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Water quality — Determination of toxicity of fresh water sediments using *Hyalella azteca*

Qualité de l'eau — Détermination de la toxicité des sédiments d'eau douce vis-à-vis de Hyalella azteca

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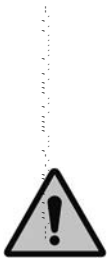

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Foreword

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.

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).

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

For an explanation on the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the WTO principles in the Technical Barriers to Trade (TBT) see the following URL: Foreword - Supplementary information

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

Introduction

Sediment in the aquatic environment serves as a reservoir for agricultural, industrial, and municipal contaminants. Contaminated sediment adversely affects the benthic community directly and acts as a source of contamination for the overlying water, often negatively impacting pelagic communities as well. Sediment toxicity tests are used globally to determine and monitor the toxic effects of discrete substances or complex mixtures that might be harmful to indigenous life in the aquatic and benthic environments. This International Standard outlines procedures for conducting 14 d and/or 28 d tests for sediment toxicity, using the fresh water amphipod *Hyalella azteca*. The biological end points for the tests include mortality and growth.

Water quality — Determination of toxicity of fresh water sediments using *Hyalella azteca*

WARNING — Persons using this International Standard should be familiar with normal laboratory practice. This International Standard does not purport to address all of the safety problems, if any, associated with its use. It is the responsibility of the user to establish appropriate safety and health practices and to ensure compliance with any national regulatory conditions.

IMPORTANT — It is absolutely essential that tests conducted in accordance with this International Standard be carried out by suitably trained staff.

1 Scope

This International Standard specifies a method for the determination of toxicity to young *Hyalella azteca* in whole sediment based on survival and growth inhibition after 14 d and/or 28 d.

The method is applicable to

a) samples of contaminated whole fresh water sediment,

b) chemical, industrial, or municipal sludge, or other solid wastes that may combine with fresh water sediments, and

c) chemicals or preparations spiked into clean sediment.

This International Standard is applicable to the testing of sediment samples from the fresh water environment. *Hyalella azteca* can be used in the testing of brackish waters up to a maximum of 15 ‰, with careful acclimation. This International Standard is not applicable to the testing of sediment samples from the marine and estuarine environment with a salinity of > 15 ‰.

This method is a 14 d and/or 28 d survival-and-growth test applicable to the sediment sample types described above.

2 Normative references

The following documents, in whole or in part, are normatively referenced in this document and are indispensable for its application. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 5814, *Water quality — Determination of dissolved oxygen — Electrochemical probe method*

ISO 6059, *Water quality — Determination of the sum of calcium and magnesium — EDTA titrimetric method*

ISO 10523, *Water quality — Determination of pH*

3 Terms and definitions

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

3.1
artificial sediment
mixture of materials that mimic the physical components of natural sediment

Note 1 to entry: See 6.2.1.

3.2
control sediment
sediment (natural or artificial) that is used to assess the performance of the test organisms and test acceptability (i.e. clean)

Note 1 to entry: The results of control sediment testing are used for comparison to response of organisms in the contaminated test sediment(s) and for evaluating test validity.

Note 2 to entry: Used routinely to assess the acceptability of a test (6.2).

3.3
test sediment
discrete portion of sediment (field collected or spiked) to be tested for possible effects on amphipods due to contamination

3.4
spiked sediment
sediment to which a material has been added for testing

3.5
reference sediment
field collected near an area of concern with properties representing sediment conditions that closely match those of the sample(s) of the test sediment except for the degree of contamination

Note 1 to entry: It is often selected from a site uninfluenced by the source(s) of contamination but within the general vicinity of the sites where samples of test sediment are collected. Test results provide a site-specific basis for evaluating toxicity.

3.6
intermittent renewal
tests in which test solutions or overlying water are renewed during the test, based on deterioration of water quality

3.7
overlying water
water placed over the layer of sediment in the test container

Note 1 to entry: Also used to manipulate the sediment, if necessary (e.g. for preparing formulated sediment or mixtures of spiked sediment).

3.8
growth
increase in dry weight of test organisms during the experiment and expressed as mean dry weight per surviving amphipod

4 Principle

Young fresh water amphipods, *Hyalella azteca*, aged 2 d to 9 d and ranging in age by 1 d to 2 d, are exposed in groups of 10 organisms to a contaminated sediment or a test-chemical spiked sediment for 14 d and/or 28 d.[1] [2] [3] The end points for the test are percent mortality and growth inhibition assessed relative to organisms exposed concurrently to control sediment. The test is performed in glass containers with the ratio of sediment to water (volume:volume) being either 1:1,75 or 1:4 (e.g. 100 ml of sediment with 175 ml of overlying water or 100 ml of sediment with 400 ml of overlying water). Comparative testing of the two recommended sediment-to-water ratios showed no significant difference in test results using *Hyalella azteca*.[4] One advantage of the 1:4 sediment-to-water ratio is greater overlying water volume for chemical analysis. The exposure is primarily static unless renewal is triggered by deterioration of water in the control treatment (e.g. shifting pH affecting the form of background ammonia).

A long-term test option (i.e. 42 d) for whole sediment toxicity testing using *Hyalella azteca* is described in Annex E. End points of this long-term test include survival (Days 28, 35, and 42), growth (Days 28 and 42), and reproduction (number of young per female produced from Days 28 to 42).

A water-only method using *Hyalella azteca* is also described in Annex F. This method is a 14-day test of survival and growth using young amphipods exposed to samples of industrial or sewage effluents, fresh waters (e.g. receiving water), aqueous extracts, or chemical substances which are soluble or which can be maintained as stable suspensions or dispersions under the conditions of the test.

5 Test environment

5.1 Facilities

The test facility shall be well ventilated, isolated from physical disturbances, and free from dust and fumes. Tests shall be carried out in a temperature-controlled room or chamber that maintains a temperature of (23 ± 2) °C in the test containers.

5.2 Lighting

All test containers shall receive direct, overhead illumination that provides normal laboratory lighting (i.e. 100 lx to 1 000 lx) at the air/water interface. Illumination should be uniform and shall have a day/night cycle (photoperiod) of 16 h of daylight and 8 h of darkness.

6 Reagents, test organisms, and materials

Use only reagents of recognized analytical grade, unless otherwise specified.

6.1 Test organism

Hyalella azteca is an epibenthic sediment-burrowing detritivore that lives in close contact with the surficial 1 cm or 2 cm of fresh water sediments. They reside in temperate lakes, ponds, and slow-flowing streams and are widely distributed on the North and South American continents.[1] [2] [3] Young *Hyalella azteca* are obtained from laboratory cultures maintained under the conditions of temperature, photoperiod, and food identical to those in the test. The species identification should be confirmed by qualified personnel experienced in identifying fresh water amphipods using the distinguishing taxonomic features described in Annex A and in previous publications.[2] [5]

6.1.1 Life stage and size

Amphipods used for the test shall be between the ages of 2 d and 9 d and shall not vary in age by more than 2 d. A method for culturing *Hyalella azteca* and for obtaining known-age test organisms is provided in Annex B. If growth is expressed as mean size at the end of the test, a mean length of organisms should be determined at test initiation.

6.1.2 Source

All amphipods used in a test shall be derived from the same population and source. Sources of animals to be used to establish cultures include government or private laboratories which are culturing *Hyalella azteca* for sediment toxicity tests or a reputable biological supply company.[1] [2] [3] A list of possible sources of *Hyalella azteca* is provided in Annex C.

6.2 Control sediment

Each sediment toxicity test shall include a control with a minimum of five replicate test containers containing control sediment. Responses of organisms exposed to control sediment during a test provide measurements for determining test validity (see 10.3), evidence of the health and normal behaviour of

the test organisms, and a basis for interpreting data derived from the test sediments. Control sediment is either natural sediment or artificial (i.e. formulated) sediment.

6.2.1 Natural sediment

Natural sediment taken from a fresh water or slightly brackish (< 15 ‰) collection site removed from known sources of contaminants, and for which there is known control performance with *Hyalella azteca*, can be used as the control sediment for a test or as a clean material for spiking a test chemical. If sediment pore water has any measureable salinity, the testing laboratory shall follow a suitable acclimation procedure to ready adult *Hyalella azteca* for use as brood organisms and to ensure salinity-adapted young amphipods are used during testing.

6.2.2 Artificial sediment

The following artificial sediment can be used as a control for fresh water sediment tests or as a clean material for spiking a test chemical. This recipe is based on the artificial sediment recommended in ISO 10872.

Mix the following components thoroughly in the given proportions.

Al_2O_3:	20 %
$CaCO_3$:	1 %
Dolomite (clay):	0,5 %
Fe_2O_3:	4,5 %
Silica sand (mean particle size 0,063 mm):	30 %
Silica sand (0,1 mm to 0,4 mm):	40 %
Peat (decomposed peat from a raised bog, untreated; finely ground and < 1 mm sieved):	4 %

There are a number of acceptable approaches to preparing and conditioning artificial sediment. In general, the following attributes should be considered when selecting a formulation for a control or test sediment.

a) should support the survival, growth, or reproduction of a variety of benthic organisms;

b) should provide consistent acceptable biological end points for a variety of species;

c) should comprise standard constituents that are readily available to test laboratories;

d) should be free from concentrations of contaminants that might cause adverse effects to test organisms.

Acceptable artificial sediment options are outlined in Environment Canada guidance on this subject, including techniques for spiking test chemicals into sediment.[6]

6.3 Overlying water

6.3.1 Natural fresh water

Natural fresh water includes an uncontaminated supply of groundwater or surface water. If the objective of testing is to simulate field site conditions, natural water can be diluted with a high purity distilled or deionized water until a desired hardness is achieved. Water taken from the site where sediment is collected can also be used. Surface water should be filtered through a fine-mesh net (e.g. 30 µm) to remove potential predators or competitors. Dechlorinated water is not recommended as overlying water because its quality is often quite variable and it could contain unacceptably high concentrations of chlorine, chloramines, fluoride, copper, lead, zinc, or other contaminants.

6.3.2 Reconstituted water

If reconstituted fresh water is used as overlying water in *Hyalella azteca* tests, the following artificial medium shall be prepared in deionized water:[7]

$CaCl_2$	110,98 mg/l
$NaHCO_3$	84,01 mg/l
$MgSO_4$	30,09 mg/l
KCl	3,728 mg/l
NaBr	1,029 mg/l

The mixture is aerated for 24 h before use to adjust the dissolved oxygen (DO) and to stabilize pH. The concentrations of salts can be adjusted to be of similar composition to a receiving water of interest. However, the Ca:Br ratio shall be kept constant because these ions are essential for *Hyalella azteca* and must be present together.[8] Na^+ and HCO_3^- are the most essential ions for *Hyalella azteca* survival, and Mg^{2+} and K^+ are needed for optimal growth and reproduction.[7] [9] Due to lack of confirmed influence, ranges for alkalinity and hardness have not been defined. Standard use of the above reconstituted water recipe in culturing and as testing water has confirmed acceptable alkalinity and hardness levels for *Hyalella azteca*. Conductivity, pH, hardness, DO, and alkalinity are measured in each batch of reconstituted water. A given batch of reconstituted water shall not be used for longer than 4 weeks.

6.3.3 Dissolved oxygen

The DO content of the water overlying the sediment is ideally to be 90 % to 100 % of the air-saturation value at test initiation and throughout the test period. This level of DO is maintained by gentle aeration using filtered, oil-free compressed air. The rate of aeration should not suspend the sediment (e.g. 2 bubbles/s to 3 bubbles/s).

6.4 Food

There are two food options for use in the *Hyalella azteca* test. Both commercial fish food and an inoculum of a mixture of yeast, Cerophyll™[1)], and trout chow (YCT) have been proven suitable for *Hyalella azteca* under the defined test conditions.[1] [2] [3] [10] [11] [12]

There are also two options for the frequency of feeding. Test organisms are fed either once daily or three times weekly (on non-consecutive days) throughout the test. An identical food ration is added to each test chamber on each feeding occasion.[11] The ration provided shall be adequate to enable acceptable survival and growth of *Hyalella azteca* during the test period, but must not be excessive.

6.4.1 Option 1: Fish food

Commercial fish food flakes (e.g. Tetrafin™, Tetramin™, or Nutrafin™)[2)] can be used as a food source for test organisms during the test. Food can be ground and sieved so that flakes are uniform in size or can be prepared as a slurry by mixing the fish flakes with clean water. Fish food flakes should be stored at room temperature in a sealed container.

If daily feeding is chosen, 2,7 mg of fish flakes (dry weight) is added to each test container on the first day of the test (the day the amphipods are placed in the test containers), as well as once per day thereafter until the day the test ends. If the option of feeding three times per week is chosen, 6,3 mg of fish flakes

1) Cerophyll™ can be obtained from Ward's Scientific as "Cereal Grass Media - Cerophyll". This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

2) Tetrafin™, Tetramin™, and Nutrafin™ are examples of fish food flakes available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of these products.

(dry weight) is added three times per week (starting on the first day of the test) to each test chamber on non-consecutive days (e.g. on Mondays, Wednesdays, and Fridays), until the day the test ends. Both rations provide approximately the same overall feeding rate; however, daily feeding might be preferred because food is then always available.[6]

6.4.2 Option 2: Yeast/Cerophyll™[3]/trout chow (YCT)

A second food combination based on the U.S. Environmental Protection Agency (U.S. EPA) and Environment Canada test methods[1] [2] is also recommended.

The formula for preparing YCT is given in Annex D. If daily feeding is chosen, an inoculum of 1,5 ml (equivalent to 2,7 mg food, dry weight) of a mixture of yeast, Cerophyll™[3], and trout chow is added daily to each test chamber on the first day of the test (i.e. the day the amphipods are placed in the test containers), as well as once per day thereafter until the day the test ends. If the option of feeding three times per week is chosen, an inoculum of 3,5 ml YCT (equivalent to ~ 6,3 mg food, dry weight) is added three times per week (starting on the first day of the test) to each test chamber on non-consecutive days (e.g. on Mondays, Wednesdays, and Fridays), until the day the test ends. Both rations provide approximately the same overall feeding rate; however, daily feeding might be preferred because food is then always available.

YCT can be stored frozen. Thawed aliquots of unused YCT can be stored in darkness at (4 ± 3) °C but shall be discarded after 14 d.

6.5 Reference substance

Cadmium chloride ($CdCl_2$), copper sulphate ($CuSO_4$), sodium chloride (NaCl), and potassium chloride (KCl) are all acceptable reference substances.[1] [2] [3] [10] Material Safety Data Sheets (MSDS) for these substances should be consulted prior to use by laboratory personnel, as necessary.

7 Apparatus

Ordinary laboratory apparatus is used for organism culturing and testing.

7.1 Temperature-controlled room, chamber, or water bath.

The system chosen shall maintain a temperature of (23 ± 2) °C in the test containers.

7.2 Measuring apparatus.

Use the apparatus and/or instruments for measuring DO, pH, hardness, conductivity, alkalinity, ammonia, light intensity, and temperature as specified in ISO 10523, ISO 5814, and ISO 6059.

7.3 Test containers.

All containers and accessories such as sieves that might contact the organisms, control or test sediment, and overlying water during sorting, handling, and testing shall be made of non-toxic materials (e.g. glass, stainless steel, Nalgene™[4] nylon, porcelain, polyethylene, polypropylene, fibre-glass) cleaned and rinsed with distilled water, deionized water, dechlorinated laboratory water, reconstituted water, or natural water from 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, overlying water, or test containers. Before initiating a test, ensure all test containers and associated labware are clean and free of all contaminants from previous use.

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3) Cerophyll™ can be obtained from Ward's Scientific as "Cereal Grass Media - Cerophyll". This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

4) Nalgene™ is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

Glass containers (beakers or wide-mouthed jars) with internal diameter of approximately 7 cm to 10 cm and a volume of 300 ml to 1 000 ml are recommended for use as test vessels. Test container shall allow a sediment depth of 1,5 cm to 2,5 cm, and a ratio of sediment to water (volume:volume) of 1:1,75 or 1:4. Each test container may be covered with a glass or a plastic lid to reduce the possibility of contamination of the contents, to reduce evaporation, and to minimize loss of volatiles from the sediment.

Prior to use, rinse the containers with the overlying water (6.3) or with deionized or distilled water.

7.4 Device, for delivering aeration of overlying water in each test container during testing, if necessary.

7.5 Pipette, for transferring *Hyalella azteca*, with a 5 mm to 6 mm diameter opening for capturing the animals while allowing transfer of only a small volume of water.

7.6 Binocular magnifying glass/dissecting microscope, with a magnification of at least 7,5 X.

7.7 Sieve(s), e.g. 300 µm or 425 µm, to remove *Hyalella azteca* from sediment at the end of the test.

7.8 Drying oven, capable of drying test organisms at 60 °C to 90 °C for 24 h prior to weighing.

7.9 Desiccator, for holding dried test organisms at test end during weighing.

7.10 Balance, capable of measuring *Hyalella azteca* to the nearest 10 µg.

8 Treatment and preparation of samples

8.1 General

Field-collect sediment from reference, control, and test sites following established practices (see ISO 5667-16 and References [1], [5], [10], [13], [14], [15], and [16]) or, if required, add a test chemical or preparation to a sample of control sediment.[2] [5] [13] [15] For site-specific investigations, similar sediment collection and handling procedures are to be used for both test and reference sediments. Sample containers are made of inert, non-toxic material and are new or thoroughly cleaned. Sample containers are filled completely to exclude air and immediately sealed and labelled or coded. Store collected sediment in a sealed container in darkness at (4 ± 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 ensured. Analysis of known chemical contaminants may be conducted on sediment samples from the field and results compared to analysis of sediment at the beginning and end of the test to quantify any changes in chemical concentration or form.

8.2 Test sediment

Collect test sediment from the site to be evaluated using apparatus such as coring (e.g. box, Phleger) or grab (e.g. Ekman, Ponar, Van Veen, Petersen, Shipek, Kajak-Brinkhurst) 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. Several litres (i.e. 5 l to 7 l) of sediment is normally required to carry out a *Hyalella azteca* toxicity test; however, the amount of sediment required will depend on the study objectives, study design, and the nature of the chemical analyses to be performed. To obtain enough sediment, it is often necessary to combine subsamples retrieved using the sampling device. For chemical analyses, 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 ± 2) °C.

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

8.3 Preparation of sediment samples

Remove large debris (> 1 cm) and indigenous organisms by hand sorting using tweezers or similar instruments. Wet sieving the sediment is not recommended because water-soluble contaminants and fine non-settling clay particles could be lost. Each sediment sample is homogenized in the laboratory before use,[1] [2] [14] [15] unless study objectives dictate otherwise. Immediately following sample mixing, subsamples of test material for chemical and physical analyses are removed and placed in labelled containers and stored appropriately for physicochemical analyses.

Chemical and physical characterization of the sediment sample is needed for the interpretation of results. Analyse a subsample of the test and reference sediment for the following: particle size distribution [percentage gravel (> 2 mm), coarse and fine sand (≤ 2 mm to > 0,063 mm), and silt (≤ 0,063 mm to > 0,004 mm) and clay (< 0,004 mm)], percent water content, total organic carbon (TOC), pore water pH, and pore water ammonia (total and unionized concentrations).[1] [2] [3] [10] Further characterization could include total inorganic carbon, total volatile solids, biochemical oxygen demand, chemical oxygen demand, cation exchange capacity, acid volatile sulphides, metals, synthetic organic compounds, oil and grease, and petroleum hydrocarbons.[2] Pore water can also be sampled and analysed for 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).[13] [14] [15]

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, the use of a chemical solvent, verification, and data analysis are available (see References [1], [2], [5], [13], and [15]). However, for this International Standard, a sediment wet-spiking procedure is recommended over dry-spiking approaches which can lead to losses of the test chemical. The sediment is spiked with the test chemical or substance, either directly to the sediment or as a sediment:water slurry, prior to the addition of the overlying water. A period of equilibration is required to allow time for test chemical concentration to stabilize in the sediment pore water spaces. The duration of this equilibration period is highly dependent on the nature of the test chemical and sediment type (e.g. equilibration period in a sandy sediment would be less than in a sediment with a significant clay content; inorganic salts would equilibrate in less time than a polar organic compound).

Information that is available on the properties of the chemical or substance to be tested is collected. This includes concentration of major ingredients and impurities, water solubility, dissociation constants, toxicity to humans and aquatic organisms, and biodegradability. Further information such as structural formulae, nature and percentage of significant impurities, presence and amounts of additives, and n-octanol:water partition coefficient is also obtained, if available. Three methods are available for spiking control sediment with chemicals: 1) wet sediment rolling technique,[16] 2) wet slurry technique,[17] or 3) sediment suspension technique.[18] [19] [20] The method chosen is contingent on the study objectives and the nature of the chemical or material being tested.[5]

If the use of an organic solvent other than water is necessary to dissolve a test chemical and keep it in solution, test concentration are spiked directly into the control sediment and the solvent carrier evaporated before the addition of overlying water. A solvent-only control sediment shall be prepared and tested concurrently with the test sediment treatments. The solvent-only control treatment shall contain the highest concentration of solvent used in preparing the test sediment treatments.[5]

9 Test procedure

9.1 Preparing the test containers

Mix each sediment sample thoroughly on the day preceding the test and add an aliquot of sediment to each test container (e.g. 100 ml). Distribute test sediment in a uniform layer, which allows amphipods to burrow (minimum 1,5 cm in depth). Prepare a minimum of five laboratory replicates for each treatment or test sample and growth measurement period (i.e. Day 14 and Day 28), plus, if necessary, one or more

replicates per treatment for monitoring chemical characteristics of the sediment and the overlying water during the test. Replicates set up for monitoring of chemistry shall receive amphipods, as in other test replicates.

Smooth the sediment surface flat in each container 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 pouring the water over a disc [polyethylene, nylon, or polytetrafluoroethylene (PTFE) sheeting of 4 mm to 6 mm thickness] lying on the sediment surface that fits the inside diameter of the test container.[1] [3] Overlying water is added to each test container such that the sediment:water ratio is 1:1,75 or 1:4. The disc is removed and rinsed with overlying water between replicates of a treatment. A separate clean disc is used for each treatment. The water overlying the sediment in each test chamber should be renewed on the day preceding the test, as well as throughout the test (9.4).

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.

9.2 Introducing the organisms

Initiate the toxicity test by placing 10 *Hyalella azteca* in each test container (i.e. 10 amphipods per replicate of each treatment).

Select individual amphipods randomly from a known-age culture container (see B.3) using a pipette or other suitable device and distribute them directly to the test vessels. Amphipods are placed below the air/water interface in the overlying water. Alternatively, 10 amphipods may be counted into a transfer chamber (e.g. 30 ml plastic cup) filled with overlying water and then recounted before their transfer into the test containers. Transfer amphipods to the test chamber by gently pouring the water and amphipods from the sorting dish. Use a disc as described in 9.1 to prevent disturbance of the sediment. Wash any amphipods remaining in the dish into the test container using more overlying water. Increase the volume in the test container to the appropriate level (water plus sediment), if necessary, remove the disc, and place covers on the test containers, if using. Because replicate containers are used, amphipods should be added at the same time to each set or block of test containers representing each treatment, following a randomized block design.

Replace any amphipods that have not buried 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.

9.3 Test conditions

Test duration is 14 d and/or 28 d. The test can be terminated at 14 d; however, it may be continued to 28 d. If the latter is chosen, an extra five replicates are prepared for each treatment prior to test initiation so that five replicates may be processed and survival and growth data collected at 14 d. The remaining five replicates remain intact for a further 14 d, for end point determination at 28 d.

The test is to be conducted at a daily mean temperature of (23 ± 2) °C. The instantaneous temperature must always be within ± 3 °C of 23 °C.[1]

Solutions overlying sediments are aerated to maintain DO concentration ideally between 90 % and 100 % saturation. Air should be delivered to the overlying water at a continuous, gentle rate as described in 6.3.3.

Lighting intensity is between 100 lx to 1 000 lx at the surface of the overlying water. Illumination should be uniform and shall have a day/night cycle as outlined in 5.2.

Food is to be provided three times a week on non-consecutive days or daily throughout the test as described in 6.4.

For testing of field-collected sediments, five replicate containers per treatment is optimum for a 14-day test and 10 replicate containers per treatment is optimum for a 28-day test (i.e. five replicates for data collection at 14 d and 5 replicates for data collection at 28 d); however, a minimum of three laboratory replicates shall be tested for each treatment or test sample and data collection episode (i.e. Day 14 and Day 28).

9.4 Renewal of overlying water

Although the test described in this International Standard is primarily a static test, renewal of overlying water may be necessary on an intermittent basis if the water overlying control sediment deteriorates or becomes fouled due to natural background levels of ammonia or build up of ammonia from uneaten food. The overlying water is typically replaced manually but could be renewed using an apparatus for timed and intermittent automatic renewals of water in each chamber at an appropriate rate.[1] Daily water renewal is initiated if ammonia in the control or reference sediment reaches a concentration of ≥ 0,2 mg/l unionized ammonia at test initiation or during the test.[2] If the overlying water needs to be renewed, usually a manual siphon system is used to remove ≤ 90 % of the water to be replaced by clean water from a similar source as the original overlying water. The end of the siphon cannot contact the sediment and care should be taken to prevent disturbance of the sediment or accidental loss of amphipods. Useful guidance on the design of suitable systems for the automated renewal of overlying water is provided elsewhere.[1]

9.5 Test observations and measurements

Measure and record the temperature and DO concentration of the overlying water at the beginning of the test in one or more replicates for each treatment and at least three times per week on non-consecutive days. Daily measurement of temperature is preferable and daily measurements of DO concentration might be advisable.[1] [2] Conductivity, pH, and ammonia concentrations, as well as hardness and/or alkalinity concentrations are measured in the overlying water at the beginning and end of the test in at least one test chamber representing each treatment exposure. For spiked sediment tests, aliquots of the spiked sediment (and optionally the overlying water) are collected at the beginning and end of the test in the high, medium, and low test concentrations at a minimum, for confirmation of the test concentrations. If amphipods are floating at the water surface and trapped due to surface tension, gently push amphipods caught in the surface film into the water using a glass rod or pipette.

At the end of the test (14 d or 28 d), sieve the contents of each test container through a 300 µm screen to remove the test organisms and determine if they are dead or alive. Additional overlying water may be used for this sieving. Determine the total number of live and dead amphipods. Some test organisms may have died early and their bodies disintegrated by test end. Animals are considered to be dead if they fail to show any movement (e.g. slight movement of pleopod) in response to gentle prodding (a low-power dissecting microscope or hand-held magnifying glass is useful for this examination). All live animals recovered from a single test chamber are counted and placed together in a numbered weighing boat or similar small holding receptacle and rinsed with overlying water to remove any sediment adhering to the carapace of the animal. The group of surviving amphipods are then transferred to a clean, aluminium (or glass) weighing boat that has been previously numbered, weighed, and held in a desiccator. It is good practice to pre-dry each of the weighing boats before adding the test animals. Weighing boats containing surviving amphipods recovered from each test chamber are placed in an oven and dried for 24 h at 60 °C to 90 °C. Upon removal, the boats are moved to a desiccator and, following cooling, each boat is randomly removed from the desiccator and weighed to the nearest 10 µg. The first boat weighed should be returned to the desiccator after the weight measurement and reweighed at the end of all of the measurements in order to check the gain of water by the boats in the desiccator. If the change is > 5 %, then all of the boats are redried for ≥ 2 h and reweighed.

10 Expression of results

10.1 Survival

Calculate the mean percentage (± standard deviation) of amphipods that survived during the 14 d and/or 28 d exposure for each treatment. Missing individuals are assumed to have died and disintegrated during the test and are included in the number of dead individuals for a replicate.

The mean values of the replicates for each test sediment or each treatment are then compared statistically with corresponding values for amphipods held in control sediment and reference sediment (if part of the study design).

10.2 Growth

The mean dry weight (± standard deviation) per surviving amphipod is calculated from the total weight of each replicate group of survivors. The mean values of the replicates for each test sediment or each treatment are then compared statistically with corresponding values for amphipods held in control sediment and reference sediment (if part of the study design). Growth can also be estimated through measurement of organism length. A recommended acceptable mean length is approximately 3,5 mm.

10.3 Validity of the test

Consider the test valid when the average percent survival for amphipods held in the control sediment for the duration of the test (14 d or 28 d) is 80 % or higher at the end of the test. In addition, the average dry weight for the replicate control groups has to be 0,1 mg or higher per individual amphipod surviving at the end of the test.

11 Analysis and interpretation of results

11.1 Data analysis

Using this biological test method, pairwise comparisons of survival and growth data for each test treatment are normally made against survival and growth data derived for a particular reference or control sediment. Initially, test all data for normality using the Shapiro-Wilk test and for homogeneity of variance using Bartlett's test or other suitable tests.[1] [2] [21]

If replicate treatments were used to compute the toxicity of a single sediment sample with the toxicity of a single reference or control sediment from a specific site, apply the Student's *t*-test.[1] [2] [21] 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 Mann-Whitney U-test or Wilcoxon Rank Sum Test[1] [2] [21] 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, use an analysis of variance (ANOVA), followed by Dunnett's test or Williams' test.[2] [21] However, if the data set does not meet the requirements for normality and homogeneity of variance and data transformation has not helped in meeting these requirements, other suitable non-parametric procedures for multiple comparisons of each test sediment versus the reference and/or control sediment can be undertaken to determine if the end point values for different treatments differ significantly (see ISO/TS 20281 or Reference [21]).

For any test that includes testing of a dilution series, it is possible to estimate the concentration which would cause x % mortality, LC_x (e.g. LC_{50}) or x % growth inhibition, EC_x (e.g. EC_{10}, EC_{20}, or EC_{50}), or IC_x (e.g. IC_{10}, IC_{20}, or IC_{50}). Many statistical methods are available for interpreting concentration-response curves and estimating a statistical end point (i.e. a concentration causing an x % effect). For further guidance on the statistical treatment of test data, see ISO/TS 20281 and Reference [21].

11.2 Non-contaminant factors

There are a number of non-contaminant factors that may influence amphipod survival and growth in this test. The most important factors include sediment particle size and pore water ammonia.[2] *Hyalella azteca* has an extremely wide tolerance of sediment particle size.[1] [2] [11] [22] [23] Concentrations of pore water ammonia can be elevated in samples of field-collected sediment. The elevated levels might be due to organic enrichment from natural and/or anthropogenic sources. The tolerance limits of the *Hyalella azteca* to ammonia depends on pH and the concentration of sodium and potassium.[2][8] In four-day water-only tests, the LC_{50} values ranged from 20 mg/l to > 200 mg/l depending on numerous factors, including water hardness and pH.[1] Although these numbers provide benchmark concentrations that may be of concern in sediment pore waters, the relationship between four-day water-only LC_{50} and those measured in sediment exposures is variable.[1] All of these factors should be considered together

with the measured levels of toxic constituents when appraising their significance in influencing the results of a test.[14]

12 Reference substance

Tests with reference substances 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.[5] [15] [24] A static four-day water-only reference toxicity test shall be routinely conducted along with sediment toxicity tests using *Hyalella azteca*.[1] The recommended frequency of testing is once per month during the period when the laboratory is conducting *Hyalella azteca* sediment testing. These reference toxicity tests can be supplemented and/or replaced with spiked sediment tests with a reference toxicant.[1] [2] [5] [15]

12.1 Water-only test

Water-only tests with one or more reference toxicants are most commonly used in conjunction with survival-and-growth tests which measure toxicity to *Hyalella azteca*.[1] [2] [3] [10] Reagent-grade cadmium (as cadmium chloride), copper (as copper sulphate), potassium chloride, and sodium chloride are recommended as reference toxicants for this test.[1] [2] [3] [24] When conducting a four-day water-only LC_{50} reference toxicity test, conditions and procedures shall be identical to those described in Clause 9 except that sediment is not added to the test vessels; some type of substrate is provided (e.g. a 2 cm^2 piece of 500 µm nylon mesh), a larger volume of test solution is used (e.g. 200 ml), feeding is reduced (i.e. 0,5 ml YCT per test vessel at test initiation and on Day 2), and only mortality is measured at test end. A minimum of five test concentrations plus a control are tested. The test is a static exposure and test vessels are normally covered to reduce evaporation. Test containers are not aerated during the test. In order for a test to be valid, there must be ≥ 90 % survival in the control containers.

A minimum of two replicates should be exposed to each concentration of reference toxicant tested. While routinely performing tests with reference toxicant(s), laboratory personnel should prepare and update a separate control chart for each reference toxicant used.

12.2 Whole sediment test

Alternatively, or in addition to the water-only reference toxicant tests, control sediment may be spiked with copper or cadmium and used to determine a 14 d or 28 d LC_{50}.[5] [15] Any test with the reference toxicant should be initiated within 1 d of starting the 14 d and/or 28 d assay with test substances. Toxicity tests using control sediment spiked with copper have been performed.[5]

When selecting control sediment for spiking with a chemical, it should be demonstrated in advance of the final selection to enable acceptable survival of *Hyalella azteca* (i.e. > 80 %) (6.2). At a minimum, candidate control sediment should be characterized for TOC content, particle size distribution, pH, and percent water. The moisture content of the control sediment shall be determined before spiking to standardize spiking on a dry weight basis. However, for spiking experiments with reference substances, the use of artificial (formulated) control sediment is recommended because properties that influence binding and bioavailability of chemicals are known. Guidance on spiking techniques and chemical equilibration is given in 8.3.

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 will confirm 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 (see References [5] and [24]). While routinely performing tests with reference toxicant(s), laboratory personnel should prepare and update a separate control chart for each reference toxicant used.

13 Test report

This test report shall contain at least the following information:

a) test method used, together with a reference to this International Standard (i.e. ISO 16303);

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

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

d) physical-chemical properties measured [e.g. particle size distribution, percent water content, TOC, pore water pH, and pore water ammonia (total and unionized), total inorganic carbon, total volatile solids, biochemical oxygen demand, chemical oxygen demand, cation exchange capacity, acid volatile sulphides, metals, synthetic organic compounds, oil and grease, petroleum hydrocarbons];

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

f) source of culture;

g) species and taxonomic verification;

h) procedures for culturing test organisms;

i) description of culture water;

j) physical conditions measured during the test (photoperiod, light intensity, temperature, pH, DO, hardness, and ammonia) and test duration;

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

l) exposure design (organisms per treatment, number of replicates, number of discreet samples);

m) description of sediment sample preparation;

n) description of overlying water and method of intermittent renewal for sediment test and test water used in reference substance test;

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

p) description of test conditions (DO, temperature, pH, hardness, TOC/dissolved organic carbon (DOC), ammonia, nitrite, sulphide);

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

r) growth of organisms results (number and dates);

s) statistical analysis used to analyse mortality and growth data; calculation of LC_{50} and EC_{50}, if sediment spiking or reference toxicant testing;

t) exposure method (water only or sediment) and results of reference toxicity test;

u) comment on any other biological effects observed or measured;

v) description of any deviations from the method and explanation.

Annex A
(informative)

Description of *Hyalella azteca*

A.1 Diagnosis of *Hyalella azteca* (Saussure, 1858), taken from Annex F Environment Canada, 2012; modified from Bousfield, 1973[4] (see Figure A.1)

Body length: Male, to 8 mm; female, to 6 mm. Body small, dorsally mucronate on pleon segments 1 and 2, occasionally on 3, or smooth (form inermis). Coxal plates very deep, fourth largest; lower margins lightly and evenly spinose. Head, eye subovate, black, slightly larger in male.

Antenna 1, peduncular segments 1 and 2 subequal, flagellum 8 to 10 segmented. Antenna 2, peduncle slender, segment 5 longer than 4; flagellum 9 to 10 segmented. Maxilliped, palp segment 2 wider than long, exceeding outer plate.

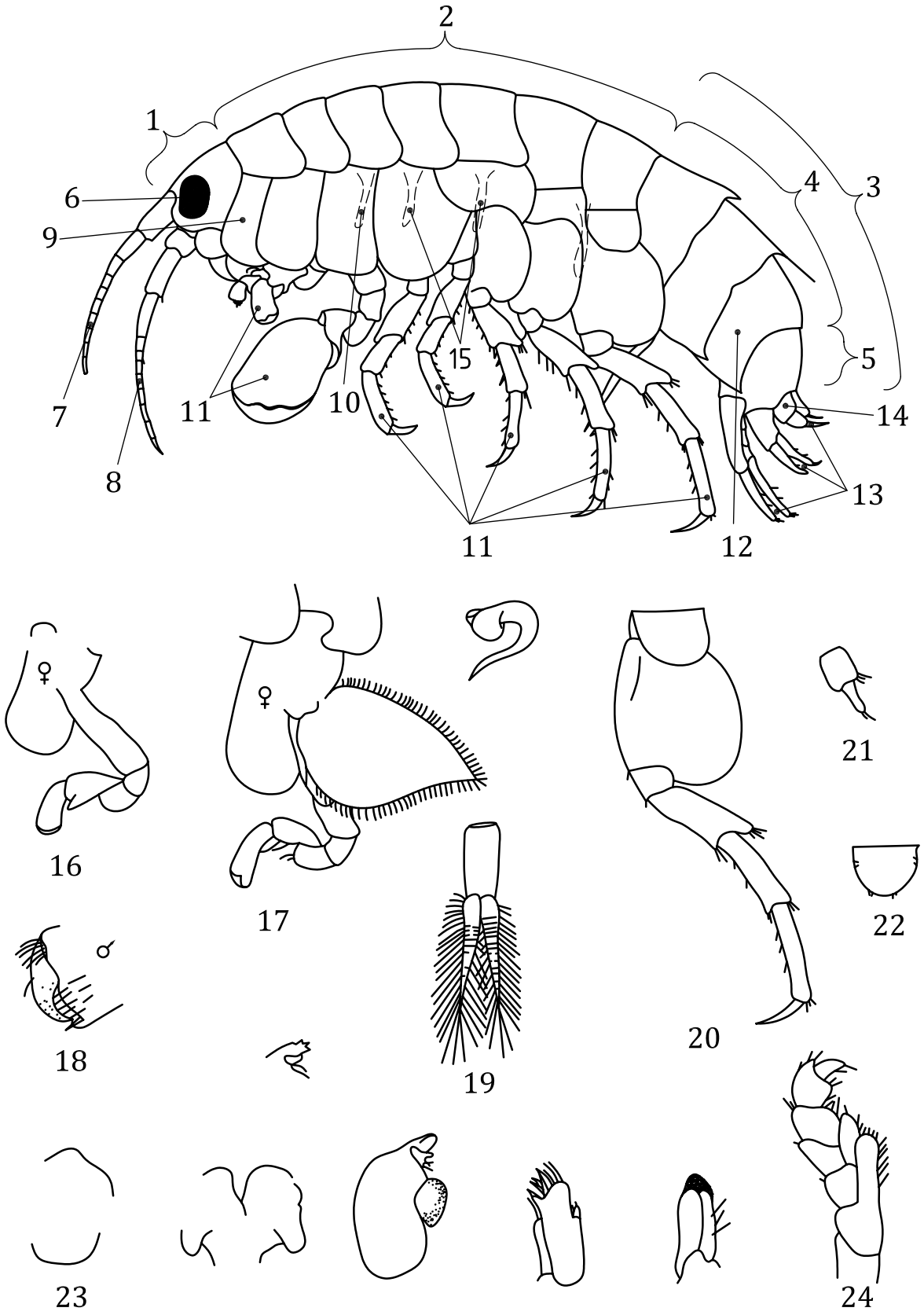
Gnathopod 1 (male), propod shorter and less deep than carpus, expanding distally; palm oblique, convex. In female, propod narrow, short; palm vertical, convex. Gnathopod 2 (male), propod very large, distally broadest; posterior margin slightly concave; palm convex, with large low tooth near hinge; carpal lobe deep. In female, propod slender, elongate, expanding distally; palm short, convex.

Peraeopods 3 and 4, posterior margins of segments 5 and 6 with 3 to 5 short, stout spines. Peraeopods 5 to 7, basis broadly expanded, posterior margin with 4 to 10 weak serrations; segments 5 and 6 lacking posterior marginal spines or setae. Abdominal side plates (epimera) 2 and 3, hind corners sharply subquadrate, not produced.

Uropods 1 and 2, both rami with two slender marginal spines. Uropod 3, ramus and peduncle subequal in length, apex with long spine(s). Telson, apex rounded, with two slender side-set spines.

Coxal gills on peraeopods 2 to 6 normal, sac-like, smallest on 6. Paired sterna gills at bases of peraeopods 3 to 5 and 7 are regular in form, not elongated or strongly curved.

With respect to other known North American species of the genus *Hyalella*, *Hyalella azteca* (Saussure) differs in usually possessing a single posterodorsal tooth or mucronation on each of pleon segments 1 and 2 (occasionally, also on 3), and in the relatively elongated, narrow form of the propod and carpus of gnathopod 2 in the female, among other items.



Key

1	head	9	buccal mass	17	gnathopod 2
2	peraeon	10	coxel gills	18	gnathopod 1
3	abdomen	11	peraeopods	19	pleopod 1
4	pleon	12	epimeron	20	peraeopod
5	urosome	13	uropods	21	uropod 3
6	eye	14	telson	22	telson
7	antenna 1	15	sternal gills	23	upper lip
8	antenna 2	16	gnathopod 1	24	maxilliped

Figure A.1 — Outline of body, appendages, and mouthparts of *Hyalella azteca*

Annex B
(informative)

Culturing *Hyalella azteca*

B.1 Culturing conditions

All equipment, containers, and accessories that might contact the organisms or water within the culturing facility are clean, rinsed as appropriate, and made of non-toxic materials. Sources of water for culturing *Hyalella azteca* may be an uncontaminated supply of groundwater, surface water, or reconstituted water (described in 6.3). In order to maintain good water quality, the water within the culture chambers should be renewed routinely either manually or automatically, using suitable apparatus and techniques for continuous or intermittent renewal. Cultures should be aerated gently (i.e. 1 bubble/s for each litre of water) using filtered, oil-free compressed air to maintain the DO at 80 % to 100 % saturation.

Overhead full-spectrum lights (fluorescent or equivalent) should illuminate the culture with a photoperiod of 16 h of light and 8 h of darkness. The light intensity adjacent to the water surface in the culture chambers should range from 100 lx to 1 000 lx. The daily average temperature of the water in the culture chambers should be (23 ± 2) °C and the instantaneous water temperature should be (23 ± 3) °C.

Substrates such as gauze strips, nylon mesh, plastic mesh, or shredded paper towels are placed in each culture chamber and replaced as necessary.

B.2 Food and feeding

A single ration diet such as commercial fish food flakes (e.g. Tetramin™[5]) or a mixed diet such as yeast, Cerophyll™[6], and trout chow (YCT) are provided to the cultures on a regular basis using a ration that maintains healthy cultures. If YCT is used, it should be prepared as described in Annex D. The amount of food distributed to each culture chamber will vary with the age of the *Hyalella azteca* and the number of organisms in the chamber.

B.3 Mixed-age and known-age cultures

Mixed-age cultures should be maintained and represent a mass culture of amphipods of various ages to provide fresh organisms for creating the known-age cultures. Known-age cultures contain individuals of a particular age class (e.g. 2 d to 14 d) and age range (e.g. 1 d to 2 d) that have been segregated and maintained in separate culture chambers until they are used in a toxicity test. There are many methods available for culturing *Hyalella azteca* for use in toxicity tests.[1] [2] [3] [10] The following method is further described in Reference [1].

To prepare a known-age culture with young *Hyalella azteca* that are within 1 d to 2 d of each other in age, mature amphipods are isolated by pouring the contents of the culture chamber(s) through a 710 µm sieve. The sieve is held in a shallow pan containing culture water overnight. Any newborn amphipods that are released after 24 h are collected by moving the sieve up and down several times to rinse the newborns into the surrounding water in the pan. The sieve is then removed from the pan and the mature amphipods are returned to the mixed-age culture or the sieve containing the adults is moved to a new pan for another 24 h for the production of more young.

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5) Tetramin™ is an example of fish food flakes available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

6) Cerophyll™ can be obtained from Ward's Scientific as "Cereal Grass Media - Cerophyll". This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

The newborn amphipods that passed through the sieve are transferred using a pipette into a fresh culture chamber and held until they are at the desired age for use in the toxicity test. Approximately 1 500 (750 mating pairs) adults will provide about 800 newborn amphipods in 24 h.

Alternatively, 50 mature amphipods (i.e. > 30 d old) are placed in 2 l beakers with 1 l of water, 10 ml of YCT, and a piece of nylon substrate. Adults are moved into fresh containers daily or every 2 d by pouring the contents of the beaker into a shallow pan and transferring the adults into fresh culture media. The young remaining in the pan will range in age by < 1 d to 2 d. Young amphipods can be held until the desired age is reached and are fed 1,0 ml YCT daily until used in the test.

B.4 Observations and health criteria

The quality of water in the culture chambers should be monitored and recorded routinely. Water temperature is measured daily and DO weekly. The hardness, alkalinity, pH, and ammonia are measured as frequently as necessary to document water quality (i.e. four times a year as a minimum, and the day before the start of a test). Other analyses, such as nitrite, suspended solids, total dissolved gases, metals, and pesticides should be carried out as frequently as necessary to document water quality. If the concentrations of nitrite or ammonia in the culture water are higher than 0,02 g/l or 0,5 g/l, respectively, a portion of the water is replaced[3] and/or more frequent renewals are necessary.

Amphipods in cultures are checked a minimum of three times per week (e.g. Monday, Wednesday, Friday), preferably daily. Individuals that appear dead or inactive are not used for testing. If more than 20 % of the amphipods in a known-age culture chamber appear dead or inactive during the 48 h period preceding the start of the test, that known-age culture chamber is discarded. Records are maintained on number of breeding pairs, number of young produced, and the survival of known-age cultures.

Annex C
(informative)

Sources of *Hyalella azteca*

United States

U.S. Geological Survey
Columbia Environmental Research Center
4200 New Haven Road
Columbus, MO, USA, 65201
website: http://ww.cerc.usgs.gov

U.S. Environmental Protection Agency
Office of Research and Development
Mid-Continent Ecology Division
6201 Congdon Blvd.
Duluth, MN, USA, 55804
website: http://ww.epa.gov/med/overview

Canada

Environment Canada
Atlantic Laboratory for Environmental Testing
Water Science and Technology Directorate
Environmental Science Centre
Moncton, NB, Canada
website: S&T@ec.gc.ca

Environment Canada
Aquatic Ecosystems Protection Research Division
Water Science and Technology Branch
867 Lakeshore Rd.

Burlington, Ontario, Canada

website: S&T@ec.gc.ca

France

INERIS

Ecotoxicology Laboratory

Chronic Risk Branch

Parc Technologique Alata, BP2

F-60550, Verneuil-en-Halatte

France

website: www.ineris.fr

Germany

ECT Oekotoxikologie GmbH

Böttgerstr. 2-14

D-65439, Flörsheim/Main

Germany

website: www.ect.de

Annex D
(normative)

Procedure for preparing YCT

D.1 Preparing digested trout chow

Use Starter No. 1 pellets. Add 5,0 g of trout chow pellets to 1 l of deionized water. Mix well in a blender and pour into a 2 l separatory funnel. Digest prior to use by aerating continuously from the bottom of the vessel for 1 w at ambient laboratory temperature. Water lost due to evaporation should be replaced during digestion. Because of the offensive odour usually produced during digestion, the vessel should be placed in a fume hood or other isolated, ventilated area.

At the end of the digestion period, place in a refrigerator and allow settling for a minimum of 1 h. Filter the supernatant through a fine mesh screen (e.g. 110 mesh or 0,100 mm). Discard the sediment.

D.2 Preparing yeast

Add 5,0 g of dry yeast, such as Fleischmann's™[7)] to 1 l of deionized water.

Stir with a magnetic stirrer, shake vigorously by hand, or mix well with a blender at low speed until the yeast is well dispersed.

Combine the yeast suspension immediately (no settling) with others supernatants.

D.3 Preparing Cerophyll™[8)] (dried, powdered cereal leaves)

Place 5,0 g of dried, powdered Cerophyll™[8)] or cereal leaves in a blender.

NOTE Dried, powdered alfalfa leaves from health food stores have been found to be a satisfactory substitute for cereal leaves.

Add 1 l of deionized water. Mix in a blender for 5 min at high speed or stir overnight at medium speed on a magnetic stir plate.

If the blender is used to suspend the material, place in a refrigerator overnight to settle. If a magnetic stirrer is used, allow settling for 1 h. Decant the supernatant and combine the equal volumes of supernatant from the trout chow and yeast preparation.

D.4 Preparing combined YCT food

Mix equal (approximately 300 ml) volumes of the three foods described previously.

Place aliquots of the mixture in small (50 ml to 100 ml) screw cap plastic bottles and freeze until needed.

Freshly prepared food can be used immediately or it can be frozen until needed. Thawed food is stored at 4 °C between feedings and is used for a maximum of two weeks. It is advisable to measure the dry weight of solids in each batch of YCT before use. The food should contain 1,7 g to 1,9 g solids per litre.

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7) Fleischmann™ is an example of yeast available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

8) Cerophyll™ can be obtained from Ward's Scientific as "Cereal Grass Media - Cerophyll". This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

Annex E
(informative)

42-day sediment reproduction test

Long-term toxicity tests may be used to identify marginally contaminated sediments, using *Hyalella azteca* to evaluate potential effects of the sediment on survival, growth, and reproduction in a 42-day test. Details on conducting a 42-day test for measuring the effects of sediment-associated contaminants on survival, growth, and reproduction are described in detail in Reference [1] and summarized in this annex.

The sediment exposure starts with 7 d to 8 d old amphipods. The test apparatus, conditions, and procedures are similar to the 28-day survival-and-growth test described in Clauses 5 to 10; however, on Day 28, amphipods are isolated (i.e. sieved) from the sediment and placed in water-only chambers (i.e. 300 ml beakers containing 150 ml to 275 ml of overlying water and a 5 cm × 5 cm piece of nylon substrate) where reproduction is measured on Days 35 and 42. A total of 12 replicate test vessels are prepared for each treatment, 4 of which are used to assess survival and growth at 28 d and 8 of which are used for the 35 d and 42 d survival, growth, and reproduction end points. More replicates may be used to reduce variability because reproduction is a more variable end point than growth or survival. Renewal of overlying water, feeding, and monitoring of the water-only phase of the test are as described for the first 28 d of the test (i.e. sediment and water exposure) in 9.3, 9.4, and 9.5. Reproduction is measured on Day 35 and Day 42 in the water-only beakers by removing and counting the adults and young in each beaker. On Day 35, the adults are then returned to the same water-only beakers. Adult amphipods surviving on Day 42 are counted and the number of adult females is determined by simply counting the adult males (mature male amphipods will have an enlarged second gnathopod) and assuming all other adults are females.

The end points of the 42-day *Hyalella azteca* test include survival (Days 28, 35, and 42), growth (Days 28 and 42), and reproduction (Day 42). Survival and growth are determined as described for the sediment-exposure portion of the test (10.1 and 10.2). Reproduction is determined as the number of young produced per female from Day 28 to Day 42 of the test. Test validity criteria for this test has not been established; however, the U.S. EPA[1] reported that laboratories participating in round-robin testing showed that after 28 d sediment exposures in a control sediment, survival was > 80 % for > 88 % of the laboratories and dry weight was > 0,15 mg/individual for > 66 % of the laboratories. Reproduction from Day 28 to Day 42 was > 2 young/female for > 71 % of the laboratories participating in the round-robin testing.[1]

Annex F
(informative)

14-day water-only survival-and-growth test

The 14-day *Hyalella azteca* water-only survival-and-growth test is based on a methodology developed by Borgmann et al.[7] for measuring survival only. This method was further refined[2][25] to create a tool that could be used in conjunction with the 14-day *Hyalella azteca* sediment test to differentiate between effects caused by sediment (e.g. historical deposition) and those caused by contaminants in the water column (e.g. wastewater). As such, the apparatus, conditions, and procedures used for this test are similar to those described for the 14-day sediment test described in Clauses 5 to 10. It can be applied to samples of industrial or sewage effluents, fresh waters (e.g. receiving water), aqueous extracts, or chemical substances which are soluble or which can be maintained as stable suspensions or dispersions under the conditions of the test.

At the end of the 14-day static exposure, both survival and growth are determined. Ten *Hyalella azteca* aged 6 d to 9 d are placed in each replicate glass vessels (e.g. 300 ml beakers) with 275 ml of test solution. A 3 cm^2 piece of 500 µm nylon mesh is added to each test vessel as substrate for the test organisms. A minimum of five replicates per test concentration are used; however, increasing replication to 10 replicates per treatment will reduce the variability in the growth end point. Test containers are aerated and test organisms are fed YCT daily (containing ~ 2,7 g/mg solids) at a rate of 1,5 ml/d/test container. Tests are conducted at a daily mean temperature of (23 ± 2) °C [instantaneous (23 ± 3) °C], a light intensity of 500 lx to 1 000 lx, and a 16 h light:8 h darkness photoperiod.

At the end of the test (Day 14), numbers of live and dead organisms are enumerated and recorded. Groups of live *Hyalella azteca* from each replicate are moved to weighing boats for dry weight measurements as described in 9.5. Test end points include mean percent survival and mean dry weight of surviving amphipods as described in 10.1 and 10.2. Test validity criteria are as described in 10.3 and data analysis is as described in 11.1.

Annex G
(informative)

Performance data

Interlaboratory performance has been assessed by the U.S. EPA in a series of round-robin tests involving 4-day water-only exposures and 10-day whole sediment toxicity tests with *Hyalella azteca*[1][12] and 10 participating laboratories from the United States and Canada. The 4-day water-only reference toxicity tests involved exposure to KCl. The between-laboratory coefficient of variation (CV) based on percent control survival was 2,1 % for the nine laboratories that met the minimum control survival criteria of ≥ 80 % and the between-laboratory CV based on the 4-day LC_{50} for KCl was 14,2 %.

An interlaboratory evaluation of a 14-day whole-sediment toxicity test, similar to the one described in this International Standard, was carried out in 1996.[26] Five laboratories, from Canada and the United States, exposed *Hyalella azteca* to four field-collected sediments for 14 d and measured survival and growth at the end of the test. The results are shown in Table G.1.

Table G.1 — Performance data for 14-day *Hyalella azteca* sediment testing using toxicity end points survival (%) and growth (increase of body weight in mg)

Test sediment	Number of laboratories	Between-lab CV for survival %	Between-lab CV for growth %
1	5	2,5	33,5
2	5	11,0	35,7
3	5	3,6	26,0
4	5	10,2	29,0

As part of an evaluation of interlaboratory precision of the long-term *Hyalella azteca* test, precision data were produced for the 28-day sediment survival-and-growth test. In total, eight laboratories from Canada and the United States participated in tests with five different sediments (including a sandy-based artificial sediment); however, not all laboratories tested all sediments. The between-laboratory CVs for the 28-day survival and growth data are shown in Table G.2.[1] The CVs include only data for sediment samples from laboratories that met the 28-d control performance acceptability.

Table G.2 — Precision data for 28-day *Hyalella azteca* sediment testing using toxicity end points survival (%) and growth (increase of body weight in mg)

Test sediment	Number of laboratories	Between-lab CV for survival %	Between-lab CV for growth %
1	7	6,8	27,8
2	7	5,9	75,2
3	7	7,5	38,2
4	3	9,9	68,6
5	3	24,1	34,0

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