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Nanotechnologies - Aquatic toxicity assessment of manufactured nanomaterials in saltwater lakes using *Artemia sp.* Nauplii

Nanotechnologies - Evaluation de la toxicité des nanomatériaux en milieu aquatique par des Artemia sp

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

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Foreword

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The 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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This document was prepared by Technical Committee ISO/TC 229, *Nanotechnologies.*

Introduction

With the increasing development and use of manufactured nanomaterials (MNMs) in consumer and other products, concern about the possible impact of MNMs on human and environmental health is increasing. Various aquatic organisms (such as fish, daphnia, algae, etc.) are currently used to predict the possible adverse effects of chemicals, including nanomaterials, on the aquatic environment. Brine shrimp (*Artemia sp.*) are found nearly worldwide in saline lakes and pools,[42] and are one of the most widespread euryhaline organisms that are suitable for ecotoxicity testing. *Artemia sp*. nauplii can be used to assess the effects of nanomaterials in salt water ecosystems, primarily salt lakes. *Artemia sp*. usually live in salt lakes, and are almost never found in an open sea. This species also adapts to a wide range of salinities (5 g/L to 300 g/L) and temperatures (6 °C to 40 °C). In fact, the physiologically optimal levels of salinity for *Artemia sp*. are about 30 g/L to 35 g/L. Due to predators at these salt levels, however, *Artemia sp*. seldom occur in natural habitats at salinities of less than 45 g/L to 80 g/L. Favoured for the absence of predators and food competitors in such places, *Artemia sp*. develop very dense populations.

There are several advantages to using *Artemia sp*. as a biological model in salt water aquatic toxicology:

a) Less concern about animal welfare than for a vertebrate species;

b) There is good knowledge of *Artemia sp*. biology and ecology;

c) *Artemia sp*. have a wide geographic distribution in salt water lakes and pools;

d) Tests performed on *Artemia sp*. nauplii are simple and cost-effective;

e) Small body size allows accommodation of *Artemia sp*. nauplii in small beakers or plates;

f) *Artemia sp*. adapt to a wide range of water salinity and temperature;

g) *Artemia sp*. are simple to maintain in the laboratory;

h) The life cycle of *Artemia sp*. is short, so it is suitable for growth, reproduction and short-term toxicity tests;

i) *Artemia sp*. cysts are commercially and readily available so that the tests can be carried out worldwide. The cysts can be stored for years under cool and dry conditions without losing viability. Upon immersion in sea water, the free swimming nauplii will hatch within approximately 24 h;

j) Hatching from cysts gives organisms of similar age, genotype and physiological condition.

In recent years, several researchers around the world have used *Artemia sp*. as a test organism in aquatic nanotoxicology (see References [1] to [35]). The lack of a standardized protocol for testing *Artemia sp*. for aquatic toxicity means that data from these studies are more likely to be non-repeatable and non-reliable.[22] The goal of this document is to provide a standard protocol intended to generate reliable aquatic toxicity data by testing *Artemia sp*., which can be used for ecotoxicity evaluation of MNMs in salt water lake ecosystems.

Nanotechnologies - Aquatic toxicity assessment of manufactured nanomaterials in saltwater lakes using *Artemia sp*. Nauplii

1 Scope

This document specifies a test method, aiming to maximize repeatability and reliability of testing, to determine whether MNMs are toxic to aquatic organisms, specifically *Artemia sp*. nauplius.

This document is intended to be used by ecotoxicological laboratories that are capable in the hatching and culturing of *Artemia sp*. and the evaluation of toxicity of nanomaterials using *Artemia sp*. nauplius.

This method uses *Artemia sp*. nauplii in a simulated environment, artificial seawater, to assess effects of nanomaterials.

This document is applicable to MNMs that consist of nano-objects such as nanoparticles, nanopowders, nanofibres, nanotubes, nanowires, as well as aggregates and agglomerates of such MNMs.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 10993-12, *Biological evaluation of medical devices — Part 12: Sample preparation and reference materials*

ISO/TS 11931, *Nanotechnologies — Nanoscale calcium carbonate in powder form — Characteristics and measurement*

ISO/TS 12805, *Nanotechnologies — Materials specifications — Guidance on specifying nano-objects*

ISO/TR 13014, *Nanotechnologies — Guidance on physico-chemical characterization of engineered nanoscale materials for toxicologic assessment*

ISO 15088, *Water quality — Determination of the acute toxicity of waste water to zebrafish eggs (Danio rerio)*

ISO/TS 16195, *Nanotechnologies — Guidance for developing representative test materials consisting of nano-objects in dry powder form*

ISO/TS 17200, *Nanotechnology — Nanoparticles in powder form — Characteristics and measurements*

ISO 26824, *Particle characterization of particulate systems — Vocabulary*

ISO/TS 80004-1, *Nanotechnologies — Vocabulary — Part 1: Core terms*

ISO/TS 80004-2, *Nanotechnologies — Vocabulary — Part 2: Nano-objects*

ISO/TS 80004-4, *Nanotechnologies — Vocabulary — Part 4: Nanostructured materials*

3 Terms and definitions

For the purposes of this document, the terms and definitions given in ISO 10993-12, ISO/TS 11931, ISO/TS 12805, ISO 15088, ISO/TS 16195, ISO/TS 17200, ISO 26824, ISO/TS 80004-1, ISO/TS 80004-2 and ISO/TS 80004-4 and the following apply.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

— IEC Electropedia: available at http://www.electropedia.org/

— ISO Online browsing platform: available at https://www.iso.org/obp

3.1
agglomerate
Note 1 to entry: collection of weakly or medium strongly bound particles where the resulting external surface area is similar to the sum of the surface areas of the individual components

Note 2 to entry: The forces holding agglomerates together are weak forces, for example van der Waals forces, or simple physical entanglement.

Note 3 to entry: Agglomerates are also termed secondary particles and the original source particles are termed primary particles.

[SOURCE: ISO/TS 80004-2:2015, 3.4]

3.2
aggregate
particle comprising strongly bonded or fused particles where the resulting external surface area is significantly smaller than the sum of surface areas of the individual components

Note 1 to entry: The forces holding an aggregate together are strong forces, for example covalent or ionic bonds, or those resulting from sintering or complex physical entanglement, or otherwise combined former primary particles.

Note 2 to entry: Aggregates are also termed secondary particles and the original source particles are termed primary particles.

[SOURCE: ISO/TS 80004-2:2015, 3.5]

3.3
hatching vessel
vessel appropriate for *Artemia sp*. cyst hatching

Note 1 to entry: Cone should be transparent or semi-translucent (preferably colourless) for ease of harvesting and light transmission.

Note 2 to entry: As shown in Figure 2, constant aeration from the bottom of the hatching vessel should be used to keep cysts in suspension, and to provide sufficient oxygen levels for the cysts to hatch.

Note 3 to entry: Hatching vessels include glass or plastic cone or "V"–bottomed container as shown in Figure 1.

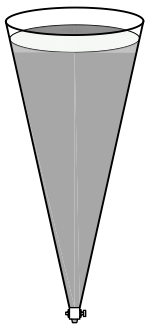

Figure 1 — Schematic of appropriate hatching vessel for *Artemia sp*

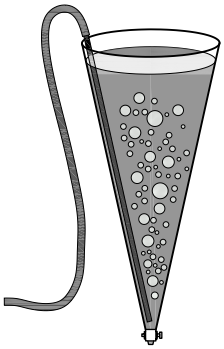

Figure 2 — Schematic of aeration from the bottom of a hatching vessel for *Artemia sp*

3.4
test vessel
vessel appropriate for *Artemia sp*. culture

Note 1 to entry: Test vessels and other apparatus that will come into contact with the test solutions should be made entirely of glass or other chemically inert material.

Note 2 to entry: Test vessels include flasks or beakers.

3.5
positive control
well-characterized material and/or substance, which, when evaluated by a specific test method, demonstrates the suitability of the test system to yield a reproducible, appropriately positive or reactive response in the test system

Note 1 to entry: Potassium dichromate ($K_2Cr_2O_7$) is suggested as a suitable treatment for the positive control in *Artemia sp*. toxicity test.

3.6
test nanomaterial
manufactured nanomaterial in a dispersion that is subjected to biological or chemical testing or evaluation

3.7
stock suspension
concentrated suspension that will be diluted to some lower concentration for actual use

3.8
nanoscale
length range approximately from 1 nm to 100 nm

Note 1 to entry: Properties that are not extrapolations from larger sizes are predominantly exhibited in this length range.

[SOURCE: ISO/TS 80004-1:2015, 2.1]

3.9
nano-object
discrete piece of material with one, two or three external dimensions in the nanoscale

Note 1 to entry: The second and third external dimensions are orthogonal to the first dimension and to each other.

[SOURCE: ISO/TS 80004-1:2015, 2.5]

3.10
nanoparticle
nano-object with all external dimensions in the nanoscale where the lengths of the longest and the shortest axes of the nano-object do not differ significantly

Note 1 to entry: If the dimensions differ significantly (typically by more than three times), terms such as nanofibre or nanoplate may be preferred to the term nanoparticle.

[SOURCE: ISO/TS 80004-2:2015, 4.4]

3.11
particle
minute piece of matter with defined physical boundaries

Note 1 to entry: A physical boundary can also be described as an interface.

Note 2 to entry: A particle can move as a unit.

Note 3 to entry: This general definition of particle applies to nano-objects.

3.12
nanofibre
nano-object with two external dimensions in the nanoscale and the third dimension significantly larger

Note 1 to entry: The largest external dimension is not necessarily in the nanoscale.

Note 2 to entry: The terms nanofibril and nanofilament can also be used.

Note 3 to entry: See nanoparticle, note 1 to entry.

[SOURCE: ISO/TS 80004-2:2015, 4.5]

3.13
nanoplate
nano-object with one external dimension in the nanoscale and the other two external dimensions significantly larger

Note 1 to entry: The larger external dimensions are not necessarily in the nanoscale.

Note 2 to entry: See nanoparticle, note 1 to entry.

[SOURCE: ISO/TS 80004-2:2015, 4.6]

3.14
Artemia sp
species of the genus of aquatic crustaceans known as brine shrimp (*Artemia*)

3.15
nauplii
newly hatched brine shrimp larvae

Note 1 to entry: The nauplius larvae of *Artemia sp*. are less than 0,4 mm in length when they first hatch.

3.16
cyst
dormant *Artemia sp*. eggs

Note 1 to entry: The cysts may be stored for long periods and hatched on demand.

3.17
hatching
process of converting cysts to nauplii under appropriate environmental conditions

3.18
control solution
test medium without sample under test

3.19
immobilization
inability of the nauplii to swim during the 15 second following gentle agitation of the test and control solutions, even if the nauplii can still move their appendages

[SOURCE: ISO 6341:2012, 3.3, modified — "organisms" has been replaced by "nauplii", and "antennae" has been replaced by "appendages".]

3.20
EC_{50}
concentration at which there is an effect on 50 % of the organisms in line with the test criterion

[SOURCE: ISO 15088:2007, 3.3]

4 Materials

4.1 Test organism

Different species of *Artemia sp.* can be used, but *Artemia salina* and *Artemia franciscana* are the preferred test species. There are many commercial sources of brine shrimp cysts. *Artemia sp.* nauplii (newborn brine shrimp) should be produced by hatching high quality cysts in the laboratory.

4.2 Chemicals

4.2.1 Artificial seawater.

4.2.2 Potassium dichromate.

4.2.3 Lugol's solution (Lugol's iodine).

4.2.4 Sodium hypochlorite (5,25 % NaOCl).

4.2.5 Sodium hydroxide solution (400 g/L NaOH).

5 Technical equipment

5.1 Adequate apparatus for temperature control.

5.2 Microscope.

5.3 Binocular stereoscope.

5.4 Centrifuge.

5.5 Air pump.

5.6 Single channel pipettes.

5.7 Laboratory balance.

5.8 Laboratory water purification system.

5.9 Laboratory oven.

5.10 Laboratory autoclave.

5.11 Centrifuge.

5.12 Sonication system.

5.13 Hot plate stirrer.

5.14 Oxygen meter.

5.15 Thermometer.

5.16 pH meter.

5.17 Salt meter (salinity meter).

5.18 Multiparameter photometer.

5.19 Light source.

5.20 Adequate apparatus for the control of the lighting regime and measurement of light intensity (lux meter).

5.21 Equipment for the determination of total organic carbon.

5.22 Equipment for the determination of chemical oxygen demand (COD).

6 Preparation and characterization of dispersion of nanomaterial

6.1 Dispersion preparation

Most nanomaterials tend to agglomerate/aggregate strongly in water, and this situation could be exacerbated in salt water. Before assessing the toxicity of a MNM using *Artemia sp*., the MNM shall be well dispersed in artificial seawater. The preparation of the MNM dispersion should be well documented, preferably via a standard operating procedure (see References [36] and [37]), as this step in the testing is known to impact on the tested material. Dispersion is often done in a two-step procedure, first a stock suspension is prepared, and then an aliquot of this is further diluted when the testing starts. Dispersion of MNMs in stock suspension can be achieved by stirring, sonication, or by means of functionalizing groups as well as using biocompatible dispersant reagents. Sonication should be carried out in a way that produces no other new materials and the effects of sonication should be evaluated. Chemicals that would have a detrimental effect on *Artemia sp.* should be avoided. When such vehicles are used, an additional control should be exposed to the same concentration of the vehicle as that used in the most concentrated suspension of the test nanomaterial. The concentration of organic solvents, emulsifiers or dispersants should not exceed 100 mg/l.

6.2 Dispersion characterization

The dispersion state of the nanomaterials should be characterized with DLS (dynamic light scattering) as described in ISO 22412 or other suitable methods such as ultrasonic attenuation spectroscopy as described in ISO 20998-1.

6.3 Dispersion stability in stock suspension

The size distribution of the nanoparticle dispersion and its stability over time shall be characterized in relevant intervals. Also the concentration of the nanoparticle in the stock suspension should be evaluated using an appropriate method. In the case of metal-based nanomaterials, which tend to convert to metal ions and vice versa, the proportions of metal ions and nanoparticles should be determined.

6.4 Dispersion stability in artificial seawater

The stability of the dispersion and the actual concentration of the MNM in the artificial seawater shall be verified at an appropriate range of concentrations during experiment (6 h, 24 h and 48 h). Since the sea water would possibly influence the physico-chemical properties of the test nanomaterials, further characterizations of the test nanomaterial are recommended. In this regard, degree of aggregation/agglomeration (or changing of particle size distribution), and the amount of metal ions in the exposure media shall be evaluated.

6.5 Preparation of exposure media for toxicity tests

All the exposure media are prepared fresh from stock suspensions (e.g. 1 000 mg/L). Appropriate volumes of stock suspension should be added directly to sterilized artificial seawater to achieve relevant concentrations of the MNM in exposure media. Although *Artemia sp*. do not need sterile conditions for hatching and growth, efforts should be made to minimize the development of unicellular algae and bacterial contamination. Sterile filtration is the best method of sterilizing artificial seawater without altering its chemistry and therefore is recommended for sterilizing artificial seawater. 0,1 µm filters can be used to yield water free of bacteria.

7 Hatching procedure

7.1 General

Thoroughly wash the hatching and culture vessels with a light chlorine solution, rinse, and allow air-drying between uses. Avoid soap, because it will leave a slight residue that will foam from aeration during hatching, and leave cysts stranded above the water level. Although complete sterility of the material is not necessary, efforts should be made to minimize the development of unicellular algae and bacterial contamination.

7.2 Dilution water

7.2.1 A standard artificial seawater with salinity of 35 ± 1 g/L should be used for the hatching as well as for all toxicity tests. After aeration and stabilization for 24 h, the dilution water should have a pH of 8,0 ± 0,5, and the oxygen content should be at least 90 % saturation. If necessary, the pH should be adjusted with concentrated HCl or NaOH. Before using the salt water, it should be filtered through a 1 µm filter.

7.2.2 The current international standard for making artificial seawater is ASTM D1141-98. It can be found at ASTM international.[38] Chemical composition of ASTM artificial seawater consists of NaCl (24,53 g/L), MgCl2 (5,20 g/L), Na2SO4 (4,09 g/L), CaCl2 (1,16 g/L), KCl (0,695 g/L), NaHCO3 (0,201 g/L), KBr (0,101 g/L), H3BO3 (0,027 g/L), SrCl2 (0,025 g/L), and NaF (0,003 g/L).

7.3 Storage of cysts

Sealed cans of *Artemia sp.* cysts can be stored for years at room temperature, but, once opened, should be used within two months. After each use, the can should be tightly covered with a plastic lid and stored in a refrigerator. If the entire contents of a can will not be used in two months, the portion that is expected to be unused should be placed in a tightly closed container and frozen until needed.

7.4 Disinfection of *Artemia sp.* cysts

7.4.1 Prepare 200 ppm sodium hypochlorite (NaOCl) solution.

7.4.2 Soak cysts for 30 min at a density of 50 g cysts per litre.

7.4.3 Thoroughly wash cysts three times with distilled water on a 125 µm screen.

7.4.4 Cysts are ready for hatching incubation.

7.5 Hatching method of *Artemia sp.* cysts

7.5.1 Use a transparent or translucent (preferably colourless) hatching vessel.

7.5.2 Supply air through an open aeration line down to the tip of the conical part of the vessel.

7.5.3 Add 1 000 ml artificial seawater to the vessel.

7.5.4 Adjust temperature to in the range of 27° C and 29 °C.

7.5.5 Adjust pH between 8 to 8,5 by adding appropriate amount of sodium bicarbonate or carbonate.

7.5.6 Apply minimum illumination of 2 000 lx at the water surface.

7.5.7 Incubate disinfected cysts at density of 2 g/l.

7.5.8 After 24 h incubation, nauplii generally are ready to harvest.

NOTE Hatching time varies with incubation temperature and the geographic strain of *Artemia sp.* used.

7.6 Harvesting of nauplii

7.6.1 Switch off the aeration for five to 10 min. Cyst shells will float and can be removed from the surface, while nauplii and unhatched cysts will concentrate at the bottom.

7.6.2 Since nauplii are positively phototactic, their concentration can be improved by shading the upper part of the hatching vessel and focusing a light source on the transparent conical part of the bottom.

7.6.3 Collect the concentrated nauplii by siphoning on a filter using a fine mesh screen (<150 µm), which should be submerged all the time so as to prevent physical damage to the nauplii.

7.6.4 Rinse collected nauplii thoroughly with artificial seawater in order to remove possible contaminants and hatching metabolites like glycerol.

7.6.5 Prepare fresh salt water for each new hatch.

7.7 Calculation of hatching percentage

7.7.1 Hatching percentage is the number of nauplii that can be produced under standard hatching conditions from 100 healthy cysts.

7.7.2 After 24 h, take 6 250 µl sub-samples out of each incubator. Pipette each into a small vial and fix samples by adding a few drops of Lugol's solution (aqueous solution containing 2 g of potassium iodide (KI), 1 g of iodine (I2) and 100 g of water (H_2O)).

7.7.3 Using a stereomicroscope, count the number of hatched nauplii in each sub-sample and calculate the mean number (N). Also, count the number of umbrella stage embryos in each sample and calculate the mean number (U).

7.7.4 Decapsulate the unhatched cysts and dissolve the empty cyst shells by adding one drop of sodium hydroxide solution and five drops of 3 % – 6 % sodium hypochlorite solution to each vial.

7.7.5 Using a stereomicroscope, count the unhatched (orange) embryos in each sub-sample and calculate the mean number (E).

7.7.6 Calculate the hatching percentage (H %) for each sub-sample: H % = (N x 100) ÷ (N + U + E). Calculate the mean of six replicates.

8 Effect of nanomaterial on *Artemia sp*. nauplii

8.1 Test groups and controls

8.1.1 Test vessels are filled with appropriate volumes of artificial salt water and suspensions of the test sample. Volume of the container should be at least 5 ml per 5 animals.

8.1.2 Nauplii are then placed into test vessels. At least 20 animals, preferably divided into four groups of five animals each, should be used at each test concentration and for the controls.

8.1.3 The test may be carried out using a semi-static renewal when the concentration of the test nanomaterial is not stable.

8.1.4 One control series and also, if relevant, one control series containing the dispersant reagents at the level used in treatments, shall be run in addition to the treatment series.

8.2 Test concentrations

8.2.1 A range-finding test may be conducted to determine the range of concentrations for the definitive test. For this purpose, the nauplii are exposed to a series of widely spaced concentrations of the test nanomaterial. At least five nauplii should be exposed to each test concentration for 48 h or less, and no replicates are necessary. The exposure period may be shortened (e.g. 24 h or less) if data suitable for the purpose of the range-finding test can be obtained in less time.

8.2.2 At least five test concentrations should be used. They should be arranged in a geometric series with a separation factor not exceeding 2,2. Justification should be provided if fewer than five concentrations are used. The highest concentration tested should result in 100 % immobilization, and the lowest concentration tested should cause no observable effect.

8.3 Exposure condition

8.3.1 The water temperature should be within the range of 25 °C and 28 °C, and for each single test it should be constant within ± 1 °C. A 16 h light and 8 h dark cycle is recommended. Complete darkness is also acceptable, especially for the light sensitive nanomaterials.

8.3.2 The nauplii should not be fed during the test.

8.4 Duration

The test duration is 48 h.

8.5 Observations

Each test vessel should be checked for immobilized nauplii at 24 h and 48 h after the beginning of the test. In addition to immobility, any abnormal behaviour or appearance should be reported.

8.6 Analytical measurements

8.6.1 At least at the beginning and end of the test, the dissolved oxygen and pH are measured in the control(s) and in the highest test nanomaterial concentration. The pH should normally not vary by more than 1,5 units in any one test. The temperature is usually measured in control vessels or in ambient air, and should be recorded continuously during the test or, at a minimum, at the beginning and end of the test.

8.6.2 At the beginning and end of the test, the total concentration of the test nanomaterial should be measured in at least the vessels that contain the highest and lowest test concentrations. Results should be based on measured concentrations. However, if evidence is available to demonstrate that the concentration of the test nanomaterial has been satisfactorily maintained within ± 20 %of the nominal or measured initial concentration throughout the test, then the results can be based on nominal or measured initial values.

8.6.3 At the beginning and end of the test, the stability of the dispersion and the size distribution of the nanoparticle shall be verified in at least the test vessels that contain the highest and lowest test concentrations.

9 Data analysis

9.1 Data should be summarized in tabular form, and show for each treatment group and control the number of nauplius used, and the percentage immobilized at each observation. The percentages immobilized at 24 h and 48 h are plotted against test concentrations. Data are analysed by appropriate statistical methods (e.g. probit analysis, etc.) to calculate the slopes of the curves and the EC_{50} with 95 % confidence limits (P = 0,95)[40] [41].

9.2 Where the standard methods of calculating the EC_{50} are not applicable to the data obtained, the geometric mean of the highest concentration causing no immobility and the lowest concentration producing 100 % immobility should be used as an approximation for the EC_{50}.

10 Test report

10.1 Test procedure

The test report shall be in accordance with the test procedures used.

10.2 Information to include in the report

10.2.1 Test nanomaterial

— Complete physical-chemical characteristics of test nanomaterial (e.g. shape, purity, size, etc.) according to ISO/TR 13014;

— Particle morphology of test nanomaterial using TEM or SEM;

— Substance of test nanomaterial (manufacturer's code, catalogue or formulation number, batch number or date of manufacture, trade-name, etc.);

— All equipment and instrumentation used (manufacturer's model or catalogue number, serial number or date of manufacture, brand-name, etc.);

— Dispersion characterization and stability of test nanomaterial in stock suspension as well as in artificial seawater.

10.2.2 Test species

— The origin of the *Artemia sp*. strain, species and, if possible, the batch number of the commercial brand of the cysts;

— The larval (naupliar) stage (Instar I, II, etc).

10.2.3 Test conditions

— Description of test vessels: type and volume of vessels, volume of solution, number of nauplii per test vessel, number of test vessels (replicates) per concentration;

— Methods of preparation of stock and test solutions, including the use of any solvents or dispersants, concentrations used;

— Details of dilution water: source and water quality characteristics (at least pH and salinity);

— Incubation conditions: temperature, light intensity and periodicity, dissolved oxygen, pH, etc;

— The nominal test nanomaterial concentrations and the results of all analyses to determine the total concentration of the test nanomaterial in the test vessels.

10.2.4 Bioassay results

— The number and percentage of nauplii that were immobilized or showed any adverse effects (including abnormal behaviour) in the controls and in each treatment group, at each observation time;

— The calculated 48 h EC_{50} with the 95 % confidence limits;

— The data confirming the validity of the results:

  — EC_{50} of potassium dichromate,

  — Mortality percentages of the controls.

11 Results validity

— The test can be considered valid if the following conditions are fulfilled:

  — In the control, including the control containing the dispersant reagent, not more than 10 % of the nauplii should have been immobilized;