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Water quality — Calanoid copepod early-life stage test with *Acartia tonsa*

Qualité de l'eau — Essai aux premiers stades de la vie de copépodes calanoïdes avec Acartia tonsia

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ISO copyright office
Ch. de Blandonnet 8 • CP 401
CH-1214 Vernier, Geneva, Switzerland
Tel. +41 22 749 01 11
Fax +41 22 749 09 47
copyright@iso.org
www.iso.org

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Foreword

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The committee responsible for this document is ISO/TC 147, *Water quality*, Subcommittee SC 5, *Biological methods*.

Water quality — Calanoid copepod early-life stage test with *Acartia tonsa*

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

1 Scope

This International Standard specifies an early-life stage test procedure for determination of the toxic effects of a chemical substance, effluent, or water sample on a cold-water marine and brackish water copepod species under semi-static conditions. The biological endpoints include survival and development of the early-life stages. The exposure starts with eggs and is continued until emergence of juvenile stages.

Copepods occur widely in marine, brackish, and fresh water ecosystems. They represent important prey items for the larvae of many fish and larger invertebrates and are increasingly used as a live food source in aquaculture. They feed on phytoplankton and, thus, are an ecologically important energy-transfer link between primary producers and higher trophic levels.

2 Normative references

There are no normative references cited in this document.

3 Terms and definitions

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

3.1
EC
effect concentration

3.2
EC_x
calculated concentration from which an effect of x % is expected

3.3
larval development ratio
LDR
fraction of animals that have turned into a copepodite stage compared to the total number of surviving nauplii and copepodites within a given period of time (5 d to 6 d)

3.4
lowest observed effect concentration
LOEC
lowest concentration within the experimental range at which a significant effect is observed

3.5
no observed effect concentration
NOEC
tested concentration just below the LOEC

[SOURCE: ISO/TS 20281:2006, 3.18]

3.6
***x* % confidence intervals**
interval of values within which the measured or calculated value is likely to be present with a probability of *x* %

3.7
dilution water
water with defined properties (e.g. salinity) or natural seawater used for stepwise dilution of the test sample or used as control

4 Principle

The test is an early-life stage test, where the organisms are exposed to various concentrations of a chemical substance, effluent, or water sample, from the egg stage to the juvenile stages. Survival and development of early-life stages [larval development ratio (LDR)] are dependent on the investigated parameters. The total test duration is about 5 d to 6 d, which is sufficient time to investigate the development from nauplii to copepodites.

The naupliar (larval) and copepodite (juvenile) stages are morphologically distinct, and therefore, the transition from the last naupliar to the first copepodite stage is easily observed. Larval development ratio (LDR) is recorded after 5 d to 6 d, when about 60 % of the control animals have reached a copepodite stage, and is expressed as the ratio of copepodites to the total number of surviving early-life stages (nauplii + copepodites) at the end of the LDR test. Hatching success and mortality of the early stages should be presented along with the LDR.

The outcome of the test is either the no observed effect concentration and the lowest observed effect concentration (NOEC-LOEC) values or the effect concentrations with a certain degree (*x* %) of inhibition (EC_x) (e.g. EC_{50} and EC_{10}).

5 Apparatus

Test vessels and other apparatus, which will come into contact with the dilution water and test solutions, should be made entirely of glass or other materials chemically inert to the test chemical.

5.1 Standard laboratory apparatus, e.g. measurements of pH, dissolved oxygen concentration, salinity, and temperature.

5.2 Glass flasks, volume 1 l, 2 l, and 5 l.

5.3 Air pumps.

5.4 Air filters, pore size 0,2 µm.

5.5 Peristaltic pump for food supply.

5.6 Temperature-controlled cabinet or room.

5.7 Low-magnifying stereomicroscope, preferably with dark field illumination.

5.8 Apparatus for membrane filtration.

5.9 Filters, 0,2 µm (6.2) and eventually filters with grids (8.5.1).

5.10 Nets, mesh sizes 50 µm and 180 µm to 200 µm, for isolation of eggs, for capture and transfer of animals, and as filters when medium is changed.

5.11 Adequate apparatus for the control of the lighting regime.

6 Reagents

6.1 Water

All water used in preparation of culture medium shall be clean natural seawater or deionized water or of equivalent purity. Take special care to avoid contamination of the water by inorganic or organic substances during preparation and storage.

Equipment made of copper shall not be used.

6.2 Culture and test media

Culture and test media are prepared from either reconstituted salt water or filtered (0,2 µm) natural marine water from an unpolluted location. An example of reconstituted salt water suitable for cultivation and testing is given in Annex A. Reconstituted salt water media with a known composition in which the copepods show suitable long-term survival, normal behaviour, development, and fecundity can be used as culture and test media, i.e. dilution water.

6.3 Dilution water

The salinity of the dilution water should be the same as that of the culture medium (see Annex A). The dilution water shall have a dissolved oxygen concentration above 70 % of the air saturation value and a pH of 8,0 ± 0,3 before being used to prepare the test solutions. If there is evidence of marked change of pH at the highest test concentration, it is advisable to adjust the pH of the stock solution/environmental sample to that of the dilution water before preparing the dilution series. The pH adjustment of the stock solution or test concentrations shall not change the concentration to any significant extent or lead to chemical reaction or precipitation of the test substance. HCl and NaOH are preferred for pH adjustments.

If the physical conditions or the salinity of the salt water to be used in the test differ more than 5 °C in temperature or 10 ‰ salinity from those used for routine culturing, it is good practice to include an adequate pre-test acclimation period at the same temperature (20 ± 1) °C and salinity (20 ± 2) ‰ of 2 to 3 weeks to avoid stressing the eggs and animals. Use of another temperature or salinity, which can be more appropriate in oceanic or brackish water situations, shall be justified in the test report.

7 Test organism

The species to be used is the marine calanoid copepod *Acartia tonsa* Dana (see Annex G and Annex H).

Eggs used in the test should be collected from a healthy stock (i.e. showing no signs of stress such as high mortality, poor fecundity, etc.). The stock animals shall be maintained in culture conditions (light, temperature, medium, and feeding) similar to those to be used in the test (culturing method for *A. tonsa* is described in Annex G).

8 Procedure

8.1 Preparation of culture medium

Natural seawater or a reconstituted medium can be used. A suitable reconstituted culture medium is described in Annex A. However, alternative reconstituted media can be used as long as the validity criteria for the test are met (see Clause 9). The defined medium described in Annex A contains a chelating agent and therefore, might not be appropriate for testing of samples that contain metals. The salinity can be varied by choosing a desired amount of the 10 % salinity solution. The salinity of natural seawater and environmental samples can be raised by using the same 10 % salinity solution (Table A.1) or lowered by adding an appropriate volume of M7 (see Reference [1] and Annex A) or deionized water.

8.2 Choice of test concentrations

Prior knowledge of the toxicity of the test substance (e.g. from an acute test[1]) or from range-finding studies) should help in selecting appropriate test concentrations. As a rule of thumb, the highest concentration in the early-life stage test should be chosen within the interval between LC_{10} and LC_{20} of the acute 48 h test to avoid significant effect on survival.

At least 5 different concentrations should be tested in a geometric series with a factor between concentrations not exceeding 3,2. Justification should be provided if fewer than five concentrations are used. Substances should not be tested above their solubility limits in dilution water. A dilution water control shall be included and, if a solvent is used, a solvent-control shall also be included (8.3), containing the same concentration of solvent as the test series.

If there are substantial reason to assume that a high concentration of a chemical or an environmental sample will have low/no toxicity at a high concentration (e.g. at 10 mg/l or 100 ml/l), the early-life stage test can be performed as a limit test, using a test concentration of, for example, 10 mg/l (or 100 ml sample/l) and the control. The usual number of replicates should be used for both the treatment and the control groups. A limit test can show that there is no statistically significant effect at the limit concentration when compared to the controls, but if significant effects are recorded, a full test will normally be required.

8.3 Preparation of test substance stock solution

The test solutions are usually prepared by diluting a stock solution of a test chemical or of an environmental sample with dilution water. Stock solutions of chemicals shall be prepared by dissolving the substance in dilution water. The preferred options for preparing test solutions are physical methods, such as stirring and sonication.[2][3] Saturation columns (solubility columns) can be used for achieving a suitably concentrated stock solution.

The use of organic solvents might be required in some cases in order to produce a suitably concentrated stock solution, but every effort should be made to avoid the use of such carrier solvents. The only recommended solvent for this test is acetone. Acetone shall be used to produce a stock solution that can be dosed accurately into water. The recommended maximum acetone concentration in the dilution water and test solutions is 0,01 ml/l. The concentration shall be the same in all test vessels. If another solvent or a higher acetone concentration is used, it shall be documented that it has no effects. Acetone will not be toxic at 0,01 ml/l and will not increase the water solubility of a substance. Acetone might be essential in handling some substances; for example, for preparing stock solutions of hydrolytically unstable or highly viscous substances.[4]

8.4 Preparation of test solutions

In the LDR test, the control should comprise of at least 10[1)] control replicates and preferably more, and as a minimum 6 replicates of each test concentration. The demand for replicates is higher if the ANOVA statistic is used whereas regression analysis generally demands more concentrations.

The number of replicates depends on the statistical endpoint (ANOVA or EC_x). When planning the test, it should be taken into consideration whether the aim is to achieve a NOEC/LOEC (by use of ANOVA) or an EC_x value (by use of regression technique).

For the use of ANOVA technique or regression analysis, see Reference [5].

In setting the range of concentrations, the following should be borne in mind:

If the aim is to obtain the NOEC, the lowest test concentration shall be low enough so that the biological endpoint at that concentration is not significantly different from that of the control. If this is not the case, the test will have to be repeated with a reduced lowest concentration. If the aim is to obtain the NOEC, the highest test concentration shall be high enough to cause a statistically significant effect when compared to the control on the biological endpoint. If this is not the case, the test will have to be repeated with an increased highest concentration.

If EC_x for effects on development is estimated, it is optimal that the lowest concentration has no effects (optimally the only one without effects), and the highest concentration is greater than EC_{50}, and that sufficient concentrations are used to define the EC_x with the appropriate level of confidence. If the highest concentration is below the EC_{50}, it is recommended also to report the EC_{10} and/or the NOEC/LOEC values.

The range of test concentrations should preferably not include any concentrations that have a significant effect on survival since the main objective of the test is to measure sublethal effects (e.g. development).

8.5 Incubation/Exposure

A recommended schedule for an early-life stage test is given in Annex B.

8.5.1 Test organisms and loading

Fresh eggs produced by the copepod stock culture are preferred but eggs stored for a maximum of one week at 4 °C can be used (see Table B.1). 60 to 90 eggs are counted by use of a stereomicroscope and added to the test solution in each test vessel. Eggs shall be counted individually on a filter with grids or in a drop of water (filters and water drops can be placed in a Petri dish with marked graduations) and after counting be flushed into the test vessel (see Table B.1).

Newly hatched nauplii present at counting can be crushed with a preparation needle while counting the eggs or if added together with the eggs, the nauplii shall be counted as well. The number of newly hatched nauplii shall not exceed 5 % of the number of added eggs.

A data collection sheet suitable for holding the recorded data are given in Annex E.

8.5.2 Control of hatching

Complementary to the LDR control vessels, an additional control for hatching may be set up. Four replicates with 60 to 90 eggs in 40 ml to 80 ml (same volume as in test replicates – see 8.5.3) of dilution water are started at the same time as the LDR test with eggs from the same batch (see Table B.1). This hatching control will only run for 2 d to 3 d and, at that time, the number of larvae and unhatched eggs are counted to check the hatching percentage.

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1) It is recommended to start with at least 12 control replicates since some will probably be used for inspection of the development at a time where the LDR is below the 60 % ±20 % criterion for the copepodite fraction. To find the optimal time to terminate the LDR test 1 to 2, controls are taken and counted at regular intervals when it is expected that the LDR approaches 60 %.

8.5.3 Larval development ratio (Early-life stages development)

Eggs (60 to 90) are exposed in each replicate vessel holding the same volume of test solution (40 ml to 80 ml). The exact number of eggs (and newly hatched nauplii) added is recorded. Test solutions are renewed on day 2 or 3 or the volume is increased by adding new test solution using the principle from Footnote 3 (see also Annex B, Table B.1). Observation of the larval development is normally recorded after 5 d to 6 d, when about 60 % of the control animals have reached a copepodite stage, and is expressed as the ratio of copepodites to the total number (sum) of larvae (nauplii) and juveniles (copepodites) alive at that point of the test. Animals dying during the test disappear quickly and all animals counted at the end of the test are assumed to be alive when Lugol's solution is added (see 8.6 and Annex D). Additional replicates of the controls should be prepared to catch the most optimal time for stopping the test as close as possible to the 60 % copepodite ratio (see Footnote 1). Mortality (animals dead and missing during the test) should be presented along with the LDR (see Annex F for calculation examples).

8.5.4 Duration

Time needed to complete (at 20 °C and 20‰ salinity) larval development test is 5 d to 6 d. At lower temperatures or higher salinities, the development might be slower and, thus, testing at these conditions might last longer. See, for example, Reference [6] which presents a study of the length of time elapsed until 50 % of the early-life stages reach a copepodite stage at different salinity and temperature regimes.

8.5.5 Handling of test vessels

Handling of the test vessels should be done in a random fashion. Failure to do this may result in bias that could be construed as being a concentration effect. In particular, if experimental units are handled in treatment or concentration order, some time-related effect, such as operator fatigue or other error, could lead to greater effects at the higher concentrations. Care should be taken that environmental conditions, such as position in the laboratory, are uniform for all test vessels independently of their physical position in the test setup. It is also important to stress that the time given for each replicate to develop is the same for all the replicates.

8.5.6 Feeding

5.0×10^4 cells · ml^{-1} of *Rhodomonas salina* should be added at the start of the test and at renewals or addition of new test solution (see 8.5.9 and Table B.1). Deviations from this should be reported. When using small volumes of test solution in semi-static tests, it is important to consider the volume of food fed and the dilution of the exposure concentration. Food should be added at a volume that does not exceed 1 % of the total volume. Specific details of the feeding regimes are given in Table B.1.

8.5.7 Light and temperature

Specific details of the light and temperature regimes to be used are described in Annex G. A photoperiod of 16:8 h light:dark is recommended at a low light intensity (5 µmol to 10 µmol · s^{-1} · m^{-2}). The temperature used shall be 20 °C ± 1 °C during the entire exposure period.

8.5.8 Aeration

If aeration is necessary to keep dissolved oxygen concentration (DO) > 70 % of the air saturation value (ASV) (see Clause 9), test vessels should be aerated as little as possible to avoid evaporation of water and stripping of test chemicals.

8.5.9 Dilution water and test solutions renewal or addition

The frequency of partial test solution renewal or addition will depend on the stability of the test substance, but should be at least once during a 5 d to 6 d test, and at least every 2 d to 3 d, if the duration is longer. If, from preliminary stability tests or from the physico-chemical properties of the test substance, concentration is evaluated not to be stable (i.e. outside the range 80 % to 120 % of nominal or falling below 80 % of the measured initial concentration) over the maximum renewal period (i.e. 2 d to 3 d),

consideration should be given to more frequent test solution renewals. There shall be evidence that the concentration of the test substance has been satisfactorily maintained (see 8.6).

When the test solution is renewed, the following are the different ways to do this:

— part (50 % to 80 %) of the old test solution can be replaced by fresh test solution[2];

— the volume can be increased gradually by adding fresh test solution[3].

Another method could be to prepare a series of test vessels with fresh test solution and transfer the animals to them by, for example, having an inner chamber supplied with fine net of suitable mesh size as a bottom.

8.6 Measurements/Observations

In the LDR test, numbers of unhatched eggs, nauplii (larvae), and copepodites (juveniles) shall be recorded at the end of the exposure period. The animals and unhatched eggs are fixed in Lugol's solution and studied (counted, measured, etc.). Since Lugol's solution may also oxidize the test chemical, samples for chemical analysis shall be taken before addition of Lugol's solution and preferably from separate vessels prepared for this purpose only (Annex D). Staining (and killing off all animals) in Lugol's solution (see Annex D) will facilitate counting of animals and unhatched eggs. Counting of different developmental stages of *A. tonsa* needs to be facilitated by the use of a stereomicroscope.

Observations made during the test should be recorded on data collection sheets. Examples are provided in Annex E.

8.6.1 Concentration of the test substance

During the test, the concentrations of test substance should be determined at regular intervals. It is recommended that, as a minimum, the highest and lowest test concentrations are analysed when freshly prepared - at the start of the test and immediately prior to renewals and at the end of the test (i.e. analyses should be made on samples from the same concentration – when freshly prepared, at renewal and at the end). To avoid biological materials and Lugol's solution in the samples for chemical analysis, it is recommended to set up three extra vessels of each concentrations for sampling purposes (without organisms and food); one to be harvested before first renewal or addition of water, another one to be harvested after first renewal or addition of water, and the last one to be harvested at the end. At the start, samples for analysis are achieved from the same portions as used to start the test. See Annex C.

If there is evidence that the concentration of the substance being tested has been satisfactorily maintained within ±20 % of the nominal/measured concentration throughout the test, then results can be based on nominal or measured values. If the deviation from nominal or measured concentration is greater than ±20 %, results should be expressed in terms of the time-weighted mean (see guidance for calculation in Annex F).

For tests, in which the concentration of the test substance is not expected to remain within ±20 % of the nominal concentration, it is necessary to sample all test concentrations (including control) when freshly prepared and at renewal. After finalizing the tests, at least samples with nominal concentrations close to EC_{10}, EC_{50}, and NOEC are analysed. In these cases, calculations of effect concentrations are based on the measured concentrations, and results should be expressed in terms of the time-weighted mean (see guidance for calculation in Annex F). Note that care should be taken when testing very lipophilic (i.e. log $K_{ow} > 5$) and hence, poorly water-soluble substances in the present test system (see Reference [3]). Using radiolabelled substances may give crucial information on the partitioning in the test system, which may facilitate the calculation of the actual concentrations.

2) Test solution can be removed with a siphon supplied with a net with an appropriate mesh size to avoid removal of animals or eggs from the test vessel.

3) Fresh test solution can be added by increasing the volume in the test vessels gradually from (for example) 40 ml at the start to 80 ml on day 2 or 3.