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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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Foreword

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The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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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.

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.

8.6.2 Physical-chemical parameters – oxygen, pH, salinity, and temperature

Dissolved oxygen, pH, salinity, and temperature should be measured in the control and all test concentrations at start and end of test, and each time dilution water is renewed. As a minimum, these measurements shall be made in the control and the highest test concentration. Temperature should preferably be monitored continuously. Additional test vessels (including animals and food) may be set up for this purpose only.

9 Validity criteria

For a test to be valid, the following performance criteria should be met:

— the dissolved oxygen concentration must have been at least 70 % of the air saturation value (ASV) throughout the exposure period;

— temperature should vary no more than ±1 °C during the entire test period;

— control pH shall not vary more than 1,0 unit from the initial control pH;

— conductivity/salinity shall not vary more than 10 % from the control start value [e.g. (20 ± 2) ‰];

— hatching success in the control shall be ≥75 %;

— in LDR tests, the average control copepodite fraction (LDR) shall be (60 ± 20) % of the counted animals at the end of the exposure;

— in LDR tests, average surviving percentage of animals in the control(s) on the day of observation of LDR shall be ≥70 % of the hatched animals;

— the EC_{50} of the reference compound 3,5-Dichlorophenol (3,5-DCP) shall be within the range of 500 µg/l ± 300 µg/l for tests performed at 20 °C and 20 ‰ salinity.

10 Expression of results

One-way nested analysis of variance (ANOVA) or regression analysis technique is used to evaluate effects of the test substance on the development of the copepods. Analysis of variance is a parametric procedure and is based on the assumptions that the observations are independent and normally distributed and that the variance of the observations is homogeneous across all toxicant concentrations and the control. These assumptions should be checked prior to using ANOVA, to determine if they have been met. Tests for validating these assumptions are Shapiro-Wilk's test for normality and Bartlett's Test for homogeneity of variance.[7] If the data do not meet the assumptions for ANOVA, non-parametric procedures such as Steel's Many-One-Rank Test or Wilcoxon's Rank Sum Test[7] might be more appropriate. Different programs are available for performing linear or nonlinear regression assuming a logarithmic normal distribution, a Weibull distribution, or a logit distribution of data.

If a limit test (comparison of control and one treatment only) has been performed and it fulfills the prerequisites of parametric test procedures (normality, homogeneity), the responses of the two groups can be evaluated by the Student test (*t*-test). If the prerequisites for the *t*-test are not fulfilled, an unequal variance *t*-test (such as Welch test[8]) or a non-parametric test such as the Mann-Whitney-U-test[9] can be used. A comparison of the control and the solvent control can be performed in the same way.

If a statistically significant difference in survival or development is detected between the control and solvent control, only the solvent control is used as the basis for the calculation of results. If no significant differences exist between control and solvent control data, these can be pooled for comparison with test substance treatments.

11 Denotion of results

Denote concentrations causing 10 % and 50 % inhibition based on a concentration-response curve as EC_{10} and EC_{50}, respectively. Quote EC_{10}, EC_{50}, NOEC, and LOEC values to two significant digits, normally in milligrams per litre (mg/l) for chemicals or in ml sample/l for environmental samples.

12 Interpretation of results

EC_{10}, EC_{50}, and NOEC values are toxicological data derived from a laboratory experiment carried out under defined conditions. They give an indication of potential hazard of the toxicant/environmental sample, but cannot be used directly to predict effects in the natural environment (see References [10] and [11]).

13 Reference substance

A reference substance (e.g. 3,5-Dichlorophenol, purity ≥99 %) can be tested periodically as a means of assuring that the test protocol and test conditions are reliable. Recommended concentrations to test at 20 °C and 20 ‰ salinity are 0 µg/l, 100 µg/l, 200 µg/l, 400 µg/l, 800 µg/l, and 1 600 µg/l.

14 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, i.e. ISO 16778:2015;

b) all information required for the complete identification of the sample or of the test substance under test including the following methods for preparation of the test samples:

   1) for effluents, waters, and aqueous extract, the method and storage time of samples etc.;

   2) for chemical substances, the method of preparation of stock and test solutions, including the following:

      — relevant physico-chemical properties;

      — chemical identification data (name, structural formula, CAS number, etc.) including purity;

      — analytical method for quantification of the test substance where appropriate;

c) the test species:

   — supplier or source (if known) and the culture conditions used;

d) the following test conditions:

   — exposure procedure used (e.g. semi-static);

   — photoperiod and light intensity;

   — test design (e.g. test concentrations used, number of replicates, number of organisms per replicate, etc.);

   — method of preparation of stock solutions and frequency of renewal (the solvent carrier and its concentration shall be given, when used);

   — the nominal test concentrations, the means of the measured values and their standard deviations in the test vessels and the method by which these were attained and evidence that the measurements refer to the concentrations of the test substance in true solution;

   — test solution characteristics (including pH, salinity, temperature, dissolved oxygen concentration, and any other measurements made);

— detailed information on feeding (e.g. type of food, source, amount given, frequency of feeding);

— analyses for relevant contaminants in water (e.g. metals, PCBs, PAHs, and organochlorine pesticides);

e) the following test results:

— results from any preliminary studies on the stability of the test substance;

— the nominal test concentrations and the results of all chemical analyses to determine the concentration of the test substance in the test vessels; the recovery efficiency of the analytical method and the limit of detection should also be reported;

— water quality within test vessels (i.e. salinity, pH, temperature, and dissolved oxygen concentration);

— a full record of all the biological effects, observed or measured, and the statistical techniques used to analyse the data;

— the lowest observed effect concentration (LOEC) and no observed effect concentration (NOEC) for all biological endpoints employed or EC_xs and statistical methods used for their determination;

— other observed effects;

— explanation of any deviation from this International Standard.

Annex A
(informative)

Defined culture and test media

Table A.1 — Composition of 10 % salinity reconstituted salt water

10 % salinity salt water	
Substance	g/l
NaCl	70,100
Na_2SO_4	11,700
KCl	2,030
KBr	0,293
$Na_2B_4O_7,10H_2O$	0,113
$MgCl_2,6H_2O$	31,700
$CaCl_2,6H_2O$	6,600
$SrCl_2,6H_2O$	0,066

Table A.2 — Bicarbonate stock solution for salt water medium

	g/l	ml stock/l	Final concentration in salt water medium mg/l
$NaHCO_3$	2,83	1,0	2,83

$NaHCO_3$ added directly to the salt water medium as addition to the 10 % salinity water will cause precipitations.

Table A.3 — Trace stock solution for M7 medium - Prepared from Stocks No 1 to No 14

Trace element stock solutions					
Stock No	Trace element compounds	In stock 1 to 14 respectively mg/l	For trace stock ml/l	In trace stock µmol	In final M7 medium µmol
1	H_3BO_3	14 297,50	1,00	231,23	11,56
2	$MnCl_2,4H_2O$	1 802,50	1,00	9,11	0,46
3	LiCl	1 530,00	1,00	36,09	1,80
4	RbCl	355,00	1,00	4,15	0,21
5	$SrCl_2,6H_2O$	760,00	1,00	2,85	0,14
6	NaBr	80,00	1,00	0,78	0,039
7	$Na_2MoO_4,2H_2O$	315,00	1,00	1,30	0,065
8	$CuCl_2,2H_2O$	83,75	1,00	0,49	0,025
9	$ZnCl_2$	260,00	1,00	1,91	0,095
10	$CoCl_2,6H_2O$	200,00	1,00	0,84	0,042

[a] Autoclaved immediately.

Table A.3 *(continued)*

Trace element stock solutions					
Stock No	**Trace element compounds**	**In stock 1 to 14 respectively**	**For trace stock**	**In trace stock**	**In final M7 medium**
		mg/l	ml/l	µmol	µmol
11	KI	65,00	1,00	0,39	0,020
12	Na_2SeO_3	43,80	1,00	0,25	0,013
13	NH_4VO_3	11,50	1,00	0,10	0,004 9
Combined Fe-EDTA solution					
14 [a]	$Na_2EDTA,2H_2O$	625,00	20,00	33,58	1,68
	$FeSO_4,7H_2O$	250,00		17,98	0,90
[a] Autoclaved immediately.					

The 14 different stock solutions are prepared and from these, a trace element stock solution is prepared.

Table A.4 — Final M7 medium

Trace stock solution (mixture of stock 1 to 14)		ml/l for M7 50,00		
Stock No	Macro nutrients	In stock mg/l	For M7 ml/l	In final M7 medium mmol
15	$CaCl_2,2H_2O$	29 380,00	10,00	1,998 4
16	$MgSO_4,7H_2O$	24 660,00	5,00	0,640 8
17	KCl	5 800,00	1,00	0,778 0
18	$NaHCO_3$	64 800,00	1,00	0,771 4
19	$NaSiO_3,9H_2O$	10 000,00	1,00	0,019 2
20	$NaNO_3$	2 740,00	0,10	0,003 2
21	KH_2PO_4	1 430,00	0,10	0,001 1
22	K_2HPO_4	1 840,00	0,10	0,001 7
Combined vitamins [b]				
23 [a]	Thiamine hydrochloride	750,00	0,10	
	Cyanocobalamine	10,00		
	Biotine	7,50		
[a] Combined vitamin stock is stored frozen at –20 °C in small aliquots.				
[b] Vitamins are added to the medium shortly before use.				

Annex B
(informative)

Specific details on renewal of test solution, feeding regime, and an example of a flow diagram for a larval development test (early-life stage test) with *Acartia tonsa*

B.1 Renewal of test solution

B.1.1 Larval development test - LDR (early-life stage test)

50 % to 80 % renewal on day 2 or 3 or increase of volume by adding fresh test solution.

B.1.2 Hatching control

No renewal of test solution.

B.2 Feeding regime

B.2.1 Larval development test - LDR (early-life stage test)

5,0 × 10^4 cells (*Rhodomonas salina*) per ml of test solution are added on day 0 and on day 2 or 3.

B.2.2 Hatching control

5,0 × 10^4 cells (*Rhodomonas salina*) per ml of test solution are added on day 0.

For details, see Table B.1.

Table B.1 — Recommended schedule for the early-life stage test – LDR test

	t = (Day)	***Rhodomonas* cells/ml in total volume**	**Organism**	**Test solution added** ml	**Total volume** ml	**Handling**
Harvest of eggs	–1	-	Eggs are harvested from the basic exposure cultures	-	-	Egg production for the test to be started on a Friday: Adult *Acartia* are filtered from the culture (14 d to 21 d old) through a 200 µm mesh. Added to 2 to 3 beakers with 500 ml to 800 ml fresh medium. *Rhodomonas* is added in surplus (until pink). Placed at culture conditions (20°C, light/dark cycle 16:8 h and gentle aeration). This is preferably done in the afternoon to prevent too many eggs from hatching before the test starts.

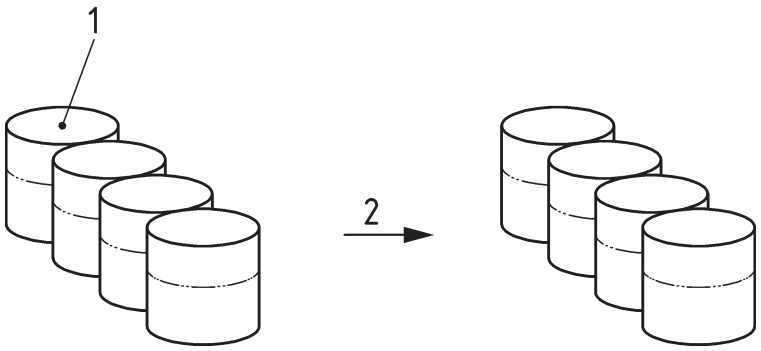
Table B.1 *(continued)*

	t = (Day)	***Rhodo-monas* cells/ml in total volume**	**Organism**	**Test solution added** ml	**Total volume** ml	**Handling**
Start (Friday)	0	50×10^3	60 to 90 eggs/vessel (incl. any newly hatched nauplii)	-	40	**Preparation of egg suspension** Females are removed by filtering through a 200 µm mesh. To concentrate the eggs, filter through a 50 µm mesh. Suspend in a small volume of sea water. The egg suspension is kept at approx. 15 °C. **Test start** Prepare the test solutions from a freshly prepared stock solution. Measure pH, oxygen, and salinity in all the test solutions. Eggs are counted either in a Petri dish or on a filter (both with gridlines). If using a filter, nauplii might be killed with a preparation needle while counting the eggs. Flush the eggs from the dish or filter into the test vessels with test solution. Make a note of the starting time as it is important that all test vessels are given the exact same time to develop. 12 controls and six vessels of each test concentration are prepared for the test and an extra vessel (with eggs – but not counted) is likewise prepared for pH, oxygen, temperature, and salinity measurements during the test. **Chemical analysis** Three additional vessels of the control and of each test concentration are prepared (without eggs). Sample of the stock solution, the control, and of each test solution are collected and frozen. **Hatching control** Four vessels with 40 ml of control solution are prepared for checking the hatching percentage after 3 d.

Table B.1 *(continued)*

	t = (Day)	***Rhodomonas* cells/ml in total volume**	**Organism**	**Test solution added** ml	**Total volume** ml	**Handling**
Monday	3	50×10^3	-	40	80	**Renewal or addition of fresh test solution** Prepare the test solutions from a freshly prepared stock solution. New test solution/algae are added. (Note: no addition of algae to the vessels for chemical analysis) pH, oxygen, temperature, and salinity are measured in old and new test solutions. **Chemical analysis** Sample of the stock solution. Samples of the control and of each test concentration are collected before and after addition of the new test solution. **Hatching control** The number of unhatched eggs and nauplii are counted in all four vessels after adding Lugol's solution. If the hatching percentage ≥ 80, the test will continue.
Wednesday	5	-	-	-	-	**Finalizing the test** One or two controls are counted Lugol's solution (1 % volume fraction) is added – the content of the vessel is filtered – and the numbers of unhatched eggs, nauplii, and copepodites on the filter are counted under a stereomicroscope. If the copepodite fraction is below 0,6 (less than 60 % copepodites), the test is allowed to run for 2 to 3 more hours before the next two controls are examined. If the copepodite fraction is close to or above 0,6, Lugol's solution is added to the test vessels at time intervals in such a way that vessels are stopped when they have run the exact same time. pH, oxygen content, temperature, and salinity are measured in the test solutions. **Chemical analysis** Samples of the control and of each test concentration are collected.

B.3 Schematic flow of preliminary and definite early-life stage test with *Acartia tonsa*

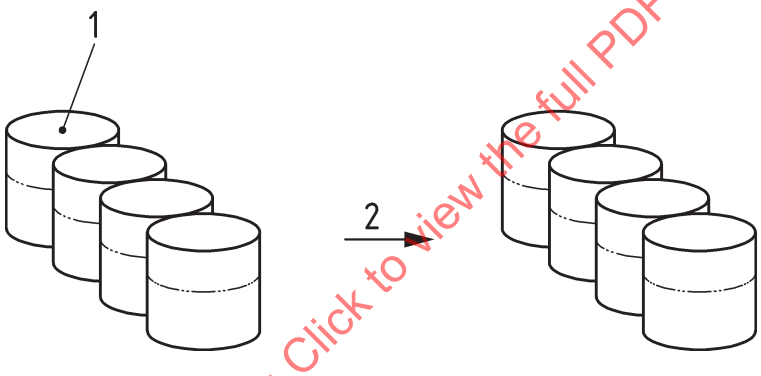


Key

1 5 animals into each

2 48 h

Figure B.1 — Acute test (as preliminary test for early-life stage test)



Key

1 60 to 90 eggs into each

2 5/6 days

Figure B.2 — F_0 generation larval survival and development test

Annex C
(informative)

Sampling for chemical analyses

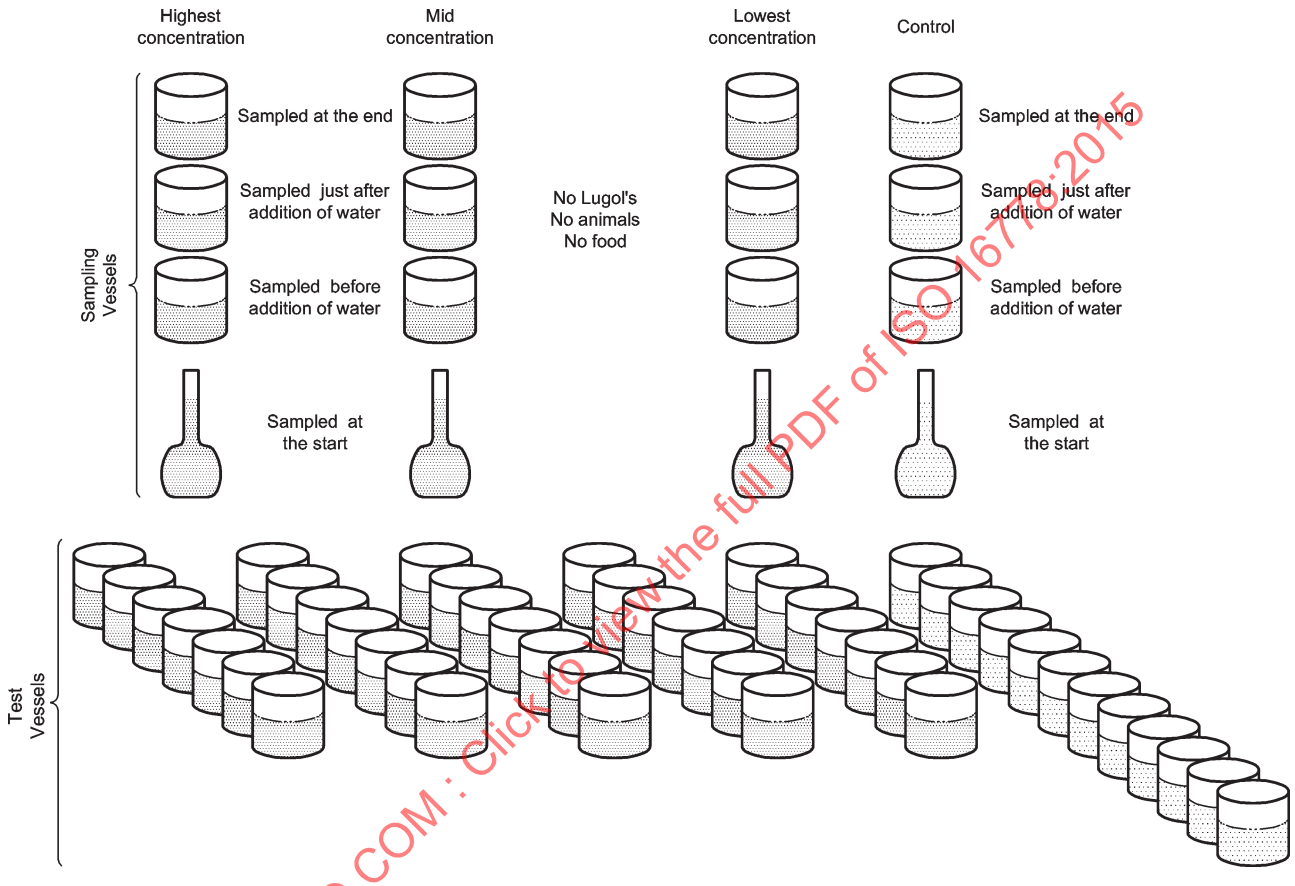


Figure C.1 — Sampling for chemical analysis

Sampling:

Step 1) At the start of the test (e.g. taken from the measuring flasks).

Step 2) Before addition of fresh test solution after 2 d or 3 d (e.g. from a "sampling replicate" which is discharged after use).

Step 3) Just after addition of fresh test solution after 2 d to 3 d (e.g. from a "sampling replicate" – do not dispose of this "sampling replicate" as it is to be used again in Step 4).

Step 4) At the end of the test (e.g. from the same "sampling replicate" as used in Step 3).

A low concentration, a concentration around EC_{50} (if possible), and a high concentration are sampled.

Annex D
(informative)

Lugol's solution

KI	100 g/l
I_2	50 g/l
Trichloro-acetic acid	100 g/l

Added to the test vessels at a concentration of approximately 1 % volume fraction.

WARNING — Lugol's solution is oxidative and samples of test solution for chemical analysis shall be taken before addition of Lugol's solution.

Different Lugol's solution products are available as commercial products on the market.

Annex E
(informative)

Data collection sheet for *Acartia tonsa* early-life stage test

TEST COMPOUND: Concentration:	Laboratory: Study director: Technician:	Date: (Start of test – Day 0)

Time schedule Day No. (after start) pH O_2 (mg /l)

	Start: No. of eggs	**End:** Unhatched eggs	Nauplii	Copepodites	Naup.+ Cop.	LDR	ELS mortality
Replicate No. 1							
Replicate No. 2							
Replicate No. 3							
Replicate No. 4							
Replicate No. 5							
Replicate No. 6							
Replicate No. 7							
Replicate No. 8							
Replica te No.							
Replicate No.							
Example	43	7	17	16	33	16/(17+16)= 0,485	1 - (17+16)/(437) = 0,083

Annex F
(informative)

Calculations

Hatching success (in %):

$$\frac{(\text{Number of eggs at start} - \text{Number of unhatched eggs}) \times 100\,\%}{\text{Number of eggs at start}} \quad \text{(F.1)}$$

Larval development ratio:

$$\frac{\text{Number of copepodites}}{(\text{Number of nauplii} + \text{Number of copepodites})} \quad \text{(F.2)}$$

Early-life stage mortality:

$$1 - \frac{\text{Number of nauplii} + \text{Number of copepodites}}{(\text{No. of eggs at start - No. of unhatched eggs})} \quad \text{(F.3)}$$

Relative response:

$$\frac{X_{\text{control}} - X_{\text{test concentration}}}{X_{\text{control}}} \quad \text{(F.4)}$$

Inhibition:

$$1 - \frac{X_{\text{control}} - X_{\text{test concentration}}}{X_{\text{control}}} \quad \text{(F.5)}$$

Time-weighted concentration mean: (Reference [1])

Given that the concentration of the test substance can decline over the period between test solution renewals, it is necessary to consider which concentration should be chosen as representative of the range of concentrations experienced by the copepods. The selection should be based on biological considerations, as well as statistical ones. For example, if hatching or first nauplii stages are the most sensitive and therefore considered to be affected mostly by the peak concentration experienced, then the maximum concentration should be used. If the accumulated or longer term effect of the toxic substance is considered more important, an average concentration is more relevant. In this case, an appropriate average to use is the time-weighted mean concentration since this takes account of the variation in instantaneous concentration over time. However, we will seldom have knowledge about the sensitivity of the different stages, thus, the use of time-weighted mean concentration is generally recommended.

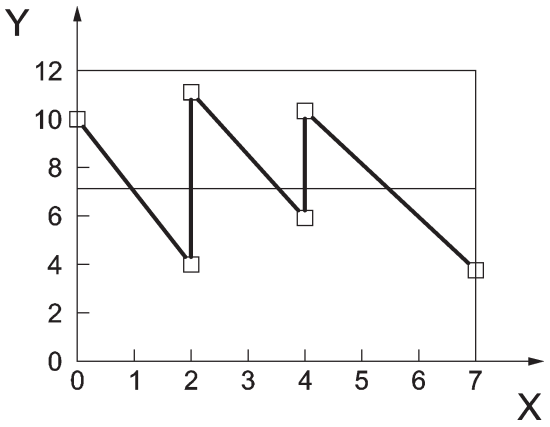
Figure F.1 shows an example of a (simplified) test, lasting 7 d with test solution renewal at days 0, 2, and 4.

Explanation to Figure F.1:

The thin zig-zag line represents the concentration at any point in time. The fall in concentration is assumed to follow an exponential decay process (first-order process).

The six plotted points represent the observed concentrations measured at the start and end of each renewal period.

The horizontal line indicates the position of the time-weighted mean.



Key

X days

Y Ln(concentration)

Figure F.1 — Example of time-weighted concentration mean

The time-weighted mean is calculated so that the area under the time-weighted mean is equal to the area under the concentration curve. The calculation for the above example is illustrated in Table F.1.

Table F.1 — Calculation example of time-weighted concentration mean (Figure F.1)

Renewal No	Days	Conc 0	Conc 1	Ln(Conc 0)	Ln(Conc 1)	Area
1	2	10,000	4,493	2,303	1,503	13,767
2	2	11,000	6,037	2,398	1,798	16,544
3	3	10,000	4,066	2,303	1,403	19,781
Total	7					50,092
					TW Mean	7,156

Days	number of days in the renewal period
Conc 0	measured concentration at the start of each renewal period
Conc 1	measured concentration at the end of each renewal period
Ln(Conc 0)	natural logarithm of Conc 0
Ln(Conc 1)	natural logarithm of Conc 1
Area	area under the exponential curve for each renewal period
TW Mean	time-weighted mean = total area divided by the total days

The area is calculated by:

$$\text{Area} = \frac{\text{Conc 0} - \text{Conc 1}}{\text{Ln(Conc 0)} - \text{Ln(Conc 1)}} \times \text{days} \qquad \text{(F.6)}$$

It is clear that when observations are taken only at the start and end of each renewal period, it is not possible to confirm that the decay process is, in fact, exponential. A different curve would result in a different calculation for Area. However, an exponential decay process is not implausible and is probably the best curve to use in the absence of other information.

However, a word of caution is required if the chemical analysis fails to find any substance at the end of the renewal period. Unless it is possible to estimate how quickly the substance disappeared from the solution, it is impossible to obtain a realistic area under the curve, and hence, it is impossible to obtain a reasonable time-weighted mean.

Annex G
(informative)

Culturing of *Acartia tonsa*

Figure G.1 — Female (top) and male of *Acartia tonsa*

G.1 The organism

Acartia tonsa Dana is a planktonic copepod belonging to the group of calanoid copepods (*Crustacea*, *Arthropoda*).

The genus *Acartia* is distributed all over the world in coastal waters and is often dominating in the zooplankton. *A. tonsa* is a common organism in European waters and at the Atlantic coast of America. It feeds on planktonic microalgae and is thus an important link between the primary producers and higher trophic levels like larger crustaceans and fish larvae.

In daytime, it stays at deeper layers but at night, it moves towards the surface where the density of algae is higher. It moves generally by jumps using its large antennae as “paddles” (waterflea).

Depending on the temperature, it reaches its adult stage after 1,5 weeks to 3 weeks at 15 °C to 20 °C.[11] The full-grown female has a length of about 1,0 mm and the male of about 0,8 mm. At copulation, the male places a spermatophore close to the opening of the egg duct of the female. After mating, the female can store the semen for fertilizations of eggs for some time. The female has no egg bag or brood pouch, and the eggs are released singly and continuously into the water and start sinking. At 15 °C to 20 °C, the eggs will hatch after approx. 24 h. In the laboratory under good food conditions, a female can produce up to 50 to 60 eggs per day.[12]

Like other crustaceans, *Acartia* grows through a number of moltings. There are two main groups of developmental stages – the nauplii and the copepodites – also named larvae and juveniles. The copepodites have the form of the adult stage while the nauplii are less differentiated and circular. There are six stages of each of the groups – the last copepodite stage being the adult stage. The sex of the animals can be determined visually from about the fourth copepodite stage.

In the U.S., a toxicity test method using *A. tonsa* has existed since 1978[13] and results from toxicity tests with *A. tonsa* were published also in 1978.[14] *A. tonsa* has been cultured in Denmark since 1981 for different purposes[15] - first as a food source for fish larvae and later, from 1985, also as a test organism for toxicity testing. The Danish strain seems to be a mixture of animals isolated at different occasions in Danish marine (North Sea) and brackish waters (the Sound).

G.2 Culturing

G.2.1 General

To be suitable for toxicity tests, cultures should have a known age, a low mortality, be well fed, and not be too crowded. The animals should exhibit normal feeding behaviour. If a batch of a culture shows high mortality in acute tests or if only few of the eggs hatch in a new culture, this culture should be discarded.

The sensitivity of the whole test system (including the sensitivity of the animals) should be checked regularly with one or more reference compound in acute tests.

Stock cultures can be kept in 1 l or 2 l (or bigger) glass vessels with 800 ml to 900 ml or 1 600 ml to 1 800 ml of medium.

Natural salt water and reconstituted salt water media can be used as cultivation medium without problems. *A. tonsa* and its food organism *Rhodomonas salina* have been cultured for years in reconstituted medium without problems. If natural sea water is used, it should be collected from a location distant from any known sources of pollution and filtered to remove indigenous organisms.

Figure G.2 — Cultivation of *Acartia tonsa* in 2 l flasks

Explanation to Figure G.2: Glass pipettes supply filtered atmospheric air and the thin tubes supply the culture with food.

G.2.2 Facilities and apparatus

A climate room or thermostated cabinet with a precise temperature regulation (±0,5 °C) is necessary for performing long-term tests with *Acartia tonsa* and also very practical for cultivation and acclimation.

Use the following apparatus:

G.2.2.1 Peristaltic pump.

G.2.2.2 Stereomicroscope.

G.2.2.3 Timers.

G.2.2.4 Light tube.

G.2.2.5 Aquaria pump.

G.2.2.6 Different filters.

G.2.2.7 Nets, mesh sizes of approximately 45 µm and 180 µm.

G.2.2.8 Tubes.

G.2.2.9 2 l flasks.

G.2.2.10 1 l flasks.

G.2.3 Lighting.

A photoperiod of 16:8 h light:dark is recommended at a low light intensity (5 µmol to 10 µmol · s^{-1} · m^{-2}). A fluorescent light tube controlled by a timer is sufficient and practical due to its low heat emission.

G.2.4 Culture media

The medium can be natural or reconstituted salt water. *A. tonsa* can live and reproduce in salinities from 15 ‰ to 35 ‰.

If natural water is used, it shall be collected at a place distant from all known sources of pollution. It shall be filtered to remove particles (coarse filter and 0,2 µm-filter) and stored at 4 °C to 8 °C until use.

Reconstituted medium can be prepared from distilled water or deionized water passed through a Millipore® unit. Basically, the reconstituted medium is a mixture of a salt solution with a salinity of 10 %[16] and a reconstituted fresh water medium M7 (used in *Daphnia* reproduction tests[1][17]). Recipes of the salt solution and the M7 medium are given in Tables A.1 to A.4.

G.2.5 Temperature

The cultures can be kept at temperatures between 15 °C to 20 °C depending on the rate of development needed/wanted.

G.2.6 Oxygen

The cultures are bubbled with filtered (0,2 µm) atmospheric air (1 to 2 bubbles per second) to keep a gentle continuous water movement, which helps keeping the food (algal cells) in suspension. This also ensures an exchange of gases between the water phase and the air.

The oxygen saturation in cultures should be above 70 % (in equilibrium with atmospheric air).

G.2.7 Food and feeding

The *Acartia* culture is fed with an algal suspension of the species *Rhodomonas salina*. A peristaltic pump controlled by a timer should provide the feed automatically three times a day. The feeding rate should be about 6×10^4 cells · ml^{-1} · day^{-1}.

G.2.7.1 Cultivation of the food alga *Rhodomonas salina*

Rhodomonas salina is a Chryptophyceae. The strain was isolated from the Sound, Denmark, in 1984 by the Marine Biology Laboratory, Copenhagen University, Elsinore, Denmark.

R. salina is cultured in a growth medium, which is prepared by adding nutrients to natural or reconstituted salt water (as the 20‰ medium based on M7 used for *Acartia*). A description of the growth medium (B medium) for *Rhodomonas*[18] is given in Table G.1.

R. salina can be cultured at 15 °C in continuous light with an intensity of 15 µmol to 20 µmol · s^{-1} · m^{-2} provided by fluorescent tubes. Cultures ready for feeding have an intensively red colour and can be stored at 4 °C for up to 3 d. The algal cells will sediment during these 3 d and it is thus possible to get a more concentrated culture by removing the top water. New cultures are prepared by adding 50 ml to 80 ml of an exponentially growing culture to 1 l of growth medium.

Table G.1 — Composition of B medium

Stock no		g/l in stock	Volume for final B medium ml/l	Concentration in final B medium µmol
1	$Na_2EDTA,2H_2O$	45,00	1,00	120,89
	$FeCl_3,6H_2O$	1,30		4,81
2	$NaNO_3$	100,00	1,00	1 176,54
3	H_3BO_3	33,60	1,00	543,41
4	NaH_2PO_4	20,00	1,00	166,70
5	$MnCl_2,4H_2O$	0,36	1,00	1,82
		g/l		nmol
6[a]	$ZnCl_2$	2,10		15,41
	$CoCl_2,6H_2O$	2,00		8,41
	$(NH_4)_6Mo_7O_{24},4H_2O$	0,90		5,10
	$CuSO_4,5H_2O$	2,00		8,01

[a] 1 ml of stock 6 is added to 1 l of stock 5.

If the B medium is made up of M7 medium, the M7 already contains vitamins.

If the B medium is made up of natural seawater, vitamins should be added.

Table G.2 — Combined vitamin solution

			In vitamin stock mg/l	For B medium ml/l
Combined vitamins[a,b]				
	Thiamine hydrochloride		750,00	0,10
	Cyanocobalamine		10,00	
	Biotin		7,50	
a Combined vitamin stock shall be stored at 4 °C in small aliquots.				
b Vitamins shall be added to the medium shortly before use.				

G.3 Handling of organisms

Cultures of *A. tonsa* are started with eggs so that the age of the animals is known. The density of the stock cultures should be 400 to 600 animals per litre.

10 d to 12 d after the start of the culture (at 20 °C), the animals have reached the adult stage and start to reproduce. During the first 10 d to 12 d, the bottom of the cultivation vessel is cleaned three to four times with a hose to remove dead algae and larvae, unhatched eggs, and faeces. When the animals start to produce eggs, the bottom should be cleaned three to five times a week to remove eggs. This is to avoid overpopulation of cultures and enables harvesting of eggs for new cultures and for experiments. A hose consisting of a tube and a glass pipette equipped with a soft piece of tube is practical. The soft tube is used to scrape the bottom of the culturing vessel.

The egg suspension is filtered through a 180 µm mesh for removing live and dead copepods and larvae. Subsequently, the eggs are collected on a 45 µm mesh, rinsed with clean medium, and transferred to small vessels, containing 20 ml of medium. Eggs can be stored at 4 °C. At this temperature, hatching is prevented and the eggs can be kept for months with only a slight, constant decrease of the hatching success.

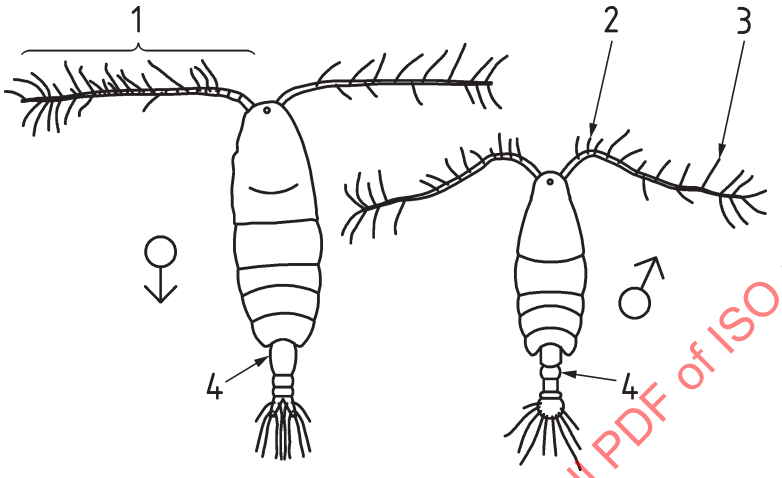
In order to isolate adult animals, they can be caught and transferred by use of a small piece (2 cm × 5 cm) of 180 µm net.

G.4 Sources of organisms

Acartia tonsa is cultured at several laboratories in Europe. Small volumes of medium with eggs can be sent by mail – at least if they are not exposed to extreme temperatures.

Annex H
(informative)

Specific details on secondary sexual characteristics for *Acartia tonsa*



Females:

— larger size (1,0 mm to 1,2 mm)

— antennae straight

— second urosome segment (of four) is bigger and prolonged

— head form rounded

Males:

— smaller size (0,8 mm to 0,9 mm)

— antennae bended and asymmetric

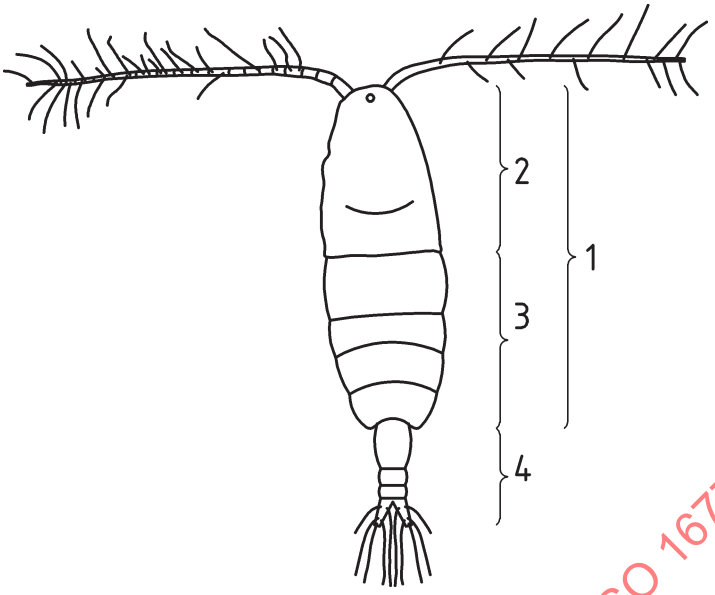
— second urosome segment (of five) is round

— head form squared

Key

1 antennae straight
2 antennae bended
3 asymmetric antennae (geniculate)
4 second urosome segment

Figure H.1 — *Acartia tonsa* - Female and male characteristics



Key

1 prosome
2 cephalosome
3 metasome
4 urosome

Figure H.2 — Segment nomenclature in copepods

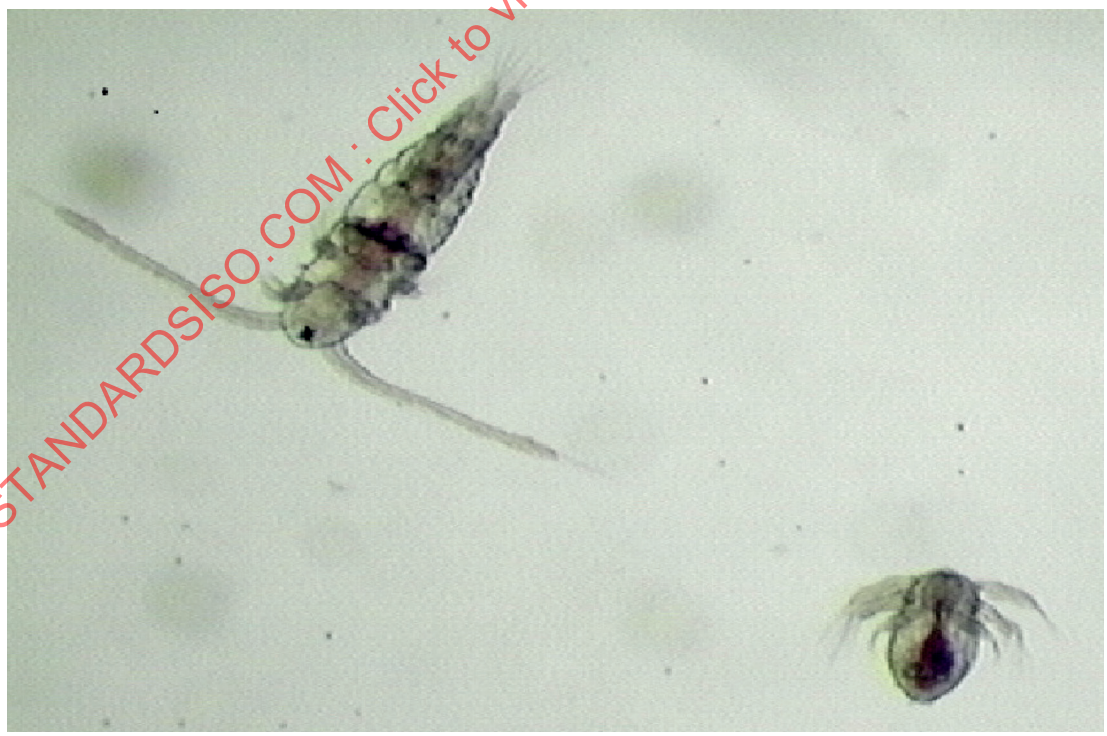


Figure H.3 — Copepodite (left) and nauplius (right)