SLOVENSKI STANDARD
SIST ISO 14669:2010

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Kakovost vode - Določevanje akutne letalne strupenosti z morskimi kopepodnimi raki (Copepoda, Crustacea)

Water quality - Determination of acute lethal toxicity to marine copepods (Copepoda, Crustacea)

Qualité de l'eau - Détermination de la toxicité létale aiguë vis-à-vis de copépodes marins (Copepoda, Crustacea)

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Water quality — Determination of acute lethal toxicity to marine copepods *(Copepoda, Crustacea)*

Qualité de l'eau — Détermination de la toxicité létale aiguë vis-à-vis de copépodes marins (Copepoda, Crustacea)

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Contents

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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 3.

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.

International Standard ISO 14669 was prepared by Technical Committee ISO/TC 147, *Water quality*, Subcommittee SC 5, *Biological methods.*

Annexes A, B and C of this International Standard are for information only.

Water quality — Determination of acute lethal toxicity to marine copepods *(Copepoda, Crustacea)*

1 Scope

This International Standard describes a method for the determination of the acute toxicity to one of three specified species of marine copepod (*Copepoda, Crustacea*) of

a) chemical substances which are soluble, or can be maintained as a stable suspension or dispersion, under the conditions of the test;

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

c) marine or estuarine waters.

2 Normative references

The following normative documents contain provisions which, through reference in this text, constitute provisions of this International Standard. For dated references, subsequent amendments to, or revisions of, any of these publications do not apply. However, parties to agreements based on this International Standard are encouraged to investigate the possibility of applying the most recent editions of the normative documents indicated below. For undated references, the latest edition of the normative document referred to applies. Members of ISO and IEC maintain registers of currently valid International Standards.

ISO 5667-2, *Water Quality — Sampling — Part 2: Guidance on sampling techniques*.

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

3 Principle

Copepods are exposed to a range of concentrations in seawater of a chemical substance, effluent or water sample. Mortality of the copepods is recorded after 24 h and 48 h.

The concentration which, in 48 h, kills 50% of exposed copepods under the conditions defined in this International Standard is determined. This concentration, known as the median lethal concentration, is designated 48 h LC50.

NOTE If possible, the concentration which kills 50% of the exposed copepods in 24 h is also determined. This concentration is designated 24 h LC50. It may be appropriate for certain purposes to extend the exposure period to 96 h and to determine the 96 h LC50.

An indication of the lowest concentration tested which kills all the copepods and the highest concentration tested which does not kill any of the copepods is desirable and provides useful information in cases where the 48 h LC50 cannot be determined.

The test is carried out in one or two stages:

— a preliminary test which determines the range of concentrations to be tested in the definitive test and gives an approximate value of the 48 h LC50 (and where appropriate, the 24 h LC50).

— a definitive test, conducted when the approximate value given by the preliminary test is not sufficient, which permits calculation of the 48 h LC50 (and where appropriate, the 24 h LC50) and determines concentrations corresponding to 0 and 100% mortality.

If the method described in this International Standard is used for chemical substances, a limit test can be performed at 100 mg/l or at a lower concentration which is the maximum at which the substance is soluble or is in stable dispersion under the conditions of the test.

4 Test environment

The procedure described in this International Standard shall be carried out in a room, incubator or water-bath controlled at 20 °C ± 2 °C and under a 16 h/8 h light/dark photoperiod. The atmosphere shall be free from vapours or dusts toxic to copepods.

5 Reagents and materials

5.1 Test organism, one of the following species of marine copepod:

a) *Acartia tonsa* Dana;

b) *Tisbe battagliai* Volkmann-Rocco;

c) *Nitocra spinipes* Boeck.

Obtain the test organisms from laboratory cultures. Guidance on identification and culture methods for each species are given in Annex B. After hatching of eggs, the lifecycle of copepods consists of naupliar, copepodid and adult stages. The age and lifestage at the start of the test shall be indicated in the test report and are as follows:

— *Acartia tonsa*: large copepodids (Stage 5) or adults;

— *Tisbe battagliai:* copepodids 6 ± 2 days old;

— *Nitocra spinipes:* adults 3 to 4 weeks old.

5.2 Dilution water.

A natural or a synthetic seawater may be used as the dilution water. If natural seawater is used, it shall be collected from a location as distant as possible from known sources of pollution and filtered to remove indigenous organisms. If synthetic seawater is used, it shall be prepared by dissolving reagents of recognized analytical grade, or a commercially available formulation, in distilled or deionized water. However, for these copepod species, there is insufficient information on the use of synthetic seawater to allow a particular example to be recommended.

The salinity of the dilution water shall be between 29×10^{-3} and 36×10^{-3}. The use of a lower salinity, which may be more appropriate for tests concerning estuarine or brackish water situations, shall be justified in the test report. *Nitocra spinipes* can be used at salinities down to 1×10^{-3} and *Tisbe battagliai* can be used at salinities down to 20×10^{-3}. Whichever salinity is employed, the test organisms shall be cultured or maintained at the same salinity ($\pm\ 3 \times 10^{-3}$) for at least 7 days before the start of the test. The dilution water shall have a dissolved oxygen concentration above 80 % of the air saturation value, and a pH of 8,0 ± 0,3 before being used to prepare the test solutions.

The dilution water shall permit survival of the copepods for at least 48 h and should be from the same source as water that has been found to support culture of the organisms through at least two generations.

5.3 Reference chemical toxicant, e.g. 3,5-dichlorophenol or a suitable alternative (8.5), of recognized analytical grade.

6 Apparatus

Ordinary laboratory apparatus, and in particular:

6.1 Apparatus for measuring dissolved oxygen, salinity and pH.

6.2 Low-power stereo microscope, preferably with darkfield illumination.

6.3 Ultrasonic device or other apparatus for the preparation of stock solutions of poorly soluble substances (7.2.1).

6.4 Test containers, of chemically inert material and of sufficient capacity (for example glass beakers or disposable rigid plastic tissue-culture well-plates). Loose-fitting lids or covers are recommended to minimize evaporation of the test solutions. Containers which are suitable for low-power microscopical observation may be necessary for nauplii or copepodid stages.

Before use, the test containers shall be carefully washed then rinsed, first with water and then with the dilution water (5.2).

7 Sampling, treatment and preparation of samples

7.1 Special precautions for sampling and transportation of samples of water or effluent

Sampling of water or effluent shall be carried out in accordance with the general procedure specified in ISO 5667-2. Bottles shall be completely filled to exclude air.

The preservation and storage of water or effluent samples shall be carried out in accordance with ISO 5667-16; the following is only a summary. The toxicity test should be carried out as soon as possible, ideally within 12 h of collection. If this time interval cannot be observed, cool the sample (0 °C to 4 °C) and test the sample within 48 h. If testing cannot be carried out within 48 h, the sample may be frozen (below −18 °C) for testing within 2 months of collection.

7.2 Preparation of solutions of substances to be tested

7.2.1 Preparation of stock solutions

Prepare stock solutions of the substance to be tested by dissolving or diluting a known quantity of the substance in a known volume of dilution water, deionized water or distilled water in a glass container. They shall be prepared at the moment of use unless the substance is known to be stable in solution, in which case the stock solution may be prepared up to two days in advance.

For substances which are poorly soluble in water, ultrasonic or other suitable devices may be used in the preparation of the stock solutions to aid solubilization or dispersion of the substance. Organic solvents of low toxicity to copepods (for example acetone) may be used provided that the concentration of the solvent in the final test solution does not exceed 0,1 ml/l and two series of control tests, one with no solvent, the other with the maximum concentration of solvent, are carried out at the same time as the test.

No single procedure for the preparation of stock solutions of poorly soluble substances can be recommended due to the differing nature of chemicals.

7.2.2 Preparation of test solutions

Prepare the test solutions by adding the stock solutions (7.2.1) or effluent (7.1) to the dilution water (5.2) in specified quantities so as to obtain the concentrations selected (8.1, 8.2) for the test.

If the stock solutions are prepared in deionized or distilled water, all the solutions, including the control, shall receive the same quantity of distilled or deionized water and the final salinity shall be within the range specified for the test (5.2).

It is recommended that the volume of test solution prepared be sufficient to allow determination of the dissolved oxygen concentration and pH at the start of the test (8.3) using the excess remaining after filling the test containers.

8 Procedure

8.1 Preliminary test

This test provides an approximate value of the 48 h LC50 and enables, if necessary, a range of concentrations to be selected for use in the definitive test. For this purpose, a wide range of concentrations (generally chosen in geometric progression) of the chemical substance, effluent or water sample is tested. Typically, a factor of 10 or 3,2 between concentrations and a minimum of five animals per concentration, without replication, is appropriate.

An example is given in annex A.

8.2 Definitive test

This test enables determination of the percentages of copepods which are killed by different concentrations and determination of the 24 h and 48 h LC50. Select a range of concentrations, based on the results of the preliminary test (8.1), but employing a smaller factor (typically 1,8 or 2) between concentrations. It is desirable that the concentrations selected result in two or three percentages of mortality between 10 % and 90 %.

An example of the selection of a range of concentrations is given in annex A.

For each concentration and each control, use a minimum of 20 copepods (for example, four replicates each containing five copepods). Replicate containers are recommended in order to facilitate counting of the copepods.

8.3 General procedure

Place equal volumes of the test solutions (7.2.2) into a series of test containers (6.4). The volume per container shall be such that, with the required number of copepods (8.1, 8.2), the density of copepods does not exceed 1 per 0,5 ml of solution. For *Acartia tonsa*, the recommended maximum density is 1 copepod per 5 ml of solution. For each series of tests, prepare control containers each having a volume of dilution water (5.2) equal to the volume of the test solutions. If a solvent is used to solubilize or disperse the substance, prepare a second set of control containers with the dilution water (5.2) containing the solvent at the maximum concentration used.

Before the start of the test determine, as a minimum, the dissolved oxygen concentration and pH of the dilution water (or control solution) and the pH of test solutions corresponding with the lowest and highest concentrations being tested.

Place the required number of copepods in each test container. It is recommended that the copepods be transferred to the test solutions using a pipette of sufficiently wide bore to avoid damage to the organisms. Minimize the quantity of water transferred to the test solutions.

The copepods shall not be fed during the test.

During the test, keep the vessels at a temperature of 20 °C ± 2 °C and under a 16 h/8 h light/dark photoperiod.

After 24 h and 48 h, count the surviving copepods in each container. It is recommended that a low-power microscope (6.2) is used to aid observation. Those which are showing no swimming or appendage movements within an observation period of 10 s are considered to be dead. Record any abnormal appearance or behaviour of the copepods in the test concentrations, compared with the control animals.

After counting the surviving copepods at 48 h, measure the dissolved oxygen concentration and pH of the solution in at least one test container for each concentration and control (if necessary, pour into one container the contents of the containers corresponding to this concentration, taking suitable precautions so as not to modify the dissolved oxygen content).

8.4 Limit test

The limit test (see clause 3) is carried out with 20 copepods at a single concentration of 100 mg/l or at a lower concentration which is the maximum at which the substance is soluble or is in stable dispersion under the conditions of the test.

8.5 Check of sensitivity of copepods and conformity with the procedure

Periodically, determine the 48 h LC50 (9.1) of an appropriate reference chemical, in order to verify the sensitivity of copepods representative of the animals used for testing other samples. The recommended reference toxicant is 3,5-dichlorophenol (5.3), but other chemicals (for example potassium dichromate) may be employed if a suitable historical database exists. Report the recent 48 h LC50 in the test report (bearing in mind that it represents the toxicity of this compound only and is not representative of the sensitivity of the copepod species to other substances).

If the 48 h LC50 of the reference chemical falls outside the range given in Table 1, verify the strict application of the test procedure, manner of breeding the copepods and, if necessary, use a new culture of the copepod species.

Table 1 — Expected sensitivity of copepods to 3,5-dichlorophenol

Species	3,5-dichlorophenol mg/l	
	48 h LC50	Concn. for 20 % to 80 % mortality
Acartia tonsa	0,5 to 1,5	1,0
Tisbe battagliai	1,1 to 3,5	2,3
Nitocra spinipes	1,9 to 5,7	3,8

An alternative procedure, suitable for checking the sensitivity of the copepods at more frequent intervals, is to determine the mortality at a single concentration of the reference chemical after 48 h. If the mortality is between 20 % and 80 % at the concentration given in Table 1, the sensitivity of the copepods is acceptable. If the mortality is not within this range, perform the full check described above.

The LC50 of chemicals may vary with the salinity of the medium. The values in Table 1 relate only to the recommended salinity of 29×10^{-3} to 36×10^{-3}.

9 Calculation and validity of the results

9.1 Calculation of the LC50

For each concentration, pool the data from the replicates and calculate the percentage mortality after 24 h and 48 h in relation to the total number of copepods used. Determine the 48 h LC50 (and where appropriate, the 24 h LC50) by an appropriate statistical method (probit analysis, moving average, binomial methods or graphical estimation on a Gaussian logarithmic diagram).

If the method described in this International Standard is used for chemical substances, and analyses of each concentration at the beginning of the test and during the test show that the relative standard deviation of the individual concentrations measured is not greater than 20 %, use these measured values to calculate the 24 h and 48 h LC50. If the standard deviation of the measured concentrations is greater than 20 % or the measured concentrations decline by more than 20 % over the course of the test, it may still be possible to calculate the LC50 based on the mean measured concentrations but use the data obtained with care.