TECHNICAL SPECIFICATION

ISO/TS 18220

First edition
2016-07-01

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
ISO/TS 18220:2016(E)

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Foreword

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

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]).

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)

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).

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.

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]).

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