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Water quality — Determination of acute toxicity of marine or estuarine sediment to amphipods

Qualité de l'eau — Détermination de la toxicité aiguë des sédiments marins et estuariens vis-à-vis des amphipodes

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ISO copyright office
Case postale 56 • CH-1211 Geneva 20
Tel. + 41 22 749 01 11
Fax + 41 22 749 09 47
E-mail copyright@iso.org
Web www.iso.org

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Foreword

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International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 2.

The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

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ISO 16712 was prepared by Technical Committee ISO/TC 147, *Water quality*, Subcommittee SC 5, *Biological methods*.

Introduction

This International Standard outlines procedures for conducting acute tests for sediment toxicity, using one or more amphipod species that are found primarily below the sediment surface in coastal marine and estuarine waters. The biological endpoint for the test is percent mortality at day 10.

Water quality — Determination of acute toxicity of marine or estuarine sediment to amphipods

1 Scope

This International Standard specifies a method for the determination of acute toxicity to amphipods exposed over a period of 10 d to

a) samples of contaminated marine or estuarine sediment,

b) chemical, industrial or municipal sludge, or other solid wastes that may combine with marine or estuarine sediments, or

c) chemicals or preparations spiked into clean sediment.

2 Principle

Marine or estuarine amphipods which typically live below the sediment surface are exposed for 10 d to contaminated sediment or to sediment spiked with a test chemical. The endpoint for the test is percent mortality. The test is performed in 1-litre vessels with 175 ml of solid-phase sediment and overlying water. Salinity and temperature are dependent on the species of amphipod used in testing.

3 Test environment

3.1 Facilities

The test facility shall be well ventilated, isolated from physical disturbances and free from dust and fumes.

3.2 Lighting

All test vessels shall receive direct, overhead illumination that provides normal laboratory lighting (i.e. 500 lx to 1 000 lx) at the water surface. Illumination should be uniform and shall be continuous throughout the test period to inhibit the nocturnal migration of amphipods into the water column[39].

4 Reagents and materials

4.1 Test organism

4.1.1 General

One of the marine or estuarine sediment-dwelling amphipod species listed in Annex B should be used as test organism for the method in this International Standard. The species identification should be conducted using taxonomic keys[18] and confirmed by a qualified taxonomist familiar with identifying marine or estuarine amphipods.

4.1.2 Life stage and size

Amphipods of uniform age and size shall be used for testing and shall not be larger than the maximum allowable species size listed in Annex B. Do not use mature females bearing embryos, nor individuals longer than the maximum length (including antennae) identified in Annex B, as they might be senescent.

4.1.3 Source

All amphipods used in a test shall be derived from the same population and source. Test organisms can be either recently collected from an area in which contaminants are at or below background levels, or organisms can be cultured in a laboratory [11], [12], [48].

4.1.4 Collection, handling and transport

Depending on species and/or collection site conditions, collect amphipods using a benthic grab[1)], a small biological dredge or, in inter-tidal zones, a shovel. If a dredge is used, a short haul ($<$ 10 m) minimizes damage to the animals[39]. Collect at least one-third more individuals than are required for the test. Choose a collection site for which the presence of abundant organisms of the correct size and age has been demonstrated previously, or by pre-collection sieving of the sediment at the site[31]. The organisms to be used as the test species should be confirmed taxonomically (e.g. references [4], [5], [32]).

Measure and record the salinity, temperature and dissolved oxygen content of water near the sediment at the collection site. Sieve sediment samples at the time of collection through a sieve of mesh 0,5 mm to 1,0 mm. The choice of sieve size depends on the size of the species to be collected and is important for determining the number of amphipods recovered. The sieve shall be made of non-toxic materials.

Use collection-site water for sieving sediment in the field, and to cover the sediment in the container(s) during specimen collection and transport. Discard detritus and predators recovered by sieving. Transport the collected amphipods either at a cool temperature, with only moist inert material such as seaweed in the transport container, or with overlying water and the amphipods returned to the sieved sediment in the transport container(s). Aerate overlying water during transport. Deliver an additional portion of the sieved sediment to the laboratory to use for holding amphipods and as control sediment. Reserve a portion of sediment for physical analysis (e.g. grain-size) and chemical analyses. Alternatively, collect and transport amphipods in bulk sediment without sieving at the field site. However, predatory organisms shall be removed by hand-picking from containers before shipment.

Efforts should be made to maintain site-water collection temperature and salinity during transport. Temperature in the transport container shall not rise above the optimum range for specific amphipod species, as outlined in Annex B. Overlying seawater shall be aerated during transit.

4.1.5 Holding and acclimation

If necessary, field-collected specimens may be re-sieved upon return to the laboratory (0,5 mm to 1,0 mm screen, depending on size of amphipods to be used in the test), if one wishes to assess their survival and condition, to confirm species, and to select and count numbers of amphipods of a size suitable for testing. However, it shall be noted that re-sieving of field-collected amphipods in the laboratory puts an additional stress on the organisms. Use seawater from the collection site, another field site or reconstituted seawater, as overlying water in the transport container, maintaining the original salinity (within $\pm$ 2 g/kg) and temperature (within $\pm$ 2 °C) of the collection-site seawater during transport.

In the laboratory, slowly agitate a sieve immersed in seawater to isolate organisms and move them using a wide-bore pipette, spoon, or fine net. Ensure that sieved organisms are submersed in seawater at all times. To minimize stress, handle organisms carefully and quickly. Amphipods that are dropped, injured, or in contact with dry surfaces shall be discarded. Only active and apparently healthy amphipods shall be transferred into the

1) Smith-McIntyre and van Veen are examples of suitable products available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of these products.

holding/acclimation containers. Depending on the species, any individuals that fail to burrow or that emerge from the sediment at any time during the holding/acclimation period and appear dead or inactive when gently prodded shall be discarded. On the day of a test, select amphipods that are active and apparently healthy, and which have an appearance and behaviour typical of that species. Discard any animals that appear or behave atypically.

Count the amphipods selected for use in tests as they are transferred into holding containers (e.g. plastic trays or glass finger bowls). Place at least 2 cm to 4 cm of previously re-sieved control sediment (free of small amphipods and other organisms) and at least 2 cm to 5 cm of overlying seawater in these containers. The density of macroorganisms in the sediment should not exceed either that observed in the field or one amphipod per cm^2 to avoid crowding.

Place holding containers with organisms in one of the following:

a) a tank or trough with flowing seawater;

b) a large aquarium (e.g. 60 l to 100 l) containing reconstituted seawater or natural, clean seawater held under static conditions;

c) a smaller aquarium (e.g. 20 l to 40 l) containing seawater held under semi-static conditions (e.g. with daily renewal of 50 % of the seawater), unless a recycled water system with proper water treatment is used, in which case daily renewal of seawater is not required; or

d) a separate room with the appropriate temperature and lighting conditions.

A photoperiod of 16 h light and 8 h dark is recommended during amphipod holding/acclimation. The seawater in which holding containers are submersed should be aerated.

Field-collected or cultured amphipods shall be acclimated to test temperature and salinity conditions for a minimum of 3 d. Upon their arrival in the laboratory, acclimate amphipods from the field salinity conditions to the test salinity conditions by changing the salinity in the holding container at a rate of 5 g/(kg × d) (or slower depending on the species to be used). Acclimation of amphipods to test temperature conditions, within the holding/acclimation container, should not occur at a rate of temperature increase greater than 3 °C per day. Once test salinity conditions have been reached, hold organisms at that salinity for at least 24 h before testing.

Temperature, salinity, pH and dissolved oxygen content shall be monitored and recorded daily during the initial acclimation period, when the amphipods are acclimating to the test conditions. Thereafter temperature, salinity, pH and dissolved oxygen content should be measured during the remaining acclimation period, and shall be measured and recorded at the end of the acclimation period. Replace the overlying water continuously or periodically (i.e. daily or every second day) with air-saturated, fresh seawater adjusted to the required temperature and salinity. While the minimum duration of the holding/acclimation period for amphipods is 3 d, the holding period for field-collected test organisms shall not exceed 14 d before use in a test. The maximum holding time does not apply to laboratory-cultured test organisms. Amphipods shall not be fed during their period of acclimation or under test conditions.

4.2 Overlying water

4.2.1 General

Amphipods are to be held and acclimated using either an uncontaminated supply of natural seawater, or reconstituted seawater. The seawater supply used should be monitored and assessed as frequently as required to document its quality. Measure the salinity, pH, dissolved oxygen content, ammonia nitrogen, nitrite, relevant pesticides and metals of the seawater used.

De-ionized or distilled water is preferred for preparing reconstituted seawater. Dechlorinated municipal drinking water, natural surface water or groundwater may also be used.

Seawater used for holding, acclimating and testing amphipods shall be free of suspended matter. It is recommended that seawater be filtered ($< 5\ \mu m$) before use to ensure the removal of suspended particles and organisms. If stored, hold natural seawater within the range of temperature appropriate for the test species (see

Annex B) and use within a few days. For laboratories that have water treatment systems such as sand-bed filters, seawater may be held for longer periods as long as the quality of the water is closely monitored.

Prepare reconstituted seawater by adding hypersaline brine (HSB) or by direct addition of dry salts to a suitable fresh water, in quantities sufficient to provide the desired salinity[22]. HSB may also be prepared using commercially available dry ocean salts or reagent-grade salts[47], or by using reagent-grade chemicals to produce reconstituted salt water (Annex A). Reconstituted water should be homogeneous and aged for 1 week to 2 weeks [1], [2], then filtered ($\leqslant$ 5 μm) shortly before use to remove suspended particles, and should be used within 24 h of filtration[46].

Reconstituted seawater is prepared by adding specified amounts of a suitable salt reagent to high-purity distilled or de-ionized water[47]. Suitable salt reagents can be reagent grade chemicals or commercial sea salts. Pre-formulated brine (e.g. 60 % to 90 %) prepared with dry ocean salts or heat-concentrated natural seawater can also be used.

4.2.2 Salinity

The choice of the appropriate test salinity conditions depends on the salinity of the pore water of the test sediment, the range of salinity tolerance for the test species[10] and the test objectives. For evaluations of marine or estuarine sediments, the acclimation and test salinity can range between 1 g/kg and 35 g/kg, depending on the test species chosen (see Annex B). Salinity can be adjusted by the addition of dry ocean salts or brine (if too brackish), or distilled water (if too saline).

4.2.3 Dissolved oxygen content

The dissolved oxygen content of the seawater overlying the sediment shall be 85 % of the air-saturation value or higher during the test organism holding or acclimation period, at test initiation and throughout the 10-d test. Maintain this level of dissolved oxygen by gentle aeration of the seawater, using filtered, oil-free compressed air, but the rate of aeration should not suspend the sediment.

5 Apparatus

Use ordinary laboratory apparatus and the following for organism holding or culturing and testing[3], [18], [31], [46]. Before initiating a test, ensure all test vessels and associated labware are clean and free of all contaminants from previous use[1], [28].

5.1 Environmental controls, apparatus to control temperature and light intensity.

5.2 Measuring apparatus and/or instruments for measuring dissolved oxygen content, pH, salinity, total organic carbon, ammonia, nitrate, light intensity and temperature.

5.3 Containers

Containers and accessories, such as sieves, that might contact the organisms, control or test sediment, and seawater during sorting, handling, holding and acclimation shall be made of non-toxic materials (e.g. glass, stainless steel, polyolefin, nylon, porcelain, polyethylene, polypropylene, fibreglass) cleaned and rinsed with distilled water, de-ionized water, dechlorinated laboratory water, reconstituted seawater or natural seawater from the collection site or an uncontaminated source.

Materials such as copper, zinc, brass, galvanized metal, lead and natural rubber shall not come in contact with this apparatus and equipment, or with samples of control, reference or test sediment, seawater, or test vessels.

1-litre glass containers (beakers or wide-mouthed jars) with internal diameter of approximately 10 cm are recommended for use as test vessels. Cover each vessel with a glass or a plastic lid to reduce the possibility of contamination of the contents and to reduce evaporation.

6 Treatment and preparation of samples

6.1 General

Collect sediment from reference, control and test sites following established practices[1], [3], [18], [19], [20] or, if required, add a test chemical or preparation to a sample of control sediment[1], [18], [20]. Similar sediment collection and handling procedures for both test and reference sediments should be used within the same testing programme. Store collected sediment in a sealed container in darkness at 4 °C ± 2 °C until required for the toxicity test. Drying, freezing and cold storage all affect toxicity and bioavailability of chemicals in sediment. Initiate sediment tests as soon as possible to maintain chemical integrity, but preferably within 5 d and not after 30 d unless chemical stability can be assured. Analysis of known chemical contaminants may be conducted on sediment samples from the field, and results may be compared to analysis of sediment at the beginning and end of the test to quantify any changes in chemical concentration or form.

6.2 Control or reference sediment

Control sediment obtained from the amphipod-collection site can be used as the negative-control sediment for a test, as a clean material for spiking a test chemical, or for organism culture. Clean reference sediment can be used as an additional experimental control.

6.3 Test sediment

Collect test sediment from the site to be evaluated using apparatus such as coring[2)] or grab[3)] devices. Sediment is taken from the middle of the sampler that has not been in contact with the apparatus. Typically, the top 2 cm to 4 cm of sediment representing the oxic zone is collected and composited from sufficient samples of the site to meet the needs of the test. A deeper depth of sediment may be collected for testing depending on the objective of the study. Transfer the sediment with a non-reactive, pre-cleaned scoop to an inert vessel, and mix the composited sample until colour and texture are uniform. Store the composited sample in a clean brown glass container (if organics are suspected contaminants), or in a clean high-density polyethylene or polycarbonate container (if metals are suspected contaminants). Fill containers to capacity and transport to the laboratory at 4 °C ± 2 °C.

Apparatus should be cleaned between sites to prevent cross-contamination (see Clause 5). Retain any solvent cleaning wastes and return to the laboratory for disposal.

6.4 Preparation of sediment samples

Remove large particles (> 1 cm) and indigenous organisms by hand sorting using tweezers or a similar instrument. Sieving the sediment for this purpose is not recommended, because water-soluble contaminants and fine non-settling clay particles could be lost. Adjust the water to the test temperature and test salinity appropriate for the test species (see Annex B) and aerate to a dissolved oxygen content of 85 % saturation or higher. If control/reference sediment is to be used at the completion of the test for determining the ability of surviving amphipods to rebury, then re-seal and refrigerate the required sediment.

Chemical and physical characterization of the sediment sample is helpful in the interpretation of results. Allow the sieved sediment to settle for at least 4 h to recover fines, before submitting it for particle size and chemical analyses. Analyse a sub-sample of the sediment for the following: total organic carbon and particle size distribution (percentage gravel, coarse and fine sands, and silt and clay), and pore water salinity (before sieving in the laboratory). Further characterization can include total volatile residue, acid-volatile sulfides (AVS)/simultaneously extracted metals (SEM), percent water content, biochemical and/or sediment oxygen

2) A Phleger box is an example of a suitable product available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of this product.

3) Ekman, Ponar, van Veen, Petersen, Shipek and Kajak-Brinkhurst are examples of suitable products available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of these products.

demand, total metals, total chlorinated organic content, chlorinated organic compounds, polycyclic aromatic hydrocarbons, and oil and grease[3], [18]. Pore water can also be sampled and analysed for pH, total ammonia, hydrogen sulfide and dissolved contaminants. Samples of pore water can be obtained from control or reference sediment by *in situ* methods (e.g. peepers) or *ex situ* methods (e.g. centrifugation or squeezing)[1], [19], [35].

Mix each sediment sample thoroughly the day preceding the test and add a 175 ml aliquot of sediment to a test vessel (see 6.3). Distribute test sediment in a uniform layer, which allows amphipods to burrow (minimum 2 cm in depth). Prepare a minimum of three laboratory replicates for each treatment or test sample, plus one or more replicate treatments for monitoring chemical characteristics of the sediment and the test water during the test. Replicates set up for monitoring of chemistry shall be seeded with amphipods, as in test replicates.

Smooth the sediment surface flat in each treatment and ensure there is minimal disturbance of the test or control sediment during the addition of overlying water. One technique for minimizing the disruption of sediment is to add the seawater by pouring over a disk (polyethylene, nylon or polytetrafluoroethylene sheeting of 4 mm to 6 mm thickness) lying on the sediment surface that fits the inside diameter of the test vessel[3], [18]. Add seawater to each test vessel to make up a total (water plus sediment) volume of 900 ml. Remove the disc and rinse with seawater between replicates of a treatment. Use a separate clean disk for each treatment.

If the objective of the study is to spike a sediment with a chemical or preparation, procedures that explain the steps involved in sediment-spiking, homogenization, chemical equilibration verification and data analysis are available (see References [1], [18], [20]). Some guidance on spiking sediments for testing is provided in Clause 9.

7 Test procedures

7.1 Preparing the exposure vessels

Each test vessel shall be clearly coded or labelled to identify the test substance/concentration, date and time the test was started. The treatments should be positioned for easy observation of amphipods. Preferably, the test treatments should be placed in randomized order, or in a random block design with one replicate treatment in each block[3], [18].

Cover each test treatment (including the controls) with seawater and allow any disturbed sediment to settle prior to beginning the test. Aerate the seawater overnight or for at least 2 h before the test amphipods are introduced, and throughout the duration of the test, using a glass or plastic pipette with the tip suspended 2 cm to 4 cm above the surface of the sediment layer. Aeration to each test treatment shall be continuous at a gentle rate, so that the sediment surface is not disturbed.

7.2 Introducing the organisms

Initiate the toxicity test by placing 20 amphipods in each test vessel (i.e. 20 amphipods per replicate of each treatment).

Remove active amphipods from the sediment in the holding container (see 4.1.5) using a sieve with mesh 0,5 mm or larger (depending on the size of amphipods to be used in test, see Annex B). Select individuals randomly using a transfer pipette or other suitable device, and distribute them sequentially among dishes containing $\leqslant$ 150 ml of test water. For example, add one amphipod to each dish, then two to each dish, and so on until each dish contains 20 individuals. Confirm the number of amphipods in each dish by recounting.

Transfer amphipods to the test vessels by gently pouring the water and amphipods from the sorting dish. Use a disk as described in 6.4 to prevent disturbance of the sediment. Wash any amphipods remaining in the dish into the test vessel using more test water. Increase the volume in the test vessel to 900 ml (water plus sediment), remove the disc, cover the test vessel and continue aeration. Because replicate vessels are used, amphipods should be added at the same time to each set or block of test vessels representing each treatment, following a randomized block design.

Depending on species, replace any amphipods that have not burrowed into the sediment within 1 h, unless they are observed to repeatedly burrow into the sediment and immediately emerge in an avoidance response to the test substrate. Amphipods displaying this avoidance behaviour during the initial hour of the test should not be replaced.

7.3 Test conditions

The test shall be static (no replacement of sediment or overlying solution during the test).

Test duration shall be 10 d.

The test shall be conducted at a temperature and a salinity appropriate for the species chosen for testing (see Annex B).

Control and test sediments (175 ml) shall be distributed in a uniform layer at least 2 cm in thickness, with overlying seawater added to increase the total volume to 900 ml.

Solutions overlying sediment shall be aerated at a continuous, gentle rate.

Lighting shall be constant overhead illumination providing between 500 lx to 1 000 lx at the surface of the test sediments.

No supplementary feeding shall be provided during the test.

For testing of field-collected sediments, five replicate vessels per treatment is optimum, but a minimum of three laboratory replicates shall be tested for each treatment or test sample. For experiments in which a control sediment is spiked with a test substance, three replicate vessels, each containing 20 test organisms, is recommended.

7.4 Test observations and measurements

Examine each test vessel daily to confirm that the airflow to the overlying solution is uninterrupted and unchanged. Measure and record the temperature, salinity, dissolved oxygen (DO) content and pH of the overlying seawater at the beginning of the test in one or more replicate exposures. Measure and record temperature daily. Note whether amphipods are swimming in the overlying solution or floating at the water surface. Gently push amphipods caught in the surface film into the water using a glass rod or pipette. Dead animals are usually not removed during the test[8], [39].

For field-collected sediment samples, measure the salinity and pH of pore water in one or more replicate vessels set up for monitoring chemical characteristics of sediment, at least at the beginning of the test. Samples of pore water can be obtained by centrifugation, vacuum filtration or squeezing[1], [19]. Measurements of the ammonia, AVS/SEM and/or hydrogen sulfide content of sediments (including control and reference sediments) in replicate vessels of individual treatments, set up for this purpose, may assist in result interpretation. This is not required for spiked-sediment testing.

For any test intended to determine if sediment exposure causes an emergence response, record daily the number of organisms located either on the sediment surface, swimming in the overlying water, or floating at the water surface for each test vessel and prior to disturbance and sieving at the end of the test.

After 10 d, sieve the contents of each test vessel through a 0,5 mm screen to remove the test organisms and determine if they are dead or alive. Additional test water with a salinity and temperature within two units of that used in the test may be used for this sieving. Determine the total number of live and dead amphipods. Animals are considered to be dead if they fail to show any movement in response to gentle prodding (a low-power dissecting microscope or hand-held magnifying glass is useful for this examination).

7.5 Expression of results

Calculate the mean ($\pm$ standard deviation) percentage of amphipods that died during the 10-d exposure for each treatment.

The mean values of the replicates for each test sediment are then compared statistically with corresponding values for amphipods held in reference and/or control sediments under otherwise identical conditions.

7.6 Reburrowing capability

If one of the test objectives is to determine the effect of 10-d exposure to test sediment on the ability of surviving amphipods to reburrow in control sediment[3], [18], [39], [45], record the number of surviving amphipods completely or partially out of the sediment before sieving. Transfer all surviving amphipods to containers holding a 2 cm layer of control sediment [previously adjusted to the temperature appropriate for the species (see Annex B) and sieved through a 0,5 mm screen using test water] and an overlying layer ($>$ 2 cm) of test water. The number of surviving amphipods that are unable to reburrow in control sediment within 1 h is recorded for each test vessel. For some species (e.g. *Monoporeia affinis*), the reburrowing test option may be more difficult since not all species reburrow immediately.

If an additional objective of the test is to determine the reburrowing rate, calculate the mean ($\pm$ standard deviation) percentage of surviving amphipods that emerged from each solid-phase test sediment in $\leqslant$ 10 d and the mean ($\pm$ standard deviation) percentage of surviving amphipods that did not reburrow in control sediment upon termination of the exposure.

7.7 Validity of the test

Consider the test valid when mean survival rate among the control sediment organisms is 85 % or higher, and when survival is 80 % or higher in any single control replicate during the 10-d exposure period.

8 Analysis and interpretation of results

8.1 Data analysis

Using this biological test method, pair-wise comparisons of survival data for each test treatment are normally made against survival data derived for a particular reference or control sediment. Initially, test all data for normality using the Shapiro-Wilk's test, and for homogeneity of variance[16], [36] using Bartlett's test or other suitable tests[46].

If replicate treatments were used to compute the acute toxicity of a sediment sample with the toxicity of a single reference or control sediment, apply the Student's t-test[37]. If a set of data cannot meet the requirements for normality and homogeneity of variance, an arcsine-square root transformation should be applied. If the transformed data still do not meet the assumptions of normality, non-parametric statistics such as the Wilcoxon Rank Sum test[46] can be applied. If the transformed data meet the assumption of normality, Bartlett's test or Hartley's F test can be used to test the homogeneity of variance assumption.

For comparison of spatial variations in sediment toxicity using multiple samples, an analysis of variance (ANOVA), followed by Dunnett's test, William's test[50] ,[51], or other suitable procedure for multiple comparisons of each test sediment versus the reference and/or control sediments, can be undertaken following the necessary arcsine transformations, to determine if the endpoint values for different treatments differ significantly[3], [18], [21], [37], [46]. For further guidance on the statistical treatment of test data, refer to ISO/TS 20281[55] or Reference [23].

8.2 Non-contaminant factors

There are a number of non-contaminant factors that may influence amphipod survival in this test. The most important factors include sediment particle size and pore-water salinity, ammonia and hydrogen sulfide[9], [25], [26], [30], [43]. For most of the amphipod species listed in Annex B, the range of sediment particle size that can be tolerated is known[21], [46]. Concentrations of pore-water ammonia and/or hydrogen sulfide can be elevated in samples of dredged material or field-collected estuarine or marine sediment. The elevated levels might be due to organic enrichment from natural and/or anthropogenic sources. The known tolerance limits of the species of test organism should be considered together with the measured levels of toxic constituents when appraising their significance in influencing the results of a test[21], [43].

9 Reference toxicant

Tests with reference toxicants are useful to assess the relative sensitivity of the population of organisms intended to be used to study the toxicity of test substances, and the precision and reliability of data produced by the laboratory[17], [20]. Reagent-grade cadmium (as cadmium chloride), copper (as copper sulfate), ammonia (as ammonium chloride) and fluoranthene are recommended as reference toxicants for this test[3], [18], [20], [33], [41], [46]. Numerous studies have reported the acute lethal tolerance of marine or estuarine amphipods to cadmium, using seawater-only tests[14], [28], [39], [42]. Another option is a 3-d static LC50 test, using a range of concentrations of ammonia (as ammonium chloride at pH 8,0 $\pm$ 0,5) in seawater only (no sediment present)[33]. When conducting a seawater-only 4-d LC50 test with cadmium or 3-d LC50 test with ammonia, conditions and procedures shall be identical to those described in 6.3, except that sediment is not added to the test vessels.

The basic requirement for three replicates of each treatment (plus at least one replicate for monitoring sediment and water quality), for all test sediments, may be relaxed for tests with reference toxicants because of space restrictions and/or because of limited numbers of amphipods for the test. A minimum of two replicates and 10 (and preferably 20) amphipods should be exposed to each concentration of reference toxicant tested.

Alternatively, control sediment may be spiked with copper, cadmium or fluoranthene, and used to determine a 10-d LC50[20]. Any test with the reference toxicant should be initiated within 1 d of starting the 10-d assay with test substances. Toxicity tests using control sediment spiked with copper have been performed[7], [20].

When selecting a control sediment for spiking with a chemical, it should be demonstrated in advance of the final selection that $>$ 90 % of the test organisms survive in this sediment. At a minimum, candidate control sediment should be characterized for total organic carbon content, particle size distribution, pH, percent water and AVS concentration (if the reference toxicant is an inorganic compound). The moisture content of the control sediment shall be determined before spiking to standardize spiking on a dry mass basis.

Wet-spiking techniques are currently the most acceptable for the preparation of a spiked-sediment. A sediment suspension method [sediment mass to water volume ratio of 1:4 (on a dry mass basis)] is recommended. The mixture should be stirred at a moderate speed for a minimum of 4 h. After the mixing procedure, enough time for equilibration of the reference toxicant shall be ensured. If the minimum time for pore-water/sediment equilibration is not known, it is recommended that the spiked sediments be allowed to equilibrate for four weeks[20]. Each test concentration shall be prepared individually. No serial dilution of a spiked-sediment should take place[6]. The nominal concentration of the spiked reference substance should be confirmed by actual measurements of the substance in the test sediment through chemical analysis. This step confirms the accuracy of the spiking technique used. Guidance on the selection and use of reference toxicants for estimating the precision of toxicity tests is provided in two companion documents[17], [20]. While routinely performing tests with reference toxicant(s), laboratory personnel should prepare and update a separate control chart for each reference toxicant used.

10 Precision

Precision data are reported for information in Annex C.

11 Test report

The test report shall include the following information:

a) a reference to this International Standard (i.e. ISO 16712:2005);

b) name and location of test facility and date of the study;

c) identification of the individual(s) responsible for the test results;

d) name(s) or identity of the sample and sample source;

e) physical-chemical properties measured (e.g. moisture content, total and dissolved organic carbon content, AVS/SEM, volatile matter, particle size distribution, biochemical oxygen demand (BOD), sediment oxygen demand (SOD), pH, Eh, content of dissolved and total metals, organics);

f) procedures for collection, handling and storage of sediment, or procedures for spiking of sediment, if conducted;

g) source or culture identification and estimation of abundance, if collected in the field;

h) species and taxonomic verification;

i) procedures for collection, acclimation, holding or culture of organisms;

j) physical conditions (photoperiod, light intensity, temperature, salinity) and exposure method;

k) methods of chemical analyses and the analytical sample design;

l) description of culture medium;

m) exposure design (organisms per treatment, number of replicates, number of discrete samples);

n) description of preparation for sediment samples;

o) results of chemical and physical analyses of test sediments;

p) description of test conditions (dissolved oxygen content, temperature, pH, salinity, TOC/DOC, ammonia, nitrite, sulfide);

q) results on mortality of organisms (number and dates);

r) statistical analysis of mortality data; calculation of LC50, if appropriate;

s) comment on any other biological effects observed or measured (e.g. emergence, reburial);

t) description of any deviations from the method, and explanation thereof.

Annex A
(informative)

Reconstitued salt water

This annex is taken from ASTM E729:1996.

The following reagent-grade chemicals are added to 890 ml of water in the amount (mass) and order listed in Table A.1. Each chemical is dissolved before the next is added.

Table A.1 — Reagent-grade chemicals

Chemical	Mass
NaF	3 mg
$SrCl_2 \cdot 6H_2O$	20 mg
H_3BO_3	30 mg
KBr	100 mg
KCl	700 mg
$CaCl_2 \cdot 2H_2O$	1,47 g
Na_2SO_4	4,00 g
$MgCl_2 \cdot 6H_2O$	10,78 mg
NaCl	23,50 mg
$Na_2SiO_3 \cdot 9H_2O$	20 mg
Na_4EDTA[a]	1 mg
$NaHCO_3$	200 mg

[a] Tetrasodium ethylenediaminetetraacetate should be omitted when toxicity tests are conducted on metals. When tests are conducted with fish or bivalve mollusc larvae, zooplankton or crustaceans, the EDTA should be omitted, and the reconstituted salt water stripped of trace metals [15].

Dilute the resulting solution to 1 l and check that the salinity is 34 g/kg ± 0,5 g/kg and the pH is 8,0 ± 0,2. If the values are outside either of these ranges, repeat the preparation, taking care that the correct masses of constituents are weighed out and the weighing balance is properly calibrated.

Annex B
(normative)

Maximum length of amphipod species, optimal salinity and temperature ranges

Table B.1 lists the species of amphipod that are used in this International Standard.

Table B.1 — Species of amphipod

	Maximum length mm	Optimal salinity range g/kg	Test temperature °C
Atlantic species			
Amphiporeia virginiana	5	15 to 35	10 ± 2
Corophium volutator	12	10 to 30	15 ± 2
Gammurus locusta	5	20 to 35	15 ± 2
Ampelisca abdita	8	10 to 30	20 ± 2
Leptocheirus plumulosus	5	1,5 to 32	25 ± 2
Corophium arenarium	10	26 to 34	15 ± 2
Monoporeia affinis	10	2 to 20	4 ± 2
Pacific species			
Eohaustorius estuarius	5	4 to 34	15 ± 2
Eohaustorius washingtonianus	5	15 to 35	15 ± 2
Foxiphalus xiximeus	6	25 to 35	15 ± 2
Rhepoxynius abronius	5	25 to 35	15 ± 2
Grandiderella japonica	6	30 to 35	17 ± 6
Ampelisca abdita	8	10 to 35	20 ± 2
Mediterranean species			
Corophium orientale	4	0 to 36	15 ± 2

Species salinity and temperature information were obtained from References [3], [13], [18], [21], [24], [27], [32], [33], [38], [46].

Maximum length of candidate test species includes their antennae (see 4.1.2).

Annex C
(informative)

Precision data

As part of the BEQUALM quality assurance programme, 14 to 16 laboratories from the European Union participated in an interlaboratory round robin concerning two substances, the bactericide Bioban™[4)] and a pesticide Ivermectin™[4)], to validate the 10-d survival test using the amphipod species *Corophium volutator*. The within-laboratory and between-laboratory coefficient of variation for each round of testing is shown in Table C.1[29].

Table C.1 — Precision data for spiked sediment testing using *Corophium volutator*

Test substance	Number of participating laboratories	Within-laboratory coefficient of variation %	Between-laboratory coefficient of variation %
Bioban™ bactericide	12	7,9	24,2
Ivermectin™ — Round 1	14	6,7	28,0
Ivermectin™ — Round 2	14	8,8	20,2

To validate the 10-d marine and estuarine survival test method, the U.S. Environmental Protection Agency sponsored an inter-laboratory round robin involving 10 testing laboratories from the United States and Canada in 1995. The test substance used in all testing rounds was a contaminated marine sediment from Black Rock Harbour, Connecticut. Three marine or estuarine amphipod species, *Ampelisca abdita, Eohaustorius estuarius* and *Leptocheirus plumulosus*, were assessed in terms of mean survival and between-laboratory variability. The results from testing of 7 % Black Rock Harbour sediment are summarized in Table C.2[34].

Table C.2 — Precision data for contaminated sediment using three amphipod species

Species evaluated	Number of participating laboratories	Mean percent survival %	Standard deviation	Between-laboratory coefficient of variation %
Ampelisca abdita	6	70,7	27,0	38,9
Eohaustorius estuarius	8	68,1	14,7	21,6
Leptocheirus plumulosus	7	46,3	27,7	59,8

In 1991, Environment Canada sponsored an inter-laboratory round robin involving three testing laboratories testing four contaminated marine sediments with six species, *Eohaustorius estuarius, Amphiporeia virginiana, Corophium volutator, Eohaustorius washingtonianus, Foxiphalus xiximeus* and *Rhepoxynius abronius*. The focus of this method validation programme was to confirm test validity criteria and non-contaminant effects such as sediment particle size for each species[28].

4) Bioban™ and Ivermectin™ are examples of suitable products available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of these products.