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Water quality — Larval development test with the harpacticoid copepod *Nitocra spinipes*

Qualité de l'eau — Essai de développement larvaire avec le copépode harpacticoïde Nitocra spinipes

Reference number
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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.

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

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Introduction

Harpacticoid copepods are predominantly benthic, occurring widely in marine, brackish and fresh water ecosystems. They represent important prey items for the benthic larvae of many fish species and larger invertebrates and constitute an ecologically important energy-transfer link between the organic phase of the sediment and higher trophic levels.

The euryhaline brackish water harpactoid *Nitocra spinipes* (Crustacea) is a common component of the benthic meiofana in shallow coastal waters around the world (see Reference [6]).

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Water quality — Larval development test with the harpacticoid copepod *Nitocra spinipes*

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

1 Scope

This Technical Specification specifies an early-life stage procedure for determination of the toxic effects of chemicals and water samples on a cold-water brackish water copepod species under semi-static conditions. The biological test variables include survival and development of the early-life stages. The exposure starts with newly hatched (<24 h) nauplii (larvae) and is continued until emergence of (c. 50 %) copepodites (juveniles) in the control.

The benthic living *Nitocra* complements the planktonic *Acartia* species in ISO 16778. These organisms represent different life-history strategies as *Nitocra* is egg-carrying, whereas *Acartia* is a broadcasting calanoid copepod and thus, different sensitivities of specific life stages. *Nitocra* is a fresh to brackish water species, which allows testing low salinity waters and is complementary to *A. tonsa*, which is of marine origin and poorly tolerates low salinities.

2 Terms and definitions

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

2.1
nauplii
larvae

2.2
copepodites
juveniles

2.3
larval development ratio
LDR
ratio of *copepodites* (2.2) to the total number of surviving early-life stages (nauplii + copepodites) at the end of the test

2.4
lowest observed effect concentration
LOEC
lowest concentration within the experimental range at which a significant effect is observed

2.5
no observed effect concentration
NOEC
tested concentration just below the LOEC (2.4)

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2.6
effect concentration
EC_x
calculated concentration from which an effect of x % is expected

2.7
mortality
calculated on dead and missing animals at the end of the test divided by animals at start

2.8
confidence interval
A x %
interval of values within which the measured or calculated value is likely to be present with a probability of x %

2.9
salinity
S
dimensionless value of which, for the purpose of checking water quality, may be regarded as an estimate of the concentration, in grams per kilogram, of dissolved salts in sea water

Note 1 to entry: It is defined algorithmically, in terms of the ratio (K15) of the electrical conductivity of the sample, at 15 °C and 1 atm, to that of defined potassium chloride solution (32,436 6 g/kg of sample) at the same temperature and pressure.

3 Principle

The test is an early-life stage test, in which the organisms are exposed to various concentrations of a test substance or water sample under semi-static test conditions from the first naupliar stage (N-1) to the first copepodite stages (C-1, C-2, etc.). Survival and development of early-life stages [larval development ratio (LDR)], are the investigated test variables. The exposure starts with newly hatched (<24 h) nauplii (larvae) and is continued until the emergence of (approximately 50 %) copepodites (juveniles) in the control. The total test duration is about 6 d to 7 d, which is sufficient time to investigate the development from N-1 to 50 % copepodites in the control. 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.

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

4 Reagents

4.1 Test organism

The species to be used is the brackish water harpacticoid copepod *Nitocra spinipes* Boeck.

Newly hatched (less than 24 h of age) nauplii 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 *N. spinipes* is described in Annex A).

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4.2 Water, deionized or of equivalent purity, to prepare artificial sea water or to dilute natural sea water.

4.2.1 Artificial sea water

An example of artificial sea water suitable for cultivation and testing is included in Annex A. Any artificial sea water with a known composition in which the copepods show suitable long-term survival, normal behaviour, development and fecundity may be used as culture and dilution medium (4.3).

4.2.2 Natural sea water

Natural sea water shall be collected from an unpolluted location. Any natural sea water with a known composition in which the copepods show suitable long-term survival, normal behaviour, development and fecundity may be used as culture and dilution medium.

Suspended particles shall be <10 mg/l and can be stored cold for approximately 6 months before preparation of culture or dilution medium.

4.3 Medium

4.3.1 Culture medium

Culture medium is used for cultivating *Nitocra spinipes* and is prepared from either natural or artificial sea water (4.2). Natural sea water shall be filtered (30 µm) and heated to 80 °C to kill undesired organisms and then conditioned (24 h) to culture temperature and oxygen saturation. The culture medium can be stored cold for several weeks.

4.3.2 Dilution medium

Dilution medium is used for diluting water samples or dissolving chemicals and is prepared from culture medium that has first been filtered (GF/C-1,2 µm) before use. Salinity of the dilution medium should be the same as the culture medium. Salinities between 3 ‰ and 25 ‰ can be used. The dilution medium shall have a dissolved oxygen concentration above 70 % of the air saturation value and a pH of 7,5 ± 1,0 before being used to prepare the test solutions. If the physical conditions or the salinity of the medium to be used in the test differ more than 5 °C or 10 % from those used for routine culturing, it is good practice to include an adequate cultivating acclimation period at the same salinity (±2 ‰) of 2 weeks to 3 weeks to avoid stressing of the larvae.

5 Cultivation

5.1 Test organism

See Annex A for detailed information.

5.2 Algae for feeding

See Annex A for detailed information.

6 Apparatus

All equipment, which will come in contact with the test medium, shall be made of glass or chemically inert material.

6.1 Glass vessels, approximately 150 ml, diameter 8 cm, and height 4,5 cm, for *Nitocra spinipes* cultivation.

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6.2 Test vessels, approximately 15 ml, diameter 2,5 cm, height 4 cm, with flat bottom.

6.3 pH meter.

6.4 Oxygen meter.

6.5 Conductivity meter.

6.6 Wide pipettes, for sampling animals, preferably salinized to prevent copepods from adhering to pipette walls.

6.7 Temperature-control cabinet or room, (22 ± 1) °C.

6.8 Low-magnifying stereo microscope.

6.9 Inverted microscope.

6.10 Apparatus for membrane filtration.

6.11 Filters, 1,2 µm and 30 µm.

7 Procedure

7.1 Production of nauplii to be used in test

Nauplii aged less than 24 h are used for initiating a test. To produce a sufficient number of nauplii, the procedure presented below should be followed.

The day before the test starts, approximately 300 females with well-developed egg sacs are sampled by pipette under a low-magnifying stereomicroscope and randomly transferred (approximately 60 in each) to six glass “hatch” vessels containing 100 ml dilution medium. The isolated female copepods are fed with a suspension of *Rhodomonas salina* to a concentration of $2,5 \times 10^5$ cells/ml.

7.2 Choice of test concentrations

The range of the 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 (i.e. development).

Prior knowledge of the toxicity of the test substance, i.e. from an acute test (see Reference [2]) 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 set at 10 % to 20 % of the acute 96 h-LC_{50} to avoid significant effect on survival.

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

The number of replicates generally depends on the statistical design (hypothesis testing or regression analysis). When planning the test, it should be taken into consideration if the aim is to achieve a NOEC/LOEC (by use of Chi-square) or an EC_x value (by use of regression analysis or none parametric alternative; see Reference [4]).

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The number of replicates in control(s) and each test concentration should not be lower than 8. For regression analysis, the number of replicates may be lowered (≥4) but such a statistical design usually requires more test concentrations as compared to a hypothesis testing design.

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

7.2.1 Hypothesis testing

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 decreased lowest concentration.

If the aim is to obtain the LOEC, the highest 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.

7.2.2 Regression analysis

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 appropriate level of confidence.

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 (i.e. development).

7.3 Preparation of solutions to be used in test

7.3.1 Stock solution

The stock solution should preferably be prepared by dissolving the substance in dilution medium. The preferred options for preparing stock solutions are physical methods, such as stirring and sonication.

NOTE See ISO 5667-16.[1]

When preparing the stock solution, the pH should be measured to assure it is in the valid range (6 to 9). The pH adjustment of the stock solution 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 and preferably used in small volumes.

The use of organic solvents may be required in some cases in order to produce a suitable concentrated stock solution of so-called “difficult substances” as described in Reference [7], but every effort should be made to avoid the use of such carrier solvents. Solvents are used to produce a stock solution that can be dosed accurately into water; the recommended maximum solvent concentration in the final test medium is 0,01 ml/l and should preferably be the same in all test vessels. If a higher solvent concentration is used, it should be documented that it has no effects on the test variables investigated in the test. Solvents may be essential in handling some substances; for example, for preparing stock solutions of hydrolytically unstable or highly viscous substances. When necessary, use of solvents of low toxicity at low concentrations is recommended to aid preparation of test solutions. Examples of suitable solvents are presented in References [7] and [8]. Care should be taken when using readily degradable agents (e.g. acetone) as these can cause problems with bacterial growth in the test vessels.

7.3.2 Test solutions

Test solutions are prepared by dilution of stock solution using dilution medium.

To be able to allocate newborn nauplii among control(s) and test concentrations in a random fashion and avoiding cross-contamination, 5 ml of dilution media is initially added to each test vessel to be used in the test. When newborn nauplii have been transferred to each test vessel (see below), either 5 ml dilution media (control) or test solution is added to the test vessels. Hence, each test solution to be used

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at start of the test shall have a concentration that is two times the intended final test concentrations. At test solution renewals (see below), the concentrations of the test solutions should, however, be the same as the chosen test concentrations.

7.4 Start of test

Experiments are preferably to be started on Wednesdays or Fridays to avoid LDR readings and feeding at weekends.

Day 0

Nauplii hatched within 24 h (see 7.1) are collected by pipette, transferred in a volume of 5 µl/nauplius to 5 ml dilution medium/test vessel and distributed evenly (so that all vessels contain nauplia from all the six hatch vessels) among the vessels. The optimal number of nauplii per test vessel is 10 and should be between 8 and 12.

Test solutions (5 ml/test vessel) are added to test vessels and dilution medium (5 ml/control vessel) is added to the control vessels as described in 7.3.2.

The vessels should be labelled according to treatment and individual identity.

Test medium levels should be marked on the test vessels to allow compensation for evaporation during the test with distilled/deionized water.

Make a final check of the number of nauplii in each vessel and register on the data sheet. Feed the nauplii in each test vessel with a suspension of *R. salina* to a concentration of $2,5 \times 10^5$ cells/ml.

7.5 Incubation/exposure

The vessels can preferably be placed in darkness. A photoperiod of 16:8 h light:dark may also be used but at a low light intensity (5 µmol to 10 µmol $\times$ s^{-1} $\times$ m^{-2}).

The time needed to complete (at 22 °C and ~6 ‰ salinity) the larval development test is 6 d to 7 d. At lower temperatures and higher salinities, the development may be slower, and thus, testing at these conditions may last longer. From day 5 and onward, the development needs to be investigated daily.

7.6 Maintenance

Day 2 or 3 and 5: Compensate for evaporation with deionized water. With a pipette, carefully transfer 7 ml from each test vessel into an additional beaker in order to control that no animals are entrapped in the pipette. Collect the extracted water from the control, lowest and highest test concentrations, respectively, and measure oxygen, salinity and pH immediately. Make notation of the parameter values on the data sheet. Add 7 ml of fresh dilution media to the control test vessels and the test solutions to treatment test vessels. Feed the copepods in each test vessel with a suspension of *R. salina* to a concentration of $2,5 \times 10^5$ cells/ml.

Day 6 or 7: Count the number of nauplii and copepodites in the control. If the ratio of copepodites to the total number of surviving early-life stages (nauplii + copepodites) is within 60 % ± 20 % (preferably close to 50 %) the test is terminated and all vessels are counted. If the ratio is lower than 40 % on day 7, the maintenance procedures described above for day 2 or 3 and 5 are repeated and the exposure period is prolonged by another 24 h (or more, if necessary). To eliminate errors from long reading times, during which animals may continue to develop, readings should be made across treatments.

7.7 Measurements/observations

7.7.1 Mortality

Dead and missing animals at the end of the test are registered and are all considered "dead" when calculating the LC_x or NOEC/LOEC.

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7.7.2 Larval development ratio (LDR)

The LDR is expressed as the ratio of copepodites to the total number of surviving early-life stages (nauplii + copepodites) at the end of the test. Mortality of the animals should be presented along with the LDR.

Observations made during the test should be recorded on data sheets, examples of which are provided in Annex B.

7.7.3 Physical-chemical parameters — oxygen, pH and salinity

During the test, dissolved oxygen, salinity and pH should be measured in the control and all test concentrations each time test medium is renewed.

As a minimum, these measurements shall be made in the control, lowest and the highest test concentration.

7.7.4 Concentration of the test substance

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. It is recommended that results be based on measured concentrations.

NOTE If there is evidence that the concentration of the substance tested has been satisfactorily maintained within ±20 % of the nominal concentration throughout the test, then results can be based on nominal or measured initial values.

For tests where the concentration of the test substance is not expected to remain within ±20 % of the nominal concentration, it is necessary to sample all tests concentrations (including control), when freshly prepared and at renewal. After finalizing the tests, only samples with nominal concentrations close to (nominal) 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 C). Note that care should be taken when testing very lipophilic (i.e. log Kow > 5) and hence poorly water-soluble substances in the present test system (see References [7] and [8]). Using radiolabeled substances may give crucial information on the partitioning in the test system, which may facilitate the calculation of the actual concentrations — especially for not readily biodegradable compounds.

8 Validity criteria

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

— The dissolved oxygen concentration shall be >70 % of the air saturation value (ASV) throughout the exposure period.

— The temperature should not vary more than ±1 °C during the entire test period.

— The pH value shall remain within 6 and 9 during the entire test.

— Conductivity/salinity shall not vary more than 10 % from the control start value.

— The average control copepodites fraction (LDR) shall be 60 % ± 20 % of surviving animals at the end of exposure;

— The average mortality of animals in the control(s) on the day of observation of LDR shall not exceed 20 %.

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9 Evaluation of results

9.1 Calculation of results

The fractionate mortality and LDR data emerging from the larval development test is considered as binomially distributed data. To assure that replicates within control/treatments are homogenous and hence can be considered as a single data set, it is necessary to use the Fisher's exact test on extreme values within each treatment/control. Given that there is no significant variation, a (two-sided) Pearson Chi-square test is used to evaluate differences in mortality and larval development ratio between treatments and the control. If, however, replicates within one or more of the treatments/control vary significantly from each other, the non-parametric Kruskal Wallis test should be used instead. Given that this test results in a significant effect of treatment, the Mann-Whitney test should be used to test for differences between specific treatments and the control. Regardless of using the Pearson Chi-square or the Mann-Whitney test for testing differences between specific treatments and the control, compounded alpha (∂) error due to multiple comparisons to the same control shall be corrected using the Bonferroni correction (∂/n), where n denotes the number of comparisons to the same control).

Different programs are available for performing linear or non-linear regression assuming a logarithmic normal-distribution, a Weibull distribution or a logit distribution of data.

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 may be pooled for comparison with test substance treatments.

9.2 Expression of results

Denote concentrations causing 10 % and 50 % inhibition based on a concentration-response curve as EC_{10} and EC_{50}. Quote EC_{10}, EC_{50}, NOEC and LOEC values to two significant digits, usually in milligrams per litre (mg/l).

9.3 Interpretation of results

EC_{10}, EC_{50} and NOEC values are toxicological data derived from a laboratory experiment carried out under defined condition. They give an indication of potential hazard of the toxicant but cannot be used directly to predict effects in the natural environment (see References [9] and [10]).

10 Reproducibility

A reference substance (e.g. 3,5-dichlorophenol) may be tested periodically as a means of assuring that the test protocol and the test conditions are reliable. As a benchmark, the LOEC for 3,5-dichlorophenol on development should be within (40 ± 20) µg/l.

11 Test report

This test report shall contain at least the following information:

a) the test method used, together with a reference to this Technical Specification, i.e. ISO/TS 18220:2016;

b) the test substance:

— relevant physicochemical properties;

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

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

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c) the test species:

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

d) the test conditions:

- — start date and duration;
- — test design (e.g. test concentration used, number of replicates, number of organisms per replicate, etc.);
- — method of preparation of stock solutions and frequency of renewal (the solubilizing agent 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;
- — medium characteristics (including pH, salinity, temperature, dissolved oxygen concentration, total organic carbon, suspended solids 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 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;
- — a full record of the biological effects, which were observed or measured and the statistical techniques used to analyse the data;
- — other observed effects;
- — the lowest observed effect concentration (LOEC) and no observed effect concentration (NOEC) for the biological end-point(s), or EC_x and statistical methods used for their determination;
- — explanation for any deviation from this Technical Specification.

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Annex A
(informative)

Biology and cultivation of *Nitocra spinipes*

A.1 The organism

Nitocra spinipes Boeck is a harpacticoid copepod, which is a common component of the benthic meiofauna in shallow coastal waters around the world.[6]

Like other crustaceans, *N. spinipes* grows through a number of moltings. There are two main forms of developmental stages — the nauplii and the copepodites — also named larvae and juveniles, respectively. Under ideal laboratory conditions, *N. spinipes* may reach sexual maturity within 10 d to 12 d at 22 °C after accomplishing 6 naupliar stages (5 d to 7 d) and 5 copepodite stages (about 5 d to 6 d). The copepodites have the form of the adult stage while the nauplii are less differentiated and circular. Depending on the stage, nauplii body lengths range from 0,09 mm to 0,2 mm and copepodites from 0,23 mm to 0,52 mm. The adult female has a body length of about 0,75 mm and is generally bigger than the male, 0,56 mm.[18] During the described conditions, the generation time is 16 d to 18 d but the life span of individual *N. spinipes* may be up to several months depending on availability of food, temperature, etc. 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 further broods. *N. spinipes* females develop single egg sacs (see Figure A.1) and can produce up to at least 6 broods. Owing to this, females can be raised individually to provide precise measurements of longevity and reproductive parameters (e.g. number of broods, brood size). These biological attributes facilitate the measurement of development, reproduction and life-table analysis, which provide sensitive and ecologically relevant endpoints that can be used to determine the potential chronic toxicity of contaminants.

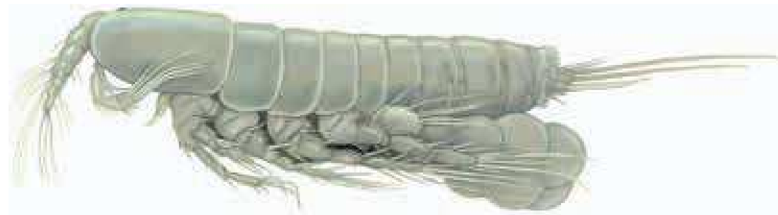

Figure A.1 — Ovigerous *Nitocra spinipes* female (by Göte Göransson, Sweden)

Observation of this copepod, especially the naupliar stages, is facilitated by using a low-power (6× to 20× magnification) binocular microscope, preferably with dark field illumination. The sex of the animals can be determined visually from about the fifth copepodite stage but owing to small differences in secondary sexual characteristics, the sex is normally not differentiated in the copepodite stages. Identification of the sexual characters requires careful microscopical analysis, which is time-consuming and difficult to perform on live animals. In the adult, the males is distinguished from the females being slightly smaller in size, more streamlined body shape and with a swollen geniculate segment on their first antenna to clasp the female. The females also have ovaries located laterally on each side of the body, which, in the microscope, further distinguishes the two sexes from each other.

Owing to its ability to acclimatize to fluctuations in salinity (0 ‰ to 30 ‰) and temperature (0 °C to 26 °C),[19][20] *N. spinipes* is easy to keep in the laboratory. Accordingly, Bengtsson[21] developed an acute toxicity test that has been established as Swedish,[5] Danish and International Standards.[2] This acute test has successively been complemented with other test methods, such as sediment[22] and reproductive tests[11][23] using the same organism.

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A.2 Culturing

A.2.1 General

To be suitable for toxicity tests, cultures should have a known age, a low mortality (i.e. mortality among control animals <10 % when placed in sea water for at least 4 d), be well fed and should not be too crowded (1 500 to 2 000 animals per 100 ml sea water). The animals should exhibit normal feeding behaviour.

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

Stock cultures may be kept in 150 ml crystallization dishes with about 100 ml of media. Natural brackish or diluted sea water are preferred as cultivation media for *N. spinipes*. Artificial sea water can be used as well but may, owing to deficiency of trace elements, etc., render in extinction of cultures over a longer period of time.

A.2.2 Facilities and apparatus

A.2.2.1 Stereomicroscope.

A.2.2.2 Oxygen meter.

A.2.2.3 Conductivity meter.

A.2.2.4 pH meter.

A.2.2.5 Dishes, total volume 150 ml, diameter 8 cm, height 4,5 cm.

A.2.2.6 Pasteur pipettes, 150 mm and slim.

A.2.2.7 Crinkled filter, 30 µm.

A.2.2.8 Climate chamber or temperature controlled room.

A.2.3 Light conditions

N. spinipes is preferably kept in darkness but a photoperiod of 16 h light/8 h darkness may also be used.

A.2.4 Culture medium

Natural or artificial sea water (1 ‰ to 35 ‰) may be used to culture *N. spinipes*. Whichever salinity is used, the test organism should be cultivated for at least 2 weeks before start of the test and the salinity should not differ more than ×1 ‰.

Natural sea water should be filtered through a crinkled filter (30 µm), heated to 80 °C and aged for 24 h before use.

Since *N. spinipes* historically has not thrived well in synthetic sea water over longer periods of time, cultures have mainly been maintained by the use of natural sea water. However, other copepod species, such as *Amphiascus tenuiremis*, are constantly kept in synthetic or artificial sea water without any signs of deficiency of trace elements, etc. The key to work with artificial sea water is allowing it to mix vigorously under aeration in a large mixing tank for about 2 weeks before using it for culture. Taking this into consideration, there is a possibility that *N. spinipes* may thrive equally well as *A. tenuiremis* in artificial sea water. However, as a general rule, care should always be taken when using synthetic sea water for cultivation.

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For shorter periods of time (e.g. for reproduction tests), the easily prepared synthetic sea water presented in Table A.1 has been proven to work well for *N. spinipes*. Distilled water (conductivity <1 mS/m) should be used when solving the chemicals. Add the chemicals in the same amount and order as given in Table A.1 to 890 ml distilled water. Each compound shall be solved before adding the next one. The total volume should be 1 000 ml. The salinity will be 34 ‰ ± 0,5 ‰ and pH 8,0 ± 0,2. This synthetic sea water should also be prepared 24 h before use. The chemicals presented in Table A.1 should be of high purity (pro analysis).

Table A.1 — Prepared synthetic sea water for shorter periods of time

Chemical	Concentration g/l
NaCl	32,5
Na_2SO_4	4,0
KCl	0,68
H_3BO_3	0,026
$MgCl_2 \cdot 6H_2O$	10,78
$CaCl_2 \cdot 2H_2O$	1,47
$NaHCO_3$	0,196
Na_2EDTA[a]	0,000 3

[a] This compound should not be added when testing metals.

Keep the prepared water (both natural and synthetic) in a glass bottle and store it cold in darkness for up to 2 months. The oxygen concentration should be above 80 % of the air-saturated value (see Table A.2). If necessary, the pH should be adjusted to 8 ± 0,3, but then the water should not be aerated again.

Table A.2 — Dissolved oxygen as a function of the salinity of the water at 20 °C

Salinity ‰	0	5	10	15	20	25	30	35
Dissolved oxygen %	91,2	88,7	86,1	83,5	81,1	78,8	76,5	74,3

A.2.5 Food and feeding

A.2.5.1 Continuous cultures

N. spinipes are preferably fed weekly with a point of a knife (~15 mg) of a dried salmon food (Start 040, EWOS AS, Postbox4-Sentrum, 5803 Bergen), which is sufficient per 100 ml culture medium (~30 mg to new cultures). The salmon food should be prepared as follows:

a) grind with help of a mortar;

b) pour into a glass tube (10 ml) with a cap;

c) keep in a freezer.

A.2.5.2 Production of nauplii to be used in tests

To obtain a more precise age cohort (i.e. nauplii <24 h old), which is needed to start a test, the following procedure may be followed. About 150 to 300 ovigerous females are collected from healthy 3 weeks to 4 weeks old continuous cultures and randomly transferred to 3 to 6 crystallizing dishes (150 ml) using a suitable pipette (preferably silanized to prevent adhesion of nauplii to the pipette). As a rule of thumb, 60 to 75 ovigerous females may be allocated to each crystallising dish, which are fed a cell suspension (10^7 cells/ml) of a chrysophyte (i.e. *R. salina*, the same should be used as food source in the test), so that the concentration of algae in each dish reach at least 2×10^5 cells/ml (see A.2.6 for further information on the use of *Rhodomonas* spp. as food item for *N. spinipes*). A further rule of thumb is that

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the low number of ovigerous females indicated above (i.e. 150) will, within 24 h, normally produce approximately 300 to 400 nauplii to start a test.

A.2.6 Cultivation of *Rhodomonas*

A.2.6.1 Autoclaving of natural sea water

In order to eliminate unwanted planktonic material, natural sea water (20 ‰ to 35 ‰) is filtered through sterile 0,2 µm mixed cellulose ester filters and diluted to 10 ‰ using MilliQ water. However, to eliminate organisms that may have passed the filter, the filtrated water, as well as an additional litre of MilliQ water, are transferred to autoclaved 1 l to 2 l flasks and autoclaved with loosened caps for 20 min at 120 °C (the pressure should be 1 kg). Use the autoclaved MilliQ water to, under sterile conditions, correct for evaporation. In addition to this, empty 1 l flasks provided with a piece of cotton wool covered with aluminium foil should also be autoclaved under the same conditions as described above.

A.2.6.2 Preparation of growth medium (f/2)

The growth medium (f/2) should be prepared as given in Table A.3.

Table A.3 — Preparation of growth medium (f/2)

Stock solution		Per litre
A	Trace metals stock solution (chelated)	
	$EDTANa_2$	4,160 g
	$FeCl_3 \cdot 6H_2O$	3,150 g
	$CuSO_4 \cdot 5H_2O$	0,010 g
	$ZnSO_4 \cdot 7H_2O$	0,022 g
	$CoCl_2 \cdot 6H_2O$	0,010 g
	$MnCl_2 \cdot 4H_2O$	0,180 g
	$Na_2MoO_4 \cdot 2H_2O$	0,006 g
B	Vitamin mix stock solution	
	Thiamine (B1)	100 mg
	Cyanocobalamine (B12)	1,0 mg
	Biotin	1,0 mg
Growth medium		**Per litre**
KNO_3		0,075 0 g
$NaH_2PO_4 \cdot H_2O$		0,005 0 g
Trace metals stock solution (A)		1,00 ml
Vitamin mix stock solution (B)		1,00 ml

Make up to 1 l with filtered and autoclaved natural sea water and adjust pH to 8,0 with 1M NaOH or HCl. Make sure to work under sterile conditions. Store all stock solutions in a refrigerator at approximately 4 °C for up to 1 week.

A.2.6.3 Inoculation

Transfer 500 ml of the f/2 growth medium to a sterile 1 l flask and add 2 ml to 5 ml *Rhodomonas* cell suspension. Place the flasks on a shaker at a light intensity of about $70 \mu Exm^2 xs^{-1}$ at 20 °C to 23 °C. At the described conditions, the algal culture should be pink in colour and ready for preparation of food suspension after about 1 week. Make sure to work under sterile conditions.

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A.2.6.4 Preparation of food suspension

The algal culture is transferred to a measure cylinder covered with a sealing film and stored at 4 °C. After 24 h, the sedimentation of the algae is finished and the growth media can be removed. Transfer the algae to a 100 ml flask and dilute up to about 100 ml with *N. spinipes* culture medium. Store the flask at 4 °C for 24 h and remove the solution. Dilute the algae with *N. spinipes* culture medium until the solution is thick and pink in colour. The density of the algal suspension is measured.

A.2.7 Handling of organisms

Cultures should be taken care of and renewed once a week as presented below. However, newly prepared cultures should be left for 2 weeks before renewal of culture medium.

a) Pour 100 ml culture medium into crystallization dish (150 ml).

b) Transfer about 20 ovigerous females with fully developed egg sacs into each dish.

c) Add the amount of about 30 mg of the food (see A.2.5).

d) Cover the dishes with transparent plastic lids.

e) Note the number of the week and day on each dish.

f) Mark the water level.

g) Adjust the level of the water in the old cultures to the marked line with deionized water and renew about one third of the culture medium.

h) Feed the old cultures with the amount of about 15 mg of the food (see A.2.5).

i) Cultivate in a climate chamber or in a temperature-regulated room.

A.3 Sources of organism(s)

N. spinipes is cultured at different laboratories in Europe. Small volumes of medium with ovigerous females can be sent by mail — at least if they are not exposed to extreme temperatures.

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Annex B
(informative)

Nitocra spinipes larval development ratio

Test compound:		Date:	(Start of test — Day 0)	Laboratory:
				Study director:
				Technician:

	Start of test	Renewal of medium		End of test
	Day 0	Day	Day	Day
pH				
Conductivity				
Oxygen				

		Day 0	End of test	End of test	End of test	End of test
Control	Replicate	Nauplii	Nauplii	Copepods	Dead %	Copepods %
	1					
	2					
	3					
	4					
	5					
	6					
	7					
	8					
Example	Total	80	25	50	5/80 × 100	50/75 × 100
					=6,25 %	=66,67 %
Concentration	Replicate					
	1					
	2					
	3					
	4					
	5					
	6					
	7					
	8					
	Total					
Concentration	Replicate					
	1					
	2					

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	3					
	4					
	5					
	6					
	7					
	8					
	Total					
		Day 0	End of test	End of test	End of test	End of test
Control	Replicate	Nauplii	Nauplii	Copepods	Dead %	Copepods %
Concentration	Replicate					
	1					
	2					
	3					
	4					
	5					
	6					
	7					
	8					
	Total					
Concentration	Replicate					
	1					
	2					
	3					
	4					
	5					
	6					
	7					
	8					
	Total					
Concentration	Replicate					
	1					
	2					
	3					
	4					
	5					
	6					
	7					
	8					
	Total					

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Annex C
(informative)

Calculations

Larval development ratio (in %):

$$\frac{(\text{Total number of living copepodites in the end of test}) \times 100}{\text{Total number of living copepodites in the end of test} + \text{total number of living nauplii in the end of test}}$$

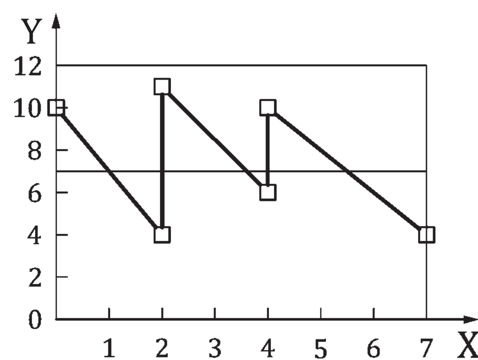
Mortality (in %):

$$\frac{\text{number of dead and missing animals at the end of test} \times 100}{\text{number of nauplii at start}}$$

Time-weighted concentration mean:

Given that the concentration of the test substance can decline over the period between medium renewals, it is necessary to consider what concentration should be chosen as representative of the range of concentrations experienced by the parent copepods. The selection should be based on biological considerations, as well as statistical ones. For example, if development is thought to be affected mostly by the peak concentration experienced, then the maximum concentration should be used. However, if the accumulated or longer term effect of the toxic substance is considered to be more important, then 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.

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



Key

X days

Y Ln(Concentration)

NOTE 1 The 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).

NOTE 2 The six plotted points represent the observed concentrations measured at the start and end of each renewal period.

NOTE 3 The horizontal line indicates the position of the time-weighted mean.

Figure C.1 — Example of time-weighted concentration mean [Ln(Concentration)]

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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 C.1.

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

Renewal no.	Days	Conc 0	Conc 1	Ln(Conc 0)	Ln(Conc 1)	Area[a]
1	2	10,000	4,493	2,303	1,503	13,676
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**

where

days is the number of days in renewal period;

Conc 0 is the measured concentration at the start of each renewal period;

Conc 1 is the measured concentration at the start of each renewal period;

Ln(Conc 0) is the natural logarithm of Conc 0;

Ln(Conc 1) is the natural logarithm of Conc 1;

area is the area under the exponential curve for each renewal period;

TW mean is the time-weighted mean = total area divided by the total days.

[a] The area is calculated by: Area = [(Conc 0 − Conc 1/Ln(Conc 0) − Ln(Conc 1)] × days.

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.

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