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Water quality — Determination of the acute toxicity to *Thamnocephalus platyurus* (*Crustacea*, *Anostraca*)

Qualité de l'eau — Détermination de la toxicité aiguë envers Thamnocephalus platyurus (Crustacea, Anostraca)

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Contents

Page

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 14380 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. Crustaceans are of interest from the ecotoxicological point of view because they are primary consumers and a major component of the zooplankton in aquatic ecosystems.

The test specified in this International Standard involves determination of the lethal effects on the fresh water fairy shrimp *Thamnocephalus platyurus* after 24 h exposure to the toxicant. A rapid test can also be carried out to determine sublethal effects after a very short exposure time (1 h).

The beavertail fairy shrimp *T. platyurus* is to date already used extensively in toxicity testing for several reasons:

a) this anostracan crustacean has a sensitivity to chemicals which is quite similar to that of the cladoceran crustacean *Daphnia magna* (see References [4][5][6][7]);

b) 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;

c) *T. platyurus* neonates are substantially smaller than neonates of *Daphnia magna*, hence the assays require much smaller test containers, and much less bench space and incubation space;

d) *T. platyurus* is very sensitive to cyanotoxins produced by algal blooms in eutrophicated waters (see References [8][9]).

Water quality — Determination of the acute toxicity to *Thamnocephalus platyurus* (*Crustacea*, *Anostraca*)

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 the lethal effects of toxicants to *Thamnocephalus platyurus* test organisms after 24 h exposure. A second method (rapid test) is described in Annex A for the determination of sublethal effects after a very short exposure time (1 h).

The methods are applicable to:

a) chemical substances which are soluble or which can be maintained as stable suspensions or dispersions under the conditions of the test;

b) industrial or sewage effluents, treated or untreated, if appropriate after decantation, filtration or centrifugation;

c) fresh waters;

d) aqueous extracts;

e) toxins of blue-green algae.

This International Standard is not applicable to the testing of unstable chemicals (hydrolysing, absorbing, etc.) in water unless exposure concentration is measured, nor to the testing of aquatic samples from the estuarine or marine environment.

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 10523, *Water quality — Determination of pH*

3 Terms and definitions

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

3.1
control batch
series of replicates containing control solution

NOTE The role of a control batch in an experimental procedure is to demonstrate the response to the detection system imposed collectively by compounds of the matrix used in the determination, in the absence of the subject of interest.

3.2
LC_{50}
concentration or dilution of the test sample which gives rise to 50 % mortality of the test organisms

3.3
EC_{50}
concentration or dilution of the sample which gives rise to 50 % effect on the test organisms

3.4
neonate
newly hatched individual

3.5
test batch
series of replicates filled with the same test solution

4 Principle

Freshly hatched *T. platyurus* larvae are exposed to a range of concentrations of the sample under analysis and the percentage mortality of the test organisms is determined after 24 h exposure, with subsequent calculation of the 24 h LC_{50}.

The test is carried out in one or two stages:

— a "range-finding test" to determine the range of concentrations or dilutions needed for calculation of the 24 h LC_{50};

— a "definitive test" conducted when the data of the range-finding test are not sufficient or adequate for calculation of the 24 h LC_{50}.

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 beavertail fairy shrimp *T. platyurus*, which are hatched from dormant eggs (cysts) of this crustacean.

Cysts of *T. platyurus* are obtained from laboratory cultures of the crustacean as described in Annex B or can be purchased from a specialized company[1)].

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

6.2 **Pure water**, 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
$CaSO_4 \cdot 2H_2O$	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 [13]). Thus prepared, the medium has a pH of 7,6 ± 0,3.

When stored in a refrigerator at (4 ± 2) °C in the dark, the solution can be used for several months.

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 sodium hydroxide or hydrochloric acid solutions. 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 **Hatching medium**. An eightfold dilution of the test medium (6.3) with pure water (6.2).

6.5 **Reference substance**. Potassium dichromate ($K_2Cr_2O_7$) is the recommended reference chemical.

7 Apparatus and materials

Usual laboratory apparatus and glassware and in particular the following.

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

7.2 **Hatching Petri dishes**, small Petri dishes, diameter 5 cm, in glass or in inert plastic material.

7.3 **Test containers**. Disposable microplates made from chemically inert material, comprising wells with a capacity >1 ml. For example, 24 (4 × 6) well microplates with a well diameter of approximately 16 mm are suitable.

7.4 **Pipette for sampling the test organisms**, with a sufficient diameter for capturing the animals while allowing sampling of only a small volume of medium.

Micropipettes of inert plastic material with a bulb at the end are very suitable for the operations.

7.5 **Stereomicroscope with incident (bottom) illumination**, with a magnification of at least eight times and, if possible, a continuous magnification.

7.6 **Light source**, providing a range of light intensity in the hatching Petri dish of 3 000 lx to 4 000 lx.

7.7 **Sample collecting bottles**, as specified in ISO 5667-16.

8 Treatment and preparation of samples

8.1 Special precautions

Special precautions are required for sampling, transportation, storage and treatment of water, effluent, or aqueous extract samples to be tested.

Sampling, transportation and storage of the samples should be performed as specified in ISO 5667-16.

Carry out the toxicity test as soon as possible, ideally within 12 h of collection. If this time interval cannot be met, cool the sample to 0 °C to 5 °C and test the sample within 24 h. If it is not possible to perform the test within 72 h, the sample may be frozen and maintained deep-frozen (below −18 °C) for testing within 2 months of collection, provided that characteristics are known to be unaffected by freezing. At the time of testing, homogenize the sample to be analysed by shaking manually, and, if necessary, allow to settle for 2 h in a container, and sample by drawing off (using a pipette) the required quantity of supernatant, maintaining the end of the pipette in the centre of the section of the test tube and halfway between the surface of the deposited substances and the surface of the liquid.

If the raw sample of the decanted supernatant is likely to interfere with the test (due to the presence of residual suspended matter, protozoa, microorganisms, etc.), filter or centrifuge the raw or decanted sample.

The sample obtained by either of these methods is the sample submitted to testing.

Measure the pH (as specified in ISO 10523) and the dissolved oxygen concentration (as specified in ISO 5814) and record these values in the test report.

If the aim of the test is to assess the acute toxicity without considering the pH effects, the test may also be carried out after adjustment of the pH value to 7,6 ± 0,3 with hydrochloric acid or sodium hydroxide solutions. Proceed, if appropriate, as indicated above, for the separation of the suspended matter formed following the adjustment of the pH. Mention any pH adjustment in the test report.

8.2 Preparation of the stock solutions of substances to be tested

Prepare the stock solution of the substance to be tested by dissolving a known quantity of substance in a specified volume of test medium (6.3) at the time of use. However, if the stock solution of the substance is stable under certain conditions, it may be prepared in advance and stored under these conditions.

For substances sparingly soluble in the test medium, refer to the specifications given in ISO 5667-16.

9 Procedure

9.1 Hatching of the cysts

9.1.1 General

T. platyurus cysts shall be hatched under the conditions specified in 9.1.2 to 9.1.4.

9.1.2 Preparation of hatching medium

Prepare 20 ml hatching medium (6.4) by adding 17,5 ml pure water (6.2) to 2,5 ml test medium (6.3) in a small glass container.

9.1.3 Prehydration of the cysts

Transfer approximately 10 mg to 15 mg dry cysts into a 1 ml tube in glass or in inert plastic material. 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. >180 nauplii). Fill the tube with hatching medium. Close the tube and shake it several times during a 30 min period to hydrate the cysts.

9.1.4 Transfer of the prehydrated cysts into the hatching Petri dish

Empty the contents of the tube with prehydrated cysts into a Petri dish (7.2). Make sure that most of the cysts are transferred by rinsing the tube with hatching medium.

Add 10 ml hatching medium to the Petri dish and swirl gently to distribute the cysts evenly.

Cover the hatching Petri dish and incubate at (25 ± 1) °C for 20 h to 22 h under continuous illumination (3 000 lx to 4 000 lx, corresponding to 40 to 55 µmol m^{-2} s^{-1}).

9.2 Selection of test concentrations

The test should comprise at least five concentrations of the sample to be tested. The dilutions shall be selected within a geometric series with a separation factor which depends on the nature of the sample to be analysed (chemical substances, effluents, waters or extracts) and of the type of assay (range finding or definitive).

For the range-finding test with chemical substances, the separation factor for the serial dilutions is usually 10 (one order of magnitude difference between two successive dilutions). A suitable concentration range is best determined by carrying out a preliminary range-finding test covering several orders of magnitude of difference in test concentration. Replication of test concentrations is not a requirement in the preliminary test.

For effluents, water or extracts a 1+1 dilution factor is normally applied (i.e. dilution of the previous concentration by half).

Dilutions series for the definitive test on chemical substances are prepared with a separation factor not exceeding 3,2.

The test is carried out with three replicates for each dilution plus a control (i.e. the test medium without sample) also in three replicates.

When using a solvent to dissolve or disperse chemical substances, the test is carried out with three replicates for the control (the test medium without sample) plus three replicates for the control with solvent.

NOTE The latter application requires the use of a second microplate at the highest concentration of solvent.

9.3 Preparation of the test and control solutions

Prepare the test solutions by mixing the appropriate volumes of the sample to be tested (Clause 8 and 9.2) or of its initial dilution, with test medium (6.3).

Control and test solutions can be prepared in 10 ml containers (e.g. tubes in glass or in inert plastic material).

The containers shall be labelled as: control, C1, C2, C3, C4 and C5, in sequence of the highest to the lowest test concentration.

Distribute the test and control solutions in the microplate in a volume of 1 ml per well and according to the spatial distribution of the solutions in the wells as shown in Figure 1.

The microplate of 24 wells has six columns (1 to 6) and four rows (A to D).

The four wells in the left column (C6) are filled with the control batch (3.1).

Those of the other columns are filled with the toxicants (test batches 3.5) as follows: the four wells in column 2 are filled with the lowest toxicant dilution (C5), those of column 3 with the second lowest toxicant dilution (C4), etc.

The wells in rows A, B and C are for the three replicates of the control batch columns and the test batch columns respectively.

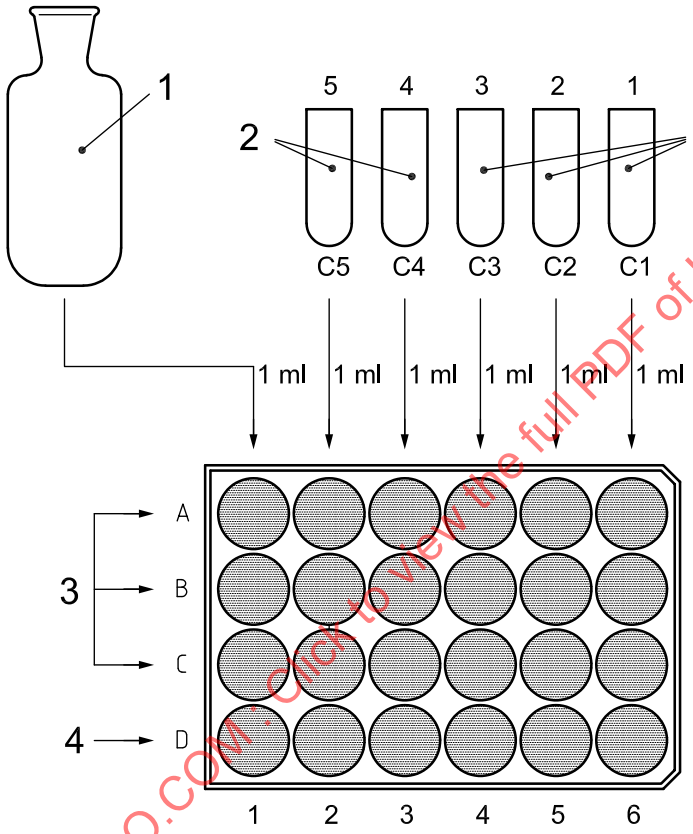
The wells in row D are “rinsing wells” intended to avoid dilution of the toxicant in the test wells during the transfer of the organisms from the hatching Petri dish to the microplate.

9.4 Introduction of the organisms

Put the hatching Petri dish on the glass stage of the stereomicroscope (7.5) and collect a number of actively swimming *T. platyurus* larvae with the pipette (7.4), taking care to suck up as little hatching medium as possible during this operation.

Transfer at least 35 test organisms into well D of column 1 (rinsing well) of the microplate and repeat this operation for the other (rinsing) wells of row D.

Put the microplate on the glass stage of the stereomicroscope and transfer 10 neonates from the (rinsing) well in column 1 (control batch) into the three wells of this column.



Key

1 test medium
2 test containers, 10 ml
3 test wells
4 rinsing wells

Figure 1 — Filling of the microplate with control and test solutions

Repeat this operation for the five other columns, going from left to right, i.e. starting with column 2 (lowest test concentration) to column 5 (highest test concentration).

The pipette should be rinsed with test medium after the organisms have been transferred from the rinsing cup to the three test cups in each individual column.

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

9.5 Incubation of the test system

Incubate the microplate at (25 ± 1) °C in the dark for 24 h.

9.6 Measurements

Take the cover and the sheet from the microplate and put the microplate on the glass stage of the stereomicroscope.

Check all the wells of rows A, B and C of the six columns, and record the number of dead larvae in each well.

NOTE The larvae are considered dead if they do not show any movement during 10 s of observation.

Insert the numbers into the data report template (see Table 1).

On completion of the count, collect the contents of the three wells (A, B, C) of the control column in a suitable container and measure the pH as specified in ISO 10523, and the oxygen concentration as specified in ISO 5814.

Transfer the contents of the three wells into a separate container and carry out the mixing of the solution with great care, in order to avoid adding oxygen from the air which could bias the oxygen measurement.

Perform the same operation and measurements for the three test wells of the most concentrated test concentration (column C1).

Explanation to Table 1:

Test dilution series

C5: (lowest test concentration)

C4:

C3:

C2:

C1: (highest test concentration)

Table 1 — Data report template (24 h exposure time)

Row	Column					
	Control	C5	C4	C3	C2	C1
A						
B						
C						
Total	/30	/30	/30	/30	/30	/30
Mortality, %						

If testing chemicals, analytical confirmation is strongly recommended in order to verify test substance concentrations.

10 Estimation of the LC_{50}

Calculate the mean percentage mortality in the control and in each test concentration.

Determine the 24 h LC_{50} (plus, if deemed necessary, other effect percentages, e.g. LC_{10} or LC_{90}) by an appropriate statistical method (see ISO/TS 20281[3] and Reference [18]), e.g. moving average or probit, depending on the mortality values in the dilution series. Other models may be used depending on the shape of the dose-response curve, as the objective is to obtain the best fit to the data (see ISO/TS 20281[3]).

11 Reference test

Periodically determine the 24 h LC_{50} of potassium dichromate (6.5) in order to verify the sensitivity of the test organisms and the conformity to the test procedure.

The following dilution series of potassium dichromate shall be prepared with test medium for the reference test.

C1: 0,32 mg/l

C2: 0,18 mg/l

C3: 0,10 mg/l

C4: 0,056 mg/l

C5: 0,032 mg/l

According to an international interlaboratory comparison (Annex C) with 23 participants, after having excluded one outlier (Mandel's h-statistic), the mean LC_{50} value is a $K_2Cr_2O_7$ concentration of 0,100 mg/l (95 % confidence limits: 0,086 to 0,113), with a repeatability standard deviation s_r (within-laboratory variability) of 0,010 (a $C_{V,r}$ of 9,74 %), and a reproducibility standard deviation s_R (between-laboratory variability) of 0,024 (a $C_{V,R}$ of 23,74 %).

Therefore, according to the data of this extensive international interlaboratory comparison, the results of a test with the reference chemical should be in the $K_2Cr_2O_7$ concentration range 0,052 mg/l to 0,148 mg/l (calculated as the mean LC_{50} 24 h $\pm$ 2 s_R).

12 Validity criteria

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

a) the percentage mortality in the controls is not higher than 10 %;

b) the dissolved oxygen concentration at the end of the test (measured as indicated in 9.6) is $\geq$2 mg/l.

13 Test report

This test report shall contain at least the following information:

a) the test method used, together with a reference to this International Standard (ISO 14380:2011);

b) all information required for the complete identification of the sample or of the substrate under test;

c) the methods of preparation of the samples:

   1) for effluents, waters and aqueous extracts, the method and the storage time of the samples, the pH and the dissolved oxygen concentration of the initial sample, if need be, the conditions in which the decantation, filtration or centrifugation of the sample and a possible adjustment of the pH were carried out,

   2) for chemicals, the method of preparation of the stock and test solutions;

d) all biological, chemical, and physical information relative to the test specified in this International Standard;

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

f) all information relative to the test (sample concentrations, pH of the test and control solutions, etc.);

g) the test results in accordance with Clause 10 and the method with which they were calculated;

h) the results obtained from the reference test (Clause 11) as well as the date of the reference test;

i) data to prove that the validity criteria (Clause 12) are met;

j) 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;

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

Annex A
(informative)

Rapid test for determination of sublethal effects on *Thamnocephalus platyurus* (1 h exposure)

A.1 General

This International Standard can be applied to testing of pure chemicals as well as to effluents, waste waters and other environmental aqueous samples.

The test procedure is based on the determination of the uptake of coloured microspheres by the test organisms in a non-toxic medium (control) as compared to the uptake (or absence of uptake) in the test solution under analysis.

Even after a very short exposure time (1 h), stressed crustaceans already either cease to take up the particles or ingest them at a much lower rate.

Comparative studies on chemicals and cyanotoxins on the sensitivity of the sublethal effect criterion after 1 h exposure versus the mortality criterion after 24 h exposure revealed a positive correlation between the 1 h EC_{50} and the 24 h LC_{50} (see References [13][14]).

The performance of the 1 h *T. platyurus* test (in terms of precision and toxicity threshold for inorganic and organic water contaminants) has been evaluated by the US EPA in the framework of the Environmental Technology Verification Program (ETV). The 1 h assay was found to be able to detect all the chemical compounds except one at, and in most cases even substantially below, the lethal dose to humans (see Reference [12]). For most compounds, the 1 h *T. platyurus* assay was also substantially more sensitive than the other "rapid" toxicity tests, which had been submitted in 2003 and 2005 to the same evaluation.

A.2 Principle

T. platyurus dormant eggs (cysts) are incubated for 30 h to 45 h at (25 ± 1) °C under continuous illumination, (3 000 lx to 4 000 lx) and the hatched larvae are exposed for 1 h to the test water, in parallel to a non-toxic control water. A suspension of coloured microspheres is then added.

In the control water, the organisms ingest the microspheres which colour the digestive tract deep red.

Stressed (intoxicated) organisms do not take up the coloured particles or ingest them at a much slower rate.

The presence or the absence of the coloured particles in the digestive tract of the larval crustaceans is observed after 15 min under a stereomicroscope at low magnification and with incident illumination.

A.3 Reagents, test organisms and media

A.3.1 Test organisms. *T. platyurus* larvae are hatched from cysts of this species (see 6.1 and 9.1).

Contrary to the mortality test specified in this International Standard, the larvae are not harvested after an incubation period of 20 h to 22 h, but after 30 h (minimum) to 45 h (maximum).

This longer incubation time for the rapid test is due to the fact that the mouth of freshly hatched nauplii (instar I) is not open and that at this stage the larvae cannot take up particles.

The nauplii, however, rapidly moult into the instar II and III stages in which the mouth and the digestive tract are fully formed.

A.3.2 Pure water. See 6.2.

A.3.3 Test medium. See 6.3.

A.3.4 Coloured microspheres in inert material (e.g. red coated polystyrene beads of diameter 5 µm) are suitable for the rapid test. Dyed microspheres can be obtained from several commercial sources.

The commercial product has to be diluted to a 5 % volume fraction stock solution with pure water.

The stock solution requires storage in a refrigerator at (4 ± 2) °C prior to use.

A.3.5 Lugol's solution used to immobilize and fix the test organisms at the end of the test, prepared by mixing 5 g iodine (I_2) and 10 g potassiuim iodide (KI) with 85 ml pure water (6.2).

Lugol's solution requires storage in a refrigerator at (4 ± 2) °C prior to use.

A.3.6 Reference substance. Potassium dichromate ($K_2Cr_2O_7$) can be used for reference testing.

A.4 Apparatus

The same equipment and materials as described in Clause 7 shall be used, with some additional items.

A.4.1 Hatching Petri dishes, diameter 10 cm.

A.4.2 Stereomicroscope, with illumination both from the bottom (transmitted light) and from the top (incident light by a ring light or fibre optic cold light).

A.4.3 Sheet of deep blue paper or **plastic sheet** of the size of the microplate.

A.4.4 Transparent sheet with rectangular counting grid, of the size of the microplate.

Such a sheet can easily be prepared by making a photocopy of a sheet of millimetric paper on a sheet of transparency film.

A.5 Treatment and preparation of samples

See Clause 8.

A.6 Procedure

A.6.1 Hatching of the cysts

A.6.1.1 Hatching medium

The hatching medium is the same as the test medium.

NOTE Contrary to the hatching medium for the 24 h mortality test, the hatching medium does not require dilution with pure water for the 1 h test.

A.6.1.2 Prehydration of the cysts

Transfer approximately 20 mg to 30 mg cysts into a 1 ml tube in glass or inert plastic and proceed further as indicated in 9.1.

A.6.1.3 Transfer of the prehydrated cysts into the hatching Petri dish

Empty the contents of the tube with prehydrated cysts into the glass Petri dish (A.4.1). Make sure that most of the cysts are transferred by rinsing the tube with hatching medium.

Add 40 ml test medium to the Petri dish and swirl gently to distribute the cysts evenly.

Cover the hatching Petri dish and incubate at (25 ± 1) °C for 30 h (minimum) to 45 h (maximum) under continuous illumination (3 000 lx to 4 000 lx).

A.6.2 Selection of test concentrations

See 9.2.

A.6.3 Preparation of the test and control solutions

The test and control solutions are prepared as described in 9.3, but in volumes of 20 ml (instead of 10 ml for the 24 h mortality test).

The microplate well-filling procedure is similar to that specified in 9.3, but 2 ml of test or control solutions are added to each well, instead of 1 ml.

A.6.4 Introduction of the organisms

This procedure is similar to that specified in 9.4. However, a larger number of organisms (about 60 to 70) shall first be put into the rinsing wells followed by transfer of 15 to 20 larvae to each test well.

A.6.5 Incubation of the test system

The microplate shall be incubated at (25 ± 1) °C for 1 h in darkness.

A.6.6 Addition of coloured microspheres

Take the cover and the sheet from the microplate and add 50 µl stock solution of coloured microspheres to each test well. The container with stock solution shall be shaken before each addition to ensure a homogenous distribution of the microspheres in the stock vial.

Cover the microplate and incubate again for 15 min to 30 min.

A.6.7 Immobilization or fixation of the test organisms

Remove the cover and the sheet from the microplate and add 50 µl Lugol's solution (A.3.5) to each test well.

Wait about 5 min for the fixed organisms to settle to the bottom of the wells.

A.6.8 Microscopic analysis of the uptake of coloured microspheres

Put the microplate on the glass stage of the stereomicroscope and switch on the bottom illumination.

Centre the visual field on a well, and carefully aspirate most of the liquid from the well with the aid of a pipette, taking care not to remove any of the test organisms during this operation.

Perform this elimination of the liquid from all the test wells.

Put the piece of transparent counting grid on top of the sheet of blue paper (A.4.3) and fix it at the edges with adhesive tape.

Put the blue sheet with the transparent counting sheet on the stage of the stereomicroscope and put the microplate on top.

Switch the illumination of the stereomicroscope from transparent (bottom) illumination to incident (top) illumination.

Centre the first well of the control column and select a magnification which allows the entire bottom surface of the well to be viewed.

Count the total number of larvae in the well and the number of larvae which have ingested coloured microspheres, as shown by their coloured digestive tracts.

Score these two numbers in the data report template (see Table A.1).

Repeat this operation for all the test wells.

A.6.9 Measurements at the end of the test

Taking into account the very short (1 h) exposure time, there is no need to perform the pH and oxygen measurements specified in 9.6.

A.6.10 Data treatment

Calculate the total number of organisms and the total number of coloured organisms for the three test wells of the control and the five toxicant dilutions, and record these figures on the data report template.

Calculate the percentage of coloured organisms for the control and the five toxicant dilutions and record these figures on the data report template.

Calculate the percentage inhibition of the particle uptake from the formula:

$$\frac{n_A - n_B}{n_A} \times 100 \qquad \text{(A.1)}$$

where

n_A is the percentage of coloured organisms in the control;

n_B is the percentage of coloured organisms in the toxicant dilution.

Insert these figures into the data report template in the appropriate cells.

Determine the 1 h EC_{50} (and if deemed necessary also other effect percentages, e.g. EC_{10} or EC_{90}) by an appropriate statistical method (see ISO/TS 20281[3], Reference [18]).

Explanation to Table A.1:

Test dilution series

C5: (lowest test concentration)

C4:

C3:

C2:

C1: (highest test concentration)

Table A.1 — Data report template (1 h exposure time)

Row	Column					
	Control	C5	C4	C3	C2	C1
A						
B						
C						
Total[a]	/*N*	/*N*	/*N*	/*N*	/*N*	/*N*
Mortality, %						

[a] *N* is the total number of larvae in the test wells.

A.7 Important remarks for correct application of the rapid test

A.7.1 Larval stage

The first larval stages of anostracan crustaceans (e.g. *T. platyurus*) live on their yolk reserves, do not have a fully developed digestive tract and do not ingest particles.

In contrast to the assay on mortality which is performed with freshly hatched larvae, the test organisms used in the rapid test should therefore only be collected, at the earliest, after 30 h from the start of the incubation of the cysts.

Hatching of the cysts is, however, not totally synchronous which means that at the time of collection, some larvae may still be in an instar stage in which they do not take up particles.

These larvae can be distinguished very easily from the older larval stages because they are smaller, orange in colour and not transparent.

These opaque (orange) larvae should be excluded from the scorings.

A.7.2 Vitality of the test organisms

The particle uptake is very poor when the larvae become too weak (due to the absence of food).

The organisms have therefore to be collected and used for the assays in the time span of 30 h to 45 h from the start of the incubation of the cysts.

A.7.3 Test population

As in all biota, a population is always a mixture of organisms with a different “vitality” ranging from weak to very strong.

Therefore, the uptake of red particles can also be different between the organisms, and even in the controls; the percentage of organisms in which red microspheres can be seen clearly is virtually never 100 %.

With a healthy population, the percentage of coloured larvae is usually between 60 % and 90 %.

A.7.4 Intensity of uptake of coloured microspheres

A “weaker” particle uptake by the test organisms in the test water compared with those in the control water is an additional sign of (toxic) stress and may also be taken into consideration in the interpretation of the results.

A.8 Reference test

By analogy to the mortality test specified in the main body of this International Standard, the performance of a periodical reference test with a selected chemical is recommended to verify the sensitivity of the test organisms and the conformity to the test procedure.

For potassium dichromate[2)], the 1 h EC_{50} should be around 10 mg/l.

A.9 Validity criteria

Tests in which less than 50 % of the test organisms in the control show a clear ingestion of coloured microspheres should be disregarded.

A.10 Test report

All the items listed in Clause 13 are also applicable to the rapid test, except for the data on the measurement of the pH and oxygen which are not required for the rapid assay.

2) The 1 h EC_{50} for potassium dichromate is substantially higher than the 24 h LC_{50} for *Thamnocephalus platyurus*, due to the slow mode of action of this chemical. For chemicals with a rapid mode of action, e.g. sodium pentachlorophenol, the 1 h EC_{50} is very near the 24 h LC_{50} (~0,75 mg/l).

Annex B
(informative)

Culturing of *Thamnocephalus platyurus* for cyst production

B.1 Life cycle of *Thamnocephalus platyurus*

The anostracan crustacean *T. platyurus* always reproduces sexually, with formation of dormant eggs (cysts) of a size of about 250 µm.

In nature, *T. platyurus* cysts can be found at various sites in different countries in temporal (dried out) fresh water pools in the western hemisphere.

When hydrated and provided with light, the embryonic development of the egg in the cyst is triggered, leading in 1 day to 2 days to the hatching of the cyst and liberation of a neonate (about 300 µm, see Figures B.1 to B.3).

Provided with food and appropriate conditions, the larvae grow through successive moultings into adult males and females, the size of which can be up to 3 cm.

Female *T. platyurus* deposit their eggs in a brood sac, where the eggs are fertilized by the sperm of the males during mating.

The fertilized eggs are subsequently surrounded by a sturdy shell (cyst). Females can produce several successive broods of cysts during their life cycle. Once the formation of the cysts is complete, they are shed by the female and sink to the bottom.

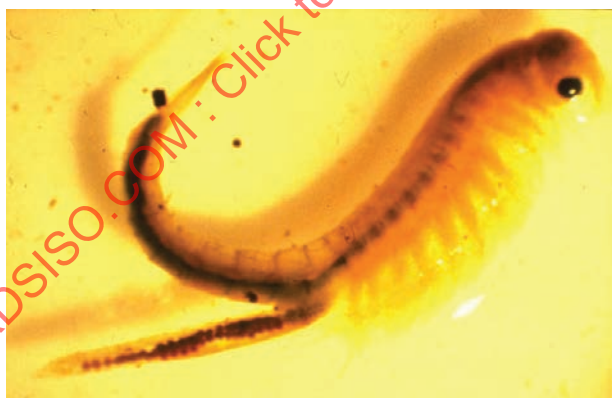


Figure B.1 — Adult *Thamnocephalus platyurus* female (2 cm) with a brood sac containing several hundred cysts