing container.

To harvest the cysts, first empty the culturing container, after which scrape the walls and the bottom of the container with the aid of a flat spatula to collect the patches of dormant eggs.

Air dry the cysts and store them in a refrigerator at (4 ± 2) °C until use.

Air-drying is needed to break the dormancy of *H. incongruens* cysts and good hatching success is only obtained after a few weeks storage of the cysts.

Under adequate storage conditions, dry *H. incongruens* cysts can remain viable for several years.

Annex B
(informative)

Precision data

B.1 General

An interlaboratory trial on the subchronic toxicity test with the fresh water ostracod crustacean *Heterocypris incongruens* was organized in 2010-05, according to ISO 5725-2[30].

Altogether, 26 laboratories from 14 countries, participated in the interlaboratory trial.

Copper sulfate $CuSO_4 \bullet 5H_2O$ was selected as the reference chemical.

B.2 Results

Three laboratories submitted results which were not acceptable, and data processing was carried out on the results of 23 laboratories.

The "survival" validity criterion, namely that the percentage mortality in the controls should not be higher than 20 %, was achieved by all the participating laboratories. The majority of the participants reported 0 % mortality and a few of them reported 3 % and 6 % mortality in the controls (i.e. only one or two dead organisms of the 30 ostracods in the test cups of the negative controls).

The "growth" validity criterion, namely the increase of the length of the test organisms in the controls at the end of the test by 1,5 times their length at the start of the test, was also achieved by all the laboratories.

All mortality data were analysed using the US EPA Benchmark Dose Software (BMDS), Version 2.1. For each laboratory, the 6 d LC_{50} and the corresponding 95 % confidence limits were calculated using the log-probit model.

The repeatability, s_r, and reproducibility, s_R, of the interlaboratory trial were calculated according to ISO 5725-2[30]. The final results are summarized in Table B.1.

Table B.1 — Computed values for the 6 d LC_{50}, after exclusion of 1 outlier (Mandel's h-statistic), for the subchronic reference toxicity test with the ostracod crustacean *Heterocypris incongruens*

l	o	LC_{50} mg/l	s_R mg/l	$C_{V,R}$ %	s_r mg/l	$C_{V,r}$ %
22	1	5,79	1,79	30,88	0,69	11,91
l	number of laboratories					
o	number of outliers					
LC_{50}	6 d LC_{50} in mg/l $CuSO_4 \bullet 5H_2O$					
s_R	reproducibility standard deviation					
$C_{V,R}$	coefficient of variation of reproducibility					
s_r	repeatability standard deviation					
$C_{V,r}$	coefficient of variation of repeatability					

According to the data of this extensive interlaboratory trial, the 6 d LC_{50} results of a test with the reference chemical copper sulfate ($CuSO_4 \bullet 5H_2O$) should be in the range 2,21 mg/l to 9,37 mg/l (calculated as the mean 6 d $LC_{50} \pm 2s_R$).

Since the percentage mortality in the highest test concentration is higher than 30 %, there was basically no need to perform length measurements on the surviving ostracods at the end of the test, to calculate the 6 d EC_{50} (see Note to Clause 10).

Data on length measurements were, however, also submitted by the participants in this ringtest, and were processed statistically to determine s_r and s_R values. From the 23 data sets, only 15 showed growth inhibition of the surviving ostracods above 50 % in the highest test concentration, from which an EC_{50} could be calculated.

The results of the data processing are summarized in Table B.2.

Table B.2 — Computed values for the 6 d EC_{50}, after exclusion of two outliers (Mandel's h-statistic), for the subchronic reference toxicity test with the ostracod crustacean *Heterocypris incongruens*

l	o	LC_{50} mg/l	s_R mg/l	$C_{V,R}$ %	s_r mg/l	$C_{V,r}$ %
15	2	6,58	1,21	18,38	0,20	3,11
l	number of laboratories					
o	number of outliers					
LC_{50}	6 d LC_{50} in mg/l $CuSO_4 \bullet 5H_2O$					
s_R	reproducibility standard deviation					
$C_{V,R}$	coefficient of variation of reproducibility					
s_r	repeatability standard deviation					
$C_{V,r}$	coefficient of variation of repeatability					

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