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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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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.

If no reasonable estimation of the 48 h LC50 is possible, the reasons shall be investigated and, if necessary, the test repeated. In cases where the data are insufficient (or it is not required) to calculate the 48 h LC50, quote the minimum concentration corresponding to 100 % mortality and the maximum concentration corresponding to 0 % mortality.

9.2 Validity of results

Consider the results as valid if the following conditions are satisfied:

a) the dissolved oxygen concentration at the end of the test (measured as indicated in 8.3) is greater than or equal to 4 mg/l;

b) the percentage mortality of the controls is less than or equal to 10 %;

c) the toxicity of the reference chemical is within the range specified in 8.5 of this International Standard.

9.3 Expression of results

Express the 24 h and 48 h LC50 and the limits corresponding to 0 % and 100 % mortality:

— as a percentage, or in millilitres per litre in the case of effluents or waters;

— in milligrams per litre, or in micrograms per litre in the case of chemical substances.

10 Precision

Precision data from interlaboratory trials are given in annex C.

11 Test report

The test report shall refer to this International Standard and shall include the following information:

a) all data necessary for identification of the sample or the substance tested;

b) methods of preparation of the samples:

   1) for effluents or waters, the manner and duration of storage of the samples and, if necessary, the conditions in which decantation and filtration of the sample and thawing were carried out,

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

c) all biological, chemical and physical information relating to the test and not specified in this International Standard, including the species, source, age and life stage of the copepods, the culture conditions, the dilution water source and characteristics;

d) the results of the definitive test in the form of the 24 h and 48 h LC50, the method of calculation, and, where appropriate, the 95 % confidence limits; if chemical analysis of a substance has been performed, the method used and the results;

e) the minimum concentration corresponding to 100 % mortality and the maximum concentration corresponding to 0 % mortality in 24 h and 48 h;

f) any abnormal behaviour or appearance of the copepods under the test conditions;

g) any operating details not specified in this International Standard and incidents which may have affected the results.

Annex A
(informative)

Example of determination of acute lethal toxicity of a chemical substance to a marine copepod

A.1 Result of the preliminary test

Table A.1 shows the result of a preliminary test, in which five copepods were tested per concentration.

Table A.1

Concentration mg/l	Number of survivors	
	24 h	48 h
0 (control)	5	5
0,1	5	5
0,32	5	5
1,0	5	5
3,2	5	3
10	0	0
32	0	0
100	0	0

The range of concentrations over which the definitive test is to be carried out is therefore 1,0 mg/l to 10 mg/l.

A.2 Definitive test

A.2.1 Results

Results of a definitive test are shown in Table A.2, in which

T is the number of surviving copepods at each concentration;

P is the percentage mortality of the copepods at each concentration.

Table A.2

Time h	Concentration mg/l	Number of survivors				*T*	*P*
		1	2	3	4		
24	0 (control)	5	5	5	5	20	0
	1,0	5	5	5	5	20	0
	1,8	5	5	5	5	20	0
	3,2	4	5	4	5	18	10
	5,6	2	3	1	2	8	60
	10	0	1	0	1	2	90
48	0 (control)	5	5	5	5	20	0
	1,0	5	5	5	5	20	0
	1,8	5	4	5	3	17	15
	3,2	3	1	1	2	7	65
	5,6	0	0	1	1	2	90
	10	0	0	0	0	0	100

A.2.2 Determination of 24 h and 48 h LC50

The results calculated by probit analysis are:

24 h LC50 = 5,4 mg/l, 95% confidence limits = 4,5 mg/l to 6,4 mg/l;

48 h LC50 = 2,9 mg/l, 95% confidence limits = 2,4 mg/l to 3,4 mg/l.

Figure A.1 illustrates how these LC50 values may be interpolated from the graph.

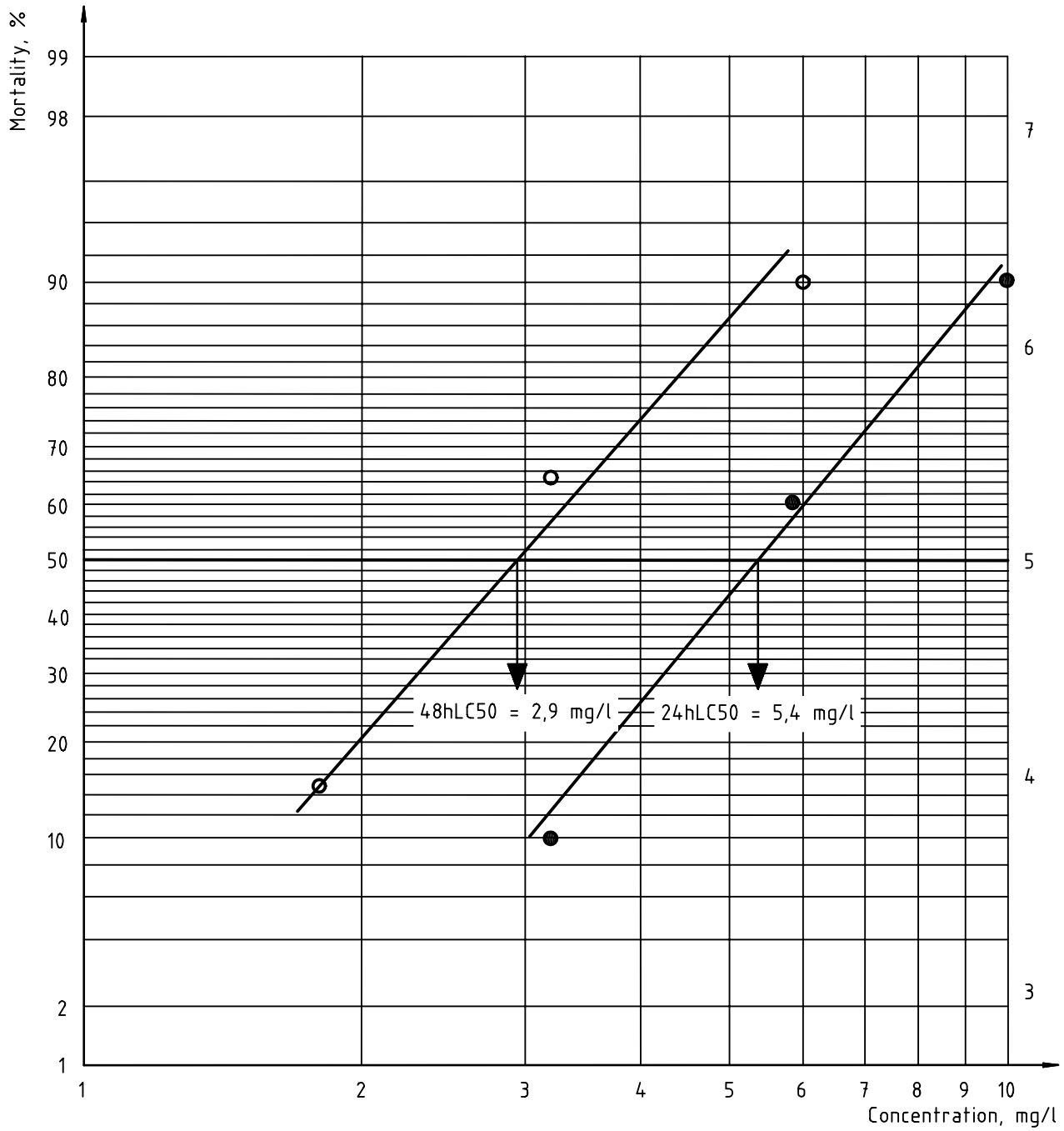

Figure A.1 — Graph of percent mortality against concentration (Gaussian logarithmic scale)

Annex B
(informative)

Culture methods for marine copepods *Acartia tonsa*, *Tisbe battagliai* and *Nitocra spinipes*

B.1 Introduction

The marine copepods *Acartia tonsa*, *Tisbe battagliai* and *Nitocra spinipes* have a wide geographic distribution, possess short lifecycles and require minimal space and equipment for culture and testing. All three species can be cultured continuously in the laboratory. Cultures shall be established using animals which have been identified reliably and preferably from cultures held in other laboratories. Identification of copepods using taxonomic keys is difficult and experts should be consulted. Taxonomic and general references for these copepods are given in the Bibliography.

Larval development proceeds through six naupliar and five copepodid stages, followed by the adult stage. At 20 °C, development from newly-hatched nauplius to adult takes 10 days to 14 days. Three or four days later, nauplius larvae are released directly from the egg-sac of the female by *T. battagliai* and *N. spinipes*; eggs, which hatch within two to three days, are released by *A. tonsa*. After fertilization, females can store sperm, enabling repeated broods of offspring to be produced from a single copulation.

B.2 Culture water

Natural or synthetic seawater (see 5.2) is used for the maintenance of stock cultures. If necessary, adjust the salinity to that required for testing (5.2).

Natural seawater should be filtered to 1 µm or less before use, to exclude indigenous algae and invertebrates. Vessels used for culturing should be fitted with loose-fitting or perforated lids to reduce evaporation but allow gas exchange. Any loss of water due to evaporation should be replaced using distilled or deionized water.

B.3 Culture conditions

Cultures are maintained at 20 °C ± 3°C in a temperature-controlled room or water bath. Provide artificial lighting at normal laboratory intensity, controlled to give a photoperiod of 16 h light:8 h dark. *N. spinipes* and *T. battagliai* should be protected from direct light. *A. tonsa* may benefit from a higher light intensity, up to 2 200 lx, at the culture surface.

Materials in contact with the cultures and culture water should be restricted to glass and unplasticized plastics.

B.4 Culture methods

B.4.1 General

The following information is intended only as guidance. For example, the volumes may need to be adjusted according to the frequency and number of tests being carried out. Also, it may be possible to increase the animal loading rate if suitable adjustments are made to the feeding rate and water renewal frequency. Although static

cultures with periodic renewal of the water are described, all three species of copepod can probably be cultured in continuous flow systems.

B.4.2 Culture of *Acartia tonsa*

B.4.2.1 Apparatus and procedure — *Acartia tonsa*

Stock cultures of *A. tonsa* are maintained in 10 l to 12 l of water. Aeration is provided centrally, near the bottom of the vessel, via a single aperture of approximately 1,5 mm diameter and controlled to give a small bubble of air about once per second. This provides gentle circulation of the water, helping to keep the algal food in suspension; the use of a spherical or round-bottomed vessel is beneficial. A number of such vessels allows cultures to be segregated by age.

The culture water is renewed at least once per week. The old water is passed through a series of different sizes of (square-weave) nylon mesh, depending on which ages of animal are to be retained or separated, as follows:

Mesh size (µm)	
60	Retains all stages
100	Retains all except eggs and 0 to 2 day old nauplii
140	Retains copepodids and adults
180	Retains adults only

Animals to be retained in culture are transferred quickly from the mesh to fresh culture water. It should be noted that eggs that have not yet hatched collect at the bottom of the vessel and can be collected by siphoning water from this point. However, these cannot easily be separated from any young nauplii that may be present, and general debris, including uneaten food, may also be collected.

For routine purposes, new cultures can be established using the offspring from adult cultures, separated at the time of the weekly water renewal, using the 180 µm and 60 µm meshes. Sequential separations over a shorter time interval can be used to provide a more closely defined age range if, for example, animals are being separated and grown on for later testing. An alternative method is to transfer separated adults to a "generation cage" [2] which allows eggs, as they are laid, to fall through a mesh into an outer container.

B.4.2.2 Culture density and management — *Acartia tonsa*

It is generally not necessary to control the density of cultures started with animals less than seven days old until the end of the first week, when the density should be reduced to approximately 25 to 50 per litre, judged "by eye". The loading should be further reduced, if necessary, to 25 per litre as the copepods reach the adult stage. Although their lifespan is longer, it is generally convenient to dispose of adults when they are five to six weeks old.

B.4.2.3 Feeding — *Acartia tonsa*

The *A. tonsa* cultures are fed a diet consisting of two species of cultured algae, *Isochrysis galbana* and *Rhodomonas reticulata*. These algae are cultured using aseptic techniques in order to exclude bacterial, algal and protozoan contaminants. Instructions for preparing culture medium for *Isochrysis galbana* and *Rhodomonas reticulata* as food for copepods are given below.

Prepare the following solutions.

a) Solution A (store in a refrigerator at approximately 4 °C)

$NaNO_3$	100,00 g
$NaH_2PO_4 \cdot 2H_2O$	20,00 g

$FeCl_3 \cdot 6H_2O$	1,30 g
$MnCl_2 \cdot 4H_2O$	0,36 g
H_3BO_3	33,60 g
$Na_2EDTA \cdot 2H_2O$	45,00 g
Trace metal solution [see c)]	1,00 ml
Distilled water to	1 litre

b) Solution B (store in a refrigerator at approximately 4 °C)

Vitamin B_{12}	0,02 g
Vitamin B_1 (Thiamine)	0,40 g
Distilled water to	1 litre

c) Trace metal solution (for preparation of Solution A)

$ZnCl_2$	2,10 g
$CoCl_2 \cdot 6H_2O$	2,00 g
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0,90 g
$CuSO_4 \cdot 5H_2O$	2,00 g
Distilled water to	100 ml

(Acidify gradually with 0,1 mol/l HCl until the solution is clear)

To 1 l of 1,0 µm filtered seawater, add 1,0 ml of Solution A and 0,05 ml of Solution B. Either add nutrients via sterilizing filter to previously autoclaved seawater, or filter-sterilize the complete medium.

Batch-culture the algae separately in round-bottomed, glass vessels containing 3 l of medium, vigorously aerated, at 20 °C ± 3 °C. Illumination is provided from "Cool White" fluorescent tubes to give an intensity of approximately 10 000 lx, with the photoperiod controlled to 16 h light:8 h dark.

Cultures are inoculated with approximately 10^7 algal cells and are harvested after 10 days when the cell density is approaching maximum (typically 10^7 cells/ml for *Isochrysis* and 5 x 10^6 cells/ml for *Rhodomonas*). The harvested algae are concentrated by centrifugation and resuspended in copepod culture water to provide a stock suspension (approximately 5 x 10^7 cells/ml for *Isochrysis*, 3 x 10^7 cells/ml for *Rhodomonas*). Algal stock suspensions can be stored for up to 1 week in a refrigerator.

After renewal of the copepod culture water, add 0,25 ml per litre of the stock suspension of each algae, to provide a total of 2 x 10^7 cells/l (1,25 x 10^7 cells/l of *Isochrysis* and 0,75 x 10^7 cells/l of *Rhodomonas*). Subsequently, the cultures are fed each day at the same level, provided that food from the previous day appears to have been cleared from the water column.

An alternative regime that has proved successful [1] employs the mixed algal diet only for the naupliar stages, with later stages receiving only *Rhodomonas* (adjust the above rate accordingly).

B.4.3 Culture of *Tisbe battagliai*

B.4.3.1 Apparatus and procedure — *Tisbe battagliai*

T. battagliai is cultured in vessels containing 2 l to 5 l of water, without aeration. A minimum of four vessels is generally necessary to allow cultures to be segregated by age and to provide a continuous supply of animals for testing.

The culture water is renewed at least once per week. The old water is passed through a series of different sizes of (square-weave) nylon mesh, depending on which ages of animal are to be separated, as follows:

Mesh size (µm)	
60	Retains all stages
100	Retains all except 0 to 2 day old nauplii
140	Retains >5 day old copepodids and adults
180	Retains late copepodids and adults
250	Retains majority of adults

Animals to be retained in culture are transferred quickly from the mesh to fresh culture water. It should be noted that copepodids and adults tend to cling to the surfaces of the vessel but can be washed onto the mesh using a gentle stream of culture water from a pipette or wash-bottle.

For routine purposes, new cultures can be established using the offspring from adult cultures, separated at the time of the weekly water renewal, using the 180 µm and 60 µm meshes. Sequential separations over a shorter time interval can be used to provide a more closely defined age range if, for example, animals are being separated and grown on for later testing.

B.4.3.2 Culture density and management — *Tisbe battagliai*

It is generally not necessary to control the density of cultures started with animals less than seven days old until the end of the first week, when the density should be reduced to approximately 100 per litre, judged "by eye". Although their lifespan is longer, it is generally convenient to dispose of adults when they are four to five weeks old.

B.4.3.3 Feeding — *Tisbe battagliai*

The cultures of *T. battagliai* are fed a diet of cultured algae. A single species, *Isochrysis galbana*, has proved successful [3], although a combination of this species with *Rhodomonas reticulata* has proved beneficial. The algae are cultured, and stock suspensions prepared, as described for *A. tonsa* (B.4.2.3).

If *Isochrysis* alone is employed, add 5 ml of algal suspension (5×10^7 cells/ml) per litre of fresh culture water, after renewal, to provide $2{,}5 \times 10^8$ cells/l. If both species are used, add 3 ml of *Isochrysis* and 2 ml of *Rhodomonas* suspension (3×10^7 cells/ml) per litre of water (providing $1{,}5 \times 10^8$ cells/l of *Isochrysis* and $0{,}6 \times 10^8$ cells/l of *Rhodomonas*).

Generally, adult cultures containing a high density of animals may require additional feeding, at the same level, one or two times per week, but only if the previous food addition has been cleared from the water. Cultures less than one week old containing nauplii and early copepodid stages will not normally require additional feeding.

B.4.4 Culture of *Nitocra spinipes*

B.4.4.1 Apparatus and procedure — *Nitocra spinipes*

N. spinipes is cultured in vessels, for example 150-ml crystallizing dishes containing 100 ml of water, without aeration. A number (approximately 15) of such vessels is necessary to allow cultures to be segregated by age and to provide a continuous supply of animals for testing.

New cultures (at least three) should be started every week by transferring 10 to 15 females, with visible egg-sacs, to dishes containing 100 ml of fresh culture water. For new cultures, renew 50 % of the culture water after two weeks. Thereafter, renew 50 % of the water at least once per week.

The adult copepods, without egg-sacs, to be used in the test should be taken from cultures that are three to four weeks old.

B.4.4.2 Culture density and management — *Nitocra spinipes*

It is generally not necessary to control the density of animals in the cultures.

Although their lifespan is longer, it is generally convenient to dispose of adults when they are four to five weeks old.

B.4.4.3 Feeding — *Nitocra spinipes*

N. spinipes is fed a diet of finely ground commercial salmon food. Small pellets, diameter about 1 mm, of salmon starter food are dried at 50 °C for 24 h. After cooling, the food is ground in a mortar and then transferred to glass tubes with screw-cap lids. These tubes are sterilised by heating at 120 °C for 24 h. Store the salmon food before and after treatment in a freezer.

To new cultures (100 ml), add approximately 20 mg of food, together with about 1 ml of the bottom deposits from an old culture. Subsequently, add approximately 10 mg per week. It is important not to give too much salmon food since oxygen depletion may occur, causing a deterioration of the culture conditions.

Annex C
(informative)

Precision data

Results from an interlaboratory test in 1991 organized by the Paris commission, published data [8] and data submitted subsequently by participating members were evaluated in accordance with ISO 5725-2. The results are summarized in Table C.1.

Table C.1 — Precision data

Chemical and species	Number of laboratories	Number of results used	Mean LC50	Standard deviation			
				Repeatability		Reproducibility	
			mg/l	Absolute	Relative %	Absolute	Relative %
3,5-DICHLOROPHENOL							
Acartia tonsa	7	14	0,95	0,22	23	0,55	58
Tisbe battagliai	4	16	2,25	0,82	36	1,07	48
POTASSIUM DICHROMATE							
Nitocra spinipes (salinity 7×10^{-3} to 9×10^{-3})	11	61	18,7	4,38	23	7,85	42
Nitocra spinipes (salinity 15×10^{-3} to 16×10^{-3})	4	23	34,6	7,61	22	16,2	47
NOTE Data for *Acartia tonsa* and *Tisbe battagliai* are for 48 h. Data for *Nitocra sinipes* are for 96 h.							

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