Environment and Climate Change Canada
Environnement et Changement climatique Canada

RECOMMENDED PROCEDURE FOR THE IMPORTATION OF TEST ORGANISMS FOR SUBLETHAL TOXICITY TESTING

Method Development and Application Section
Environmental Technology Centre
Environment Canada

September 1999

Canada

Note that this document was originally released in 1999, in print format only. No modifications were made to its content during the creation of this electronic version, except for updating the front cover page and certain details on the inside cover page.

Cat. No.: En84-258/2021E-PDF
ISBN: 978-0-660-38909-7
EC: 21039

Environment and Climate Change Canada
Public Inquiries Centre
12th Floor, Fontaine Building
200 Sacré-Coeur Boulevard
Gatineau QC K1A 0H3
Telephone: 819-938-3860
Toll Free: 1-800-668-6767 (in Canada only)
Email: ec.enviroinfo.ec@canada.ca

Aussi disponible en français

RECOMMENDED PROCEDURE FOR THE IMPORTATION OF TEST ORGANISMS FOR SUBLETHAL TOXICITY TESTING

1.0 Application

The following procedures for the importation of test organisms are recommended by Environment Canada (EC) for sublethal tests performed using test organisms obtained from outside the testing laboratory. In this application, test organisms are imported into the testing laboratory for immediate use (i.e., without substantial acclimation and without in-house culturing) in sublethal toxicity tests.

This guidance document applies to test methods using test organisms which are purchased and transported from an external supplier (e.g., a commercial supplier) for immediate use in toxicity testing. Test methods which fall under this guidance include:

(a) the 7-day larval survival-and-growth test with fathead minnows *Pimephales promelas*, performed according to Environment Canada (EC, 1992a);

(b) the 7-day larval survival-and-growth test with the inland silverside fish *Menidia beryllina*, performed according to United States Environmental Protection Agency (USEPA,1994);

(c) the 7-day larval survival-and-growth test with the topsmelt fish *Atherinops affinis*, performed according to USEPA (1995);

(d) the 10-day survival-and-growth sediment test with freshwater midge larvae *Chironomus tentans* or *Chironomus riparius*, performed according to EC (1997a); and

(e) the 14-day survival-and-growth sediment test with the freshwater amphipod *Hyalella azteca*, performed according to EC (1997b).

This guidance document does not apply to the following two categories of test methods:

(1) those toxicity methods designed to use test organisms obtained from external sources, where adequate guidance on the importation of test organisms is already provided in the individual test method documents, including:

- organisms that are collected from wild populations and imported to the testing laboratory for immediate use in toxicity tests (e.g., the 10-day acute sediment test with marine or estuarine amphipods, performed according to EC, 1992b or 1998a);

- gametes or juveniles that are imported to the testing laboratory for use in toxicity tests (e.g.,

toxicity tests using early life stages of salmonid fish (rainbow trout), performed according to EC, 1998b); and acute lethality test using rainbow trout, performed according to EC,1990a); and

- adult organisms imported by the testing facility to provide gametes for use in toxicity tests (e.g., fertilization assay using echinoids (sea urchins or sand dollars), performed according to EC, 1992c).

(2) those toxicity test methods where the importation of organisms for immediate use in toxicity tests is not practical (i.e., cost effective) and in some cases, not possible, due to the nature and/or specificity of the quality, age, and/or acclimation requirements of the organisms to be used in a test. For these methods, test organisms must be cultured in the laboratory that is conducting the toxicity tests. Examples of such methods include:

- the 7-day growth-inhibition test using the freshwater macrophyte *Lemna minor*, performed according to EC (1999);

- the 72-hour growth-inhibition test using the freshwater alga *Selenastrum capricornutum*, performed according to EC (1992d);

- the reproduction and survival test using the cladoceran *Ceriodaphnia dubia*, performed according to EC (1992e);

- the sexual-reproduction test using the red macroalga, *Champia parvula*, performed according to USEPA (1994); and

- the acute lethality test using *Daphnia* spp., performed according to EC (1990b).

Guidance on the importation of broodstock organisms purchased or collected for the purpose of in-house culturing of toxicity test organisms is not provided in this document. Procedures related to in-house culturing of test organisms can be found in the respective test method documents.

Table 1 provides a summary of the required conditions and procedures for the importation of toxicity test organisms. Some of these items are in addition to those conditions and procedures detailed in specific test method documents.

Table 1 List of Required Conditions and Procedures for Importing Toxicity Test Organisms

Test Organisms	
Age	- must be known by the testing laboratory
Species	- must be identified by a qualified taxonomist for each species provided by a supplier on at least one occasion
Source	- must be from a dedicated culture which has met the health criteria and quality assurance requirements outlined in the test method
	- must be purchased from commercial suppliers or laboratories that maintain ongoing cultures of these organisms
Permits	- procurement, shipment and transfer of test organisms must be approved by provincial or federal authorities, where required
Organism Culturing	
Morbidity, Mortality and Treatment	- test organisms to be shipped to a laboratory for use in toxicity tests must be in good health (e.g., appear healthy and behave normally), be disease free, and show minimum mortality (as required in USEPA and EC test method documents)
	- any test organisms recovered from disease or previous exposure in a test must not be used in further sublethal toxicity tests
	- test organisms must be carefully observed by both supplier (prior to shipment) and testing laboratory (prior to use in a toxicity test) for a length of time which is reasonable for the species to ensure that they are disease free, behave normally, and in good physical condition.
	- individual(s) or batches of organisms where health is questionable must not be used in sublethal toxicity tests
Transport and Handling	
Temperature	- temperature of the test organisms must not change by more than 3EC in any 24-hour period while being transported
Dissolved Oxygen	- must be maintained at or above the minimum level required for organism culturing during transportation (e.g., $\exists$4.0 mg/L for inland silversides; > 6.0 mg/L for topsmelt; $\exists$80% saturation for *Hyalella azteca*, freshwater midges, and fathead minnows)
Crowding	- excessive crowding of animals must be avoided to minimize stress and prevent oxygen deficiency in transit
Handling	- organisms that are dropped, touch dry surfaces, or are injured during handling must be discarded and not used in testing

Acclimation and Holding

General	- upon arrival at the testing laboratory, the organisms must be acclimated as gradually as possible to the laboratory holding and/or testing conditions such that the test organisms are not stressed

Observations and Monitoring

General	- any requirements for monitoring water quality characteristics and other culture conditions such as temperature and salinity must be followed by the supplier, as specified in test method documents
	- the testing laboratory must establish an in-house system for evaluating the health of each shipment of organisms

Quality Control

Health Criteria	- test-organism health criteria, specified in individual test method documents must be followed
	- organisms must not be used for a test if they appear to be unhealthy, discolored, or otherwise stressed; fish larvae must not be used if they are not actively feeding and if their swimbladders are not inflated
	- mortality in a given batch of test organisms imported for use in toxicity tests must not exceed 10% for larval fish and freshwater midges and 20% for *Hyalella azteca* in the 24-hour period immediately preceding the test
Reference Toxicity Testing	- by the testing laboratory, on each batch of organisms imported for testing; reference toxicity testing procedures and the reference toxicant(s) used must meet the requirements outlined in specific test method documents
	- the testing laboratory must report any invalid reference toxicity test conducted concurrently with sample toxicity test(s) and must report the initiation and/or findings of a test system review
Test Control Performance	- organisms imported to the laboratory for testing must demonstrate acceptable performance in control solutions at the end of each test conducted, as specified in each specific test method document

2.0 Rationale

Test organisms should be cultured in the laboratory where the toxicity tests will be conducted (i.e., in-house), wherever it is practical and cost effective. If, however, in-house cultures cannot be maintained, test organisms should be purchased from a facility experienced in culturing organisms for use in toxicity tests (e.g., commercial supplier, government or private laboratory, etc.).

Organism sensitivity might vary with source, genetic history, age, sex, state of health, and stress associated with any recent environmental change (e.g., shipping) (EC, 1998c). Therefore, Environment Canada has decided to provide guidance herein on the use of test organisms that are not cultured by the laboratory conducting the toxicity tests, but are supplied by a facility independent of the testing laboratory for immediate use in sublethal toxicity tests.

3.0 Test Organisms

3.1 Species and Life Stage

The species used to characterize the toxicity of a sample will depend on the toxicity test method being followed, the requirements of the regulatory authority, and/or the objective of the test (USEPA, 1994).

Organisms used in a given toxicity test must be of known age, and should all be approximately the same age. Toxicity test organisms must be positively identified to species. Organisms that are purchased from a commercial supplier should be supplied with certification of the organisms' species identification, and the taxonomic reference (citation and page) or name(s) of the taxonomic expert(s) consulted. The identification of a representative specimen must be confirmed by a qualified taxonomist at least once for each species provided by the supplier. Thereafter, periodic confirmation of the species of test organism shipments can be conducted by the testing laboratory. This can be done by comparing an organism in a given batch to a representative specimen, previously confirmed by a taxonomist, which is kept on file (i.e., mounted on glass slides or preserved in ethanol) at the testing laboratory.

3.2 Source

All organisms used in a given toxicity test must be from a dedicated culture at a supplier's facility which has met the health criteria and quality assurance requirements outlined in the test method.

The source and condition of test organisms may affect the quality of the toxicity test data. Therefore, it is the responsibility of the testing laboratory to ensure that test organisms are purchased from commercial suppliers, culture collections, or other facilities experienced in the culturing of the specific test organisms required. The supplier must maintain an ongoing

program of quality assurance to provide high quality organisms. The program should incorporate quality control practices which include: regular health monitoring; performance evaluation of organisms through the use of standard reference toxicants; independent laboratory inspections; and adequate record keeping.

Larval fathead minnows, inland silversides, and topsmelt; chironomid instars or eggs; and juvenile *Hyalella* that are imported for immediate use in sublethal toxicity tests must be purchased from commercial suppliers or laboratories that maintain ongoing cultures of these organisms (EC, 1992a; 1997a; b; USEPA, 1994; 1995).

3.3 Permits

Moving animals and plants from one location to another raises serious questions of introducing non-native species and of transporting diseases and parasites. Any proposed procurement, shipment and transfer of test organisms must be approved, if required by provincial or federal authorities. Provincial governments might require a permit to import organisms whether or not the species is native to the area, or movements of aquatic organisms might be controlled by a Federal-Provincial Introductions and Transplant Committee. Advice on contacting the committee or provincial authorities and on sources of test organisms, can be obtained from the regional Environmental Protection Office (EC, 1992a). For example, in areas where fathead minnows are not native (B.C., P.E.I., N.S., Nfld., and parts of other provinces and territories), application for a permit must be made to the above-mentioned committee, to the appropriate provincial agency, or to the Regional Director-General of the Department of Fisheries and Oceans (DFO), depending on procedures in place locally (EC, 1992a).

Testing laboratories might be required to set-up a quarantine section within their facilities where imported organisms or gametes can be isolated and all equipment and fluids that come in contact with the test organisms or gametes can be sterilized and disposed of according to provincial or federal regulations. Standard operating procedures detailing quarantine operations and procedures might also be required by provincial agencies or DFO.

4.0 Organism Culturing

4.1 General

Instructions for culturing and/or holding each species of test organism are included in test-specific USEPA and amended Environment Canada sublethal toxicity test method documents. Some test methods (e.g., EC, 1992a) provide detailed guidance on culturing and/or maintaining toxicity test organisms (e.g., fathead minnow), whereas others (e.g., survival-and-growth tests using *Hyalella azteca* and freshwater midges) provide only general guidance and recommendations (EC, 1997a; b), leaving explicit directions regarding many aspects of culturing to the discretion and experience of the laboratory personnel. Where explicit culturing instructions are not provided, performance-based criteria are used to evaluate the suitability of the cultured organisms for tests and the acceptability of the test results (see Section 8.0).

Regardless of the level of detail provided in the test method documents, the testing laboratory should ensure that the test organism supplier is aware of, and follows, all of the required and recommended conditions for test organism culturing specified in the respective toxicity test method documents. The testing laboratory must maintain on file the information on the culturing practices of the supplier. This may be accomplished in several ways. For example, the supplier could provide the laboratory with a standard operating procedure on their culturing procedures or a signed confirmation that they have been supplied with the relevant section of the applicable method document and that the methods are being followed. Another option is for the laboratory to audit the supplier's facility, collecting all of the culturing information to be maintained on file.

4.2 Facilities and Apparatus

The testing laboratory should ensure that the facilities and apparatus used by the supplier for culturing test organisms meet the "must" requirements specified in the test method documents. As required by Environment Canada (EC, 1997a; b), freshwater midges and *Hyalella azteca* must be cultured in a controlled temperature facility. Equipment for temperature control (i.e., incubator, recirculating water bath, or constant temperature room) must be adequate to maintain the temperature of the test organisms within the recommended limits (i.e., 23 ± 1EC for *Hyalella azteca* and *Chironomus riparius* or *C. tentans*). The culturing area must be isolated from any activities that might lead to contamination of the cultures, and must also be designed and constructed to prevent contamination of cultures (e.g., elimination of copper or galvanized piping or fixtures that could drip metal-contaminated condensates; use of filtered, oil-free air for aerating organism cultures) (EC, 1997a; b). These culturing conditions are also recommended for rearing larval fish (i.e., fathead minnows, topsmelt and inland silversides)(EC, 1992a; USEPA, 1994; 1995).

All equipment, containers, and accessories that might contact the organisms, or water within the culturing facility must be made of nontoxic materials (e.g., glass, type 316 stainless steel, porcelain, fibreglass, Teflon™, nylon, Nalgene™, polyethylene, polypropylene). Toxic materials such as copper, zinc, brass, galvanized metal, lead and natural rubber must not come in contact with apparatus, equipment, or culture water (EC, 1992a; 1997a; b; USEPA, 1994; 1995).

All culture chambers, handling equipment and supplies should be dedicated for use in culturing activities. All new materials should be leached in culture water before use. After use, all culture materials should be thoroughly cleaned and rinsed as appropriate. Cleaning procedures may range from simply washing with detergent to more complex procedures which include rinses with dilute acid (to remove scale, metals and bases) and/or acetone (to remove organic compounds), depending on what the degree of equipment contamination. In all cases, the final rinse should be with the same water in which the organisms are to be cultured. Recommended cleaning procedures are provided by USEPA (1994; 1995).

Organism holding tanks and associated handling equipment should be disinfected between individual batches. However, since disinfectants are toxic, equipment should be thoroughly rinsed with water from the same source as that which will be used for culturing (EC, 1991;

1992a). Regular schedules for feeding and cleaning of organism enclosures should be established and should follow recommendations in specific test method documents (EC, 1991; 1992a; 1997a; b; USEPA 1994; 1995).

4.3 Water Quality

The quality of the water used for test organism culturing and for toxicity test dilution and control is extremely important. Water for these two uses should, ideally come from the same source (USEPA, 1994), however, in the case of test organism importation, this might not be feasible, and in most cases, is not possible. Most Environment Canada and USEPA test method documents outline the types of water recommended for test organisms culturing. Testing laboratories should ensure that the organism supplier maintains test organisms according to any recommended culture water conditions, specified in test method documents.

Where detailed culturing water quality characteristics are not specified in test method documents or where the organism supplier conducts water quality analyses on an infrequent basis, information (e.g., copy of supplier's water quality records) must be requested by the testing laboratory to ensure that culture water quality is sufficiently high to sustain satisfactory survival (EC, 1992a) and where applicable, growth and reproduction of the test species (EC, 1997a; b) (see Section 8.1).

Sources of water for culturing freshwater toxicity test organisms may be "uncontaminated" ground, surface, reconstituted, or, if necessary, may be dechlorinated municipal tap water. Culture water may also be prepared by diluting natural water with a high purity distilled or deionized water until a desired hardness is achieved (EC, 1992a; 1997a; b).

For marine toxicity test organisms, culture water may be natural seawater, or water made up from hypersaline brine derived from natural seawater, or artificial sea water prepared from reagent grade chemicals (GP2), or commercial artificial sea salts, as specifically recommended or required in respective toxicity test method documents (USEPA, 1994; 1995) and specific guidance on salinity adjustment prepared by Environment Canada (EC, 1997c).

Dechlorinated water is not recommended for use as culture and/or test water since its quality is often variable and it could contain unacceptably high concentrations of chlorine, chloramines, fluoride, copper, lead, zinc or other contaminants (EC, 1997a; b). In particular, dechlorinated municipal water should not be used for hatching fish embryos or rearing larvae since it is difficult to remove the last traces of residual chlorine and chlorinated organic substances which could be toxic to the larval fish (EC, 1992a). If, however, municipal drinking water is effectively dechlorinated to remove any harmful concentration of residual chlorine or chloramine (i.e., total residual chlorine (TRC) #0.002 mg/L, as recommended by the Canadian Council of Resource and Environment Ministers (CCREM, 1987) for the protection of freshwater aquatic life; and/or TRC #0.01 mg/L recommended by USEPA (1994; 1995) for marine organisms) and the water has been previously demonstrated to support acceptable test organism performance, then dechlorinated water can be used.

If reconstituted water is used for test organism culturing, it should be prepared according to the recipes provided in test-specific method documents. If reconstituted water is to be used as dilution and control water for the 7-day fathead minnow survival and growth test, then adult fathead minnows providing embryos for the test must be acclimated to that reconstituted water or to a similar water for at least the five days immediately before embryos are obtained for the test. The similar water could be: (a) a natural water with hardness within 20% of the reconstituted water; (b) a harder natural water adjusted downwards to the desired hardness with deionized water; or (c) a softer natural water adjusted upwards with the appropriate quantities and ratio of reagent-grade salts. Without such acclimation, the benefit of a standardized dilution water might be lost (EC, 1992a).

If surface water is used for culturing, it should be filtered through a fine mesh net (i.e., #60µm) to remove potential predators or competitors. Water that might be contaminated with pathogens may be sterilized by passing it through an ultraviolet sterilizer (EC, 1992a; USEPA, 1994; 1995).

Water varies substantially from facility to facility in critical factors which can strongly affect test organisms and test responses. Water supply within a facility might also change periodically (EC, 1998c). Regular chemical monitoring and assessment of the supplier's culture water should be conducted to demonstrate the maintenance of acceptable water quality. Variables such as pH, total organic carbon, suspended solids, dissolved oxygen, total dissolved gases, temperature, ammonia nitrogen, nitrite, metals, pesticides, as well as residual chlorine (if municipal water is used); hardness, alkalinity, and conductivity (if freshwater is used); and salinity (if saltwater is used), should be routinely monitored in the culture water. Analyses should be conducted as frequently as necessary to document water quality (e.g., quarterly) and/or whenever difficulty is encountered in meeting minimum acceptability criteria for test organism health and/or control performance (EC, 1992a; 1997a; b; USEPA, 1994; 1995). Some parameters, such as temperature, dissolved oxygen, pH and salinity should be measured more regularly (preferably daily). For each analytical method used, the detection limit should be appreciably (e.g., 3 - 10 times) below either (a) the concentration in the water, or (b) the lowest concentration that has been shown to adversely affect the survival, growth, or development of the test species or other sensitive freshwater animals (EC, 1992e).

Detailed records of analyses conducted on the supplier's water quality should be maintained for comparison to maximum contaminant acceptance criteria established by the laboratory or the supplier (EC, 1998c; USEPA, 1994). Culture water-quality-criteria established by the supplier and/or the testing laboratory must be based on any existing criteria specified in each test method document (EC, 1991). Water supply treatment by activated carbon filtration, particle filtration, ultraviolet irradiation, ion exchange or reverse osmosis filtration might be required to meet culture water criteria established by the laboratory or the supplier. Water treatments and their rationale should always be identified by the supplier (e.g., in a Standard Operating Procedure)(EC, 1992a; 1997a; b; USEPA, 1994; 1995).

The water within the culture chambers should be renewed routinely. This can be accomplished manually or automatically using suitable apparatus and techniques for continuous or intermittent renewal (EC, 1992a; 1997a; b; USEPA, 1995).

4.4 Food Quality

The nutritional quality of the food used in culturing and testing fish and invertebrates is an important factor in the quality of the toxicity test data. Problems with the nutritional suitability of the food will be reflected in the survival, growth, and reproduction of the test organisms in cultures and toxicity tests. For example, the inconsistent fatty acid content of brine shrimp nauplii, *Artemia* that are used to feed newly hatched fathead minnows, inland silversides and topsmelt (EC, 1992a; USEPA, 1994; 1995) lead the USEPA (1994; 1995) to require each new batch of *Artemia* cysts be evaluated for size and nutritional suitability against known suitable reference cysts by performing a side-by-side larval growth test using the "new" and "reference" cysts.

For the culturing of invertebrates (freshwater midges and amphipods), the choice and quantity of commercial fish food to be added to each culture chamber and feeding frequency is left to the discretion and experience of the laboratory personnel, although the Environment Canada methods do provide some guidance on feeding invertebrate cultures (EC, 1997a; b).

If a batch of any food being used to maintain test organism cultures (fish or invertebrates) is suspected of being defective, the performance of organisms fed with the new food should be compared with the performance of organisms fed with a food of known quality in side-by-side tests. Short-term sublethal tests should be conducted which will determine the effect of food quality on growth or reproduction of each of the relevant test species in culture, using four replicates with each food source (USEPA, 1994; 1995). Suppliers maintaining cultures or test organisms should ensure that the nutritional quality of the food is supporting acceptable survival, growth and reproduction of the test organisms.

The nutritive quality of the food being used to culture test organisms can be tracked by maintaining records of food sources and success of rearing organism cultures, and by sharing information on any measured nutritive values with other culture facilities or laboratories (EC, 1992a).

It is also desirable to assess toxic contaminants in fish and invertebrate food, but particularly dry flake food and brine-shrimp eggs. Toxicants of concern are bioaccumulative metals and pesticides. New batches of food used in culturing should be analyzed for toxic organics and metals or whenever difficulty is encountered in meeting minimum acceptability criteria for control survival and reproduction of growth (USEPA, 1994). Guidance on which foods are acceptable and which are not can often be obtained from the experience of other laboratories, and the measurements that they have done.

Food supplies should be routinely monitored for trace contaminants, and records maintained for comparison to minimum nutritional and maximum contaminant acceptance criteria (EC, 1998c). Criteria specified in test method documents (e.g., USEPA, 1994) should be considered in establishing laboratory criteria.

When foods other than the recommended food are used to maintain test organism, the foods must be evaluated. Side-by-side reference toxicity tests with the required food and the alternative food should be conducted. The results of these tests should meet the test acceptability requirements and should fall within the reference toxicant control limits for each species (see Section 8) if the food is to be considered adequate for culturing (USEPA, 1994; 1995).

4.5 Morbidity, Mortality and Treatment

Test organisms to be shipped to a testing laboratory for use in sublethal toxicity tests must be in good health, be disease free and show minimum mortality in culture tanks in the period of time preceding the test (i.e., as required in species-specific USEPA and EC test method documents). Test organisms must be carefully observed by the supplier, prior to shipment and by the testing laboratory, prior to use in a toxicity test, for a length of time which is reasonable for the species to ensure that they are disease free, behave normally, and in good physical condition (USEPA, 1994; 1995). Any organism(s) or batches of organisms where health is questionable must not be used in sublethal toxicity tests.

Treatment of test organisms with chemicals for disease prevention or control should be avoided, if possible. If the use of chemically-treated broodstock organisms cannot be avoided, a minimum amount of time (as specified in respective test method documents) must follow their treatment before their offspring can be used for tests (EC, 1992a).

In most toxicity testing laboratories, diseased animals are sacrificed immediately. Any test organisms recovered from disease or previous exposure in a test must not be used for further testing (EC, 1991).

5.0 Transport and Handling

All information needed to properly identify the test organisms transported to a testing laboratory must be provided with each shipment. Records accompanying each batch of test organisms must include, as a minimum: the quantity and source of test organisms in each shipment, supplier's name, age of the test organisms (i.e., date and time of hatching), date of arrival at the testing laboratory, arrival condition, and species identification. Data records of water quality and culture conditions should also be included.

The transportation of test organisms imported for immediate use in sublethal toxicity tests should be done in such a way to ensure that all required characteristics of the water and/or sediment, in which the organisms are shipped, are maintained during the transportation process.

To ensure that test organism health is maintained during transit, test organisms must be transported to the laboratory under proper temperature, dissolved oxygen, and where applicable, salinity conditions, as required by respective test method documents. For test organisms that will be used within the first 24 to 48 hours after arrival at the testing facility, the temperature of the water and/or sediment (and where applicable, the salinity) in which the organisms are being

transported must be maintained at or near the required test conditions since there is little, if any time for acclimation upon arrival at the testing laboratory (see Section 6.0). The temperature of the test organisms must not change by more than 3EC in any 24-hour period during transportation.[1] Shipping containers should be insulated to minimize changes in water temperature during transit and depending on transport conditions and time, it might be necessary during transit to chill or warm the contents of the transport container(s) (EC, 1992 a). If the test organisms cannot be delivered on the same day that they are shipped, the transport containers containing the test organisms should be stored in such a way that the temperature of the test organisms is maintained. The temperature of the test organisms must be recorded upon departure from the supplier's facility and upon arrival at the testing laboratory.

Water used for transporting animals must be well oxygenated (e.g., 90 - 100% saturation) before shipment (EC, 1997a; b). During transportation, the dissolved oxygen level must be maintained at or above the minimum level required for organism culturing in the respective test method documents (e.g., ∃4.0 mg/L for inland silversides; > 6.0 mg/L for topsmelt; ∃80% saturation for *Hyalella*, freshwater midges, and fathead minnows). Adequate dissolved oxygen can be maintained by replacing the air above the water in the bags with oxygen from a compressed gas cylinder, and sealing the bags. Another method commonly used to maintain sufficient dissolved oxygen during shipment over short distances is to aerate with an airstone which is supplied from a portable pump (USEPA, 1994).

There are an infinite number of ways in which organisms might be transported to a testing laboratory from a commercial supplier (e.g., plastic bags or large-mouth screw-cap (500 mL) plastic bottles, coolers). Organisms should be transported to the laboratory using the source of water in which the organisms have been reared. Where applicable (i.e., for invertebrates), a suitable substrate should be provided (EC, 1997a; b). Excessive crowding of animals during shipment must be avoided to minimize stress (i.e., organisms must not appear stressed) and prevent oxygen deficiency (i.e., oxygen levels must be at or above the minimum level required for organism culturing, see previous paragraph) in transit.[2] For further information on the specifics of transportation and handling of test organisms, laboratories should consult reputable organism suppliers.

Some sublethal test method documents (e.g., USEPA 7-day larval survival-and-growth tests with topsmelt and inland silverside) provide detailed guidance on importing test organisms for immediate use in toxicity tests.[3] In these cases, any required transport and handling conditions

[1]For overnight transportation, temperature recorders can be used to monitor the temperature of shipped organisms. The BOXCAR system is a computer programmable temperature recorder which provides a temperature profile of the shipped contents (G. van Aggelen, personal communication).

[2]Where 1- to 8-day old *Hyalella azteca* are being transported to a testing laboratory, there should be less than 500 organisms per liter of water in the shipping container. Also, organisms should not be in sealed shipping containers for >24 hrs.

[3]Silverside larvae are very sensitive to handling and shipping during the first week after hatching. For this reason, if organisms must be shipped to the test laboratory, it may be impractical to use larvae less than 11 days old because the sensitivity of younger organisms might result in excessive mortality during shipment. If organisms are to be shipped to a test facility, they should be shipped only as (1) early embryos, so that they hatch after arrival, or (2) after they are known to be feeding well on *Artemia*

must be met, and, where provided, recommendations should be followed.

Organisms should be handled as little as possible. When handling is necessary, it should be done as gently, carefully, and quickly as possible to minimize stress (USEPA, 1994; 1995). Organisms that are dropped or touch dry surfaces or are injured during handling must be discarded (EC, 1997a; b; USEPA, 1994; 1995).

6.0 Acclimation and Holding

In order to ensure that the quality of imported test organisms is maintained upon arrival at the testing laboratory, proper conditions for the housing, handling and care of the organisms should be established and maintained at the testing facility. Where specified in test method documents, culturing/holding conditions must be the same as test conditions with respect to critical factors such as temperature, light, and photoperiod.

Test organisms that will be used within the first 24 to 48 hours after arrival at the testing facility should be cultured by the supplier in water that has similar qualities (e.g., temperature, pH, alkalinity, hardness, salinity) as the laboratory's control/dilution water, if possible.

Fathead minnows imported for use in sublethal toxicity tests should not be subjected to water temperature changes of > 3EC per day, whereas topsmelt, inland silversides, *Hyalella azteca* and freshwater midges should not be subjected to temperature changes > 2EC per day. For salinity, topsmelt and inland silverside should not be exposed to changes of more than 3 g/kg in any 12-hour period (EC 1992a; 1997a; b; USEPA, 1994; 1995). Adequate acclimation periods and rates, where required in test method documents must be followed.

Upon arrival at the laboratory the organisms must be acclimated to the laboratory holding conditions as gradually as possible, so that the stress of acclimation does not influence test results. Organisms should be held in the water and sediment used in transit while temperature adjustments are made. Alternatively, they may be transferred to well-oxygenated water similar in character to that from which they originated (i.e., were cultured), at the temperature of the water in the shipping container. Gradual exposure of organisms to the testing laboratory's culture water is recommended in all cases, but especially in instances where there is a marked difference in quality (e.g., hardness, pH, conductivity) from that to which they were previously acclimated. This should minimize any stress on the animals caused by different water quality characteristics. A useful procedure for acclimating test organisms is to hold them for 2 h in a 50:50 mixture of culture water:test water, then for 2 h in a 25:75 mixture of culture water:test water, followed by a final 2h in 100% test water before their introduction to test chambers (Ingersoll and Nelson, 1990). Another useful procedure is to siphon off 20 to 30% of the shipping water every 2 to 3 hours and replacing it with laboratory control/dilution water,

---

nauplii (8 - 10 days of age). Larvae shipped at 8 - 10 days of age would be 9 to 11 days old when the test is started. Larvae that are hatched and reared in the test laboratory can be used at 7 days of age (USEPA, 1994).

ensuring that the temperature remains constant over this acclimation period. Acclimation should be started upon arrival at the testing facility. Ideally, test organisms (excluding fathead minnow larvae) should be in the laboratory's control/dilution water for 2 days prior to setting up a test.

7.0 Observations and Monitoring

As discussed in Section 4.3, the quality of water in culture chambers should be routinely monitored and recorded by the organism supplier. Any test method requirements for monitoring water quality characteristics and other culture conditions (e.g., temperature, salinity) must be followed by the supplier. Recommendations on culture observation and monitoring should also be carried out in accordance with the respective test method documents. As with culture water quality (see Section 4.3) acceptance criteria for environmental conditions such as temperature, light, water change rates and holding densities should be established based on conditions specified in the test method documents, and compliance should be monitored by the test-organism supplier on a regular basis.

The testing laboratory must establish an in-house system for evaluating the health (e.g., in the form of an SOP) of each shipment of organisms. The organisms should be observed carefully each day for signs of disease, contamination with other organisms, stress, physical damage, or mortality. Dead and abnormal organisms should be removed as soon as observed. It is not uncommon for some mortality (5 - 10%) to occur among imported test organisms during the first 48h in a holding tank because of individuals that refuse to feed (USEPA, 1994), therefore, it may be more appropriate to start monitoring pre-test mortality rates 24 hours after the organisms arrive at the testing facility (see Sections 4.5 and 8.1).

8.0 Quality Control

Performance-based criteria include: (a) those related to the survival and condition of cultured animals intended for use in the test (i.e., organism health criteria); (b) the performance of groups of animals in reference toxicity tests; and (c) criteria that must be met by control organisms during a test (i.e., test control performance) in order for that test to be valid. These performance-based criteria are often used to evaluate the suitability of the cultured organisms for tests, and the acceptability of test results, particularly where detailed organism culturing requirements are not specified in test method documents (EC, 1997a; b).

To be suitable for use in tests, supplier and/or in-laboratory cultures must have low mortalities, and the cultured organisms must appear healthy, exhibit normal feeding and other behavior, and be of the appropriate age and size before shipment to a laboratory and prior to initializing a test. Tests properly utilizing randomization procedures along with required and suggested quality control standards retain many built-in checks of typical organism response. A commonly used indicator of organism health includes individual test acceptability criteria. The acceptability of the culture should also be demonstrated by concurrent or ongoing tests using one or more reference toxicants.

8.1 Test-organism Health Criteria

Both subjective and objective indicators of organism health are available in the toxicity test method documents (USEPA, 1990). Test-organism health criteria, specified in individual test method documents must be followed. Indicators of good health include: normal activity and behavior, active feeding, and good color. A group of organisms must not be used for a test if they appear to be unhealthy, discolored, or otherwise stressed. Fish larvae must not be used if they are not actively feeding and if their swimbladders are not inflated. Mortality in a given batch of test organisms imported for immediate use in sublethal toxicity tests must not exceed 10% for larval fish and freshwater midges and 20% for *Hyalella azteca* in the 24-hour period immediately preceding the test. If a batch of organisms fails to meet these criteria, it should be humanely destroyed and a new batch obtained (OECD, 1992; EC, 1997a; b; USEPA, 1994; 1995). Organisms should be treated with due care and where applicable, humanely destroyed according to guidelines established by the Canadian Council on Animal Care (CCAC, 1984; 1993).

8.2 Reference Toxicity Testing

In addition to test-organism health criteria, the health of test organisms must be documented by periodic use of reference toxicity testing (USEPA, 1990). The routine use of a reference toxicant or toxicants is necessary to assess, under standardized test conditions the relative sensitivity of the organism culture(s), and the precision and reliability of data produced by the laboratory (EC, 1992a; 1997a; b). It is the responsibility of the testing laboratory to demonstrate the relative sensitivity of the individual batch of organisms used in a given toxicity test as well as its ability to obtain consistent precise results using one or more reference toxicants.

The quality of test organisms from outside sources must be verified by conducting reference toxicity tests. When a laboratory imports test organisms from an outside source for immediate use in sublethal toxicity tests, the sensitivity of each batch of test organisms must be determined with a reference toxicity test.[4] Reference toxicity testing procedures and the reference toxicant(s) used must meet the requirements outlined in specific test method documents. The test using the reference toxicant is most useful when carried out simultaneously with an actual toxicity test. Therefore, when toxicity tests are performed with effluents, receiving waters, chemicals, or sediments using test organisms obtained from outside the test laboratory, reference toxicity tests of the same type (except for sediment tests where the reference toxicity tests consist of 96-hour water-only tests) should be performed concurrently.

An organism supplier who maintains a culture of the test organisms should provide a warning

[4]This frequency of reference toxicity testing differs from the monthly reference tests specified in test method documents for organisms being cultured in-house, since culture conditions at a commercial supplier are not necessarily controlled/monitored as thoroughly as they would be if cultured in-house (i.e., in the testing facility). Also, transport and handling conditions will differ every time test organisms are shipped to the testing laboratory, thereby introducing unknown variability with each batch of test organisms received at the testing facility.

chart with data from at least the last five monthly sublethal toxicity tests using the same reference toxicant and an outline of the test conditions used in the reference testing.

If a routine reference toxicity test performed in the testing laboratory fails to meet test acceptability criteria (e.g., control survival, growth, reproduction), or falls outside the control limits of the warning chart, the laboratory should undertake an investigation into the cause of the test results falling outside the control limits (EC, 1990c). The investigation should include a review of the test system and further reference toxicity testing (if possible). The laboratory's investigation should also include, as a minimum, a review of: the quality data provided by the organism supplier; the methodology and conditions under which the organisms were cultured; and the shipping conditions. If the failed reference toxicity test was being performed concurrently with effluent, receiving water, chemical, or sediment toxicity tests, then the laboratory must report the failed reference toxicity test, the results of a repeated reference toxicity test (if performed), and the findings of the test system investigation.

8.3 Test Control Performance

The organisms imported to the laboratory for testing must also demonstrate "acceptable" performance in control solutions, at the end of each test conducted. Specific control acceptance criteria are outlined in each specific test method document. Control performance criteria might include maximum levels of test-organism mortality and abnormal behavior, and minimum levels of sublethal endpoints such as growth, reproduction, and egg fertilization.

If test organisms have been cultured in water that is different from test control/dilution water, most test methods (e.g., Environment Canada's 7-day survival and growth test using fathead minnows) require the inclusion of a second set of controls using culture water (EC, 1992a).

9.0 Reporting and Record-Keeping

Specific information on the test organisms used in a given sublethal toxicity test must be obtained from an organism supplier in accordance with the reporting requirements sections of USEPA and amended Environment Canada test method documents. The "Reporting Requirements" section of the amended Environment Canada biological test method documents includes two lists of information items relevant to test organisms. The first list that appears in the "Minimum Requirements for a Test-specific Report" section contains information items pertaining to test organisms that must be included in each test-specific report and therefore must be obtained from the test-organism supplier with every batch of test organisms. Information pertaining to test organisms listed in the "Additional Reporting Requirements" section must be obtained from the organisms supplier for inclusion in test-specific reports or for inclusion in the testing laboratory's records. For USEPA methods, Environment Canada has published minimum reporting requirement documents that outline the specific test-organism information that is required in each test report under Environmental Effects Monitoring programs for the pulp and paper and metal mining industries (EC, 1998d; e).

9.1 Minimum Reporting Requirements

In addition to the information required in the "Minimum Requirements for Test-specific Report" section of specific test method documents, the following list of items must be provided by the test organism supplier and/or obtained by the testing laboratory with every batch of test organisms shipped to the testing laboratory.

9.1.1 Test Organism

- name and address of test organism supplier
- age of test organism (i.e., date and time of hatching)

9.1.2 Culturing

- any deviations from specific test method "must" requirements for culturing of test organisms; facilities and apparatus used for culturing test organisms; and culture/holding water conditions
- any unusual appearance or behavior, and mortality rates of broodstock and test organisms observed by supplier; any disease occurrence

9.1.3 Transport and Handling

- temperature, DO, and salinity (where applicable) of water and/or sediment in which organisms are shipped, prior to being shipped and upon arrival at the testing laboratory
- any unusual appearance or behavior of test organisms upon arrival at the testing laboratory

9.1.4 Acclimation

- brief description of test organism acclimation rate and procedure (at the testing laboratory)

9.1.5 Quality Control

- data showing that required test organism health criteria were met
- any unusual appearance or behavior of organisms in the period immediately preceding a toxicity test (record the period of time relative to test initiation in which the observations were made); for fish larvae, brief confirmation of inflated swimbladders and normal feeding behavior

- data showing test organism mortality rates upon arrival of test organism at the test laboratory, as well as during the period (e.g., 24 hours) immediately preceding a toxicity test

- the results and duration of the toxicity test with a reference toxicant(s) for each batch of test organisms used in a sublethal toxicity test, together with the geometric mean value (± 2 SD) for the same reference toxicant(s) as derived at the test facility in previous tests

9.2 Additional Reporting Requirements

In addition to the information required in the "Additional Reporting Requirements" section of specific test method documents, the following list of items must be obtained from the organisms supplier and/or maintained by the testing laboratory on file for a period of five years.

9.2.1 Test Organism

- certification of species identification; taxonomic reference (citation and page) or name(s) of the taxonomic expert consulted in the identification of test organisms

- any health certificates

- origin of broodstock, for laboratory cultured test organisms

- detailed description of health care and disease treatment

- permit from provincial or regional authorities for procurement, shipment and transfer of test organisms, where required;

9.2.2 Culturing

- detailed description of supplier's culturing facilities and procedures

- acceptance criteria for organism food, water quality and other environmental conditions such as temperature, pH, light, DO, water change rates and holding densities

- results of food(s) evaluation, if food(s) differs from that recommended in test method document(s) or from food(s) previously evaluated and found to be acceptable

- results of chemical analyses conducted on culture/holding water, organism food and other environmental conditions

- any broodstock or test organisms treated with or exposed to chemicals (i.e., for disease treatment or preventative treatment)

9.2.3 Transport and Handling

- number of organisms, size, and volume of water and/or sediment per shipping container

9.2.4 Acclimation

- details of acclimation rate and procedures, including conditions under which organisms were held by the supplier, laboratory condition to which organisms were acclimated and the rate and method of acclimation

9.2.5 Observation and Monitoring

- nature and timing of all monitoring performed

- records of required monitoring of organism cultures (e.g., water quality characteristics, temperature, salinity)

- description of laboratory's system for evaluating the health of each shipment of test organisms

9.2.6 Quality Control

- a copy of the organism supplier's reference toxicant warning chart for test organisms maintained in culture; an outline of the test conditions used in the reference testing

10.0 Acknowledgments

This procedure document was written by J.A. Miller (Miller Environmental Sciences Inc., Stroud, ON). Mr. R.P. Scroggins (Environmental Technology Centre, Environment Canada, Gloucester, ON) acted as Scientific Authority and provided technical input and guidance throughout the work. Special acknowledgment is made of the many useful comments and suggestions provided by the following persons who reviewed the draft document: A. Borgmann (Environment Protection Branch, Ontario Region - EC, Toronto, ON) for her guidance on invertebrate transport and handling; J. Bruno (Pacific Environmental Science Centre, Pacific and Yukon Region - EC, North Vancouver, BC); K. Doe (Environmental Conservation Branch, Atlantic Region - EC, Moncton, NB); E. Jonczyk (Beak International Inc., Brampton, ON); C. McPherson (EVS Environment Consultants, North Vancouver, BC); J. Pickard (BC Research, Vancouver, BC); D. Rodrigue (Environment Canada, Gloucester, ON); J. Stewart (EVS Environment Consultants, North Vancouver, BC); B.J. Unis (HydroQual Laboratories Ltd., Calgary AB); and G. van Aggelen (Pacific Environmental Science Centre, Pacific and Yukon Region - EC, North Vancouver, BC.

11.0 References

CCAC (Canadian Council on Animal Care), "Guide to the Care and Use of Experimental Animals", Vol 2, Ottawa, ON (1984).

CCAC (Canadian Council on Animal Care), "Guide to the Care and Use of Experimental Animals", Vol 1, 2nd Edition, Ottawa, ON (1993).

CCREM (Canadian Council of Resource and Environment Ministers), "Canadian Water Quality Guidelines", Task Force on Water Quality Guidelines, Environment Canada, Ottawa, ON (1987).

EC (Environment Canada), "Biological Test Method: Acute Lethality Test Using Rainbow Trout", Conservation and Protection, Ottawa, ON, Report EPS 1/RM/9, 51p. (1990a).

EC (Environment Canada), "Biological Test Method: Acute Lethality Test Using *Daphnia* spp.", Conservation and Protection, Ottawa, ON, Report EPS 1/RM/11, 57p. (1990b).

EC (Environment Canada), "Guidance Document on Control of Toxicity Test Precision Using Reference Toxicants", Conservation and Protection, Ottawa, ON, Report EPS 1/RM/12, 85 p. (1990c).

EC (Environment Canada), "Quality Assurance Guidelines for Biology in Aquatic Environment Protection", (Prepared by Beak Consultants Ltd.) National Water Research Institute, Burlington, ON, (1991).

EC (Environment Canada), "Biological Test Method: Test of Larval Growth and Survival Using Fathead Minnows", Conservation and Protection, Ottawa, ON, Report EPS 1/RM/22, 70 p. (1992a), Amended in November, 1997.

EC (Environment Canada), "Biological Test Method: Acute Test for Sediment Toxicity Using Marine or Estuarine Amphipods", Conservation and Protection, Ottawa, ON, Report EPS 1/RM/26, 83 p. (1992b), Amended in October 1998.

EC (Environment Canada), "Biological Test Method: Fertilization Assay Using Echinoids (Sea Urchins and Sand Dollars", Conservation and Protection, Ottawa, ON, Report EPS 1/RM/27, 97 p. (1992c), Amended in November 1997.

EC (Environment Canada), "Biological Test Method: Growth Inhibition Test Using the Freshwater Alga *Selenastrum capricornutum*", Conservation and Protection, Ottawa, ON, Report EPS 1/RM/25, 41p. (1992d), Amended in November, 1997.

EC (Environment Canada), "Biological Test Method: Test of Reproduction and Survival Using the Cladoceran *Ceriodaphnia dubia*", Conservation and Protection, Ottawa, ON, Report EPS 1/RM/21, 73 p. (1992e), Amended in November, 1997.

EC (Environment Canada), "Biological Test Method: Test for Survival and Growth in Sediment Using Larvae of Freshwater Midges (*Chironomus tentans* or *Chironomus riparius*)", Conservation and Protection, Ottawa, ON, Report EPS 1/RM/32, 131 p. (1997a).

EC (Environment Canada), "Biological Test Method: Test for Survival and Growth in Sediment Using the Freshwater Amphipod *Hyalella azteca*", Conservation and Protection, Ottawa, ON, Report EPS 1/RM/33, 123 p. (1997b).

EC (Environment Canada), "Recommended Procedure for Adjusting Salinity of Effluent Samples for Marine Sublethal Toxicity Testing Conducted Under Environmental Effects Monitoring (EEM) Programs", Conservation and Protection, Ottawa, ON, 5 p. (1997c).

EC (Environment Canada), "Biological Test Method: Reference Method for Determining Acute Lethality of Sediment to Marine or Estuarine Amphipods", Conservation and Protection, Ottawa, ON, Report EPS 1/RM/35, 57p. (1998a).

EC (Environment Canada), "Biological Test Method: Toxicity Tests Using Early Life Stages of Salmonid Fish (Rainbow Trout)", 2nd Edition, Conservation and Protection, Ottawa, ON, Report EPS 1/RM/28, 102 p. (1998b).

EC (Environment Canada), "Technical Guidance Document for Aquatic Environmental Effects Monitoring Related to Federal *Fisheries Act* Requirements", Environment Canada, Hull, P.Q., 282 p. (1998c).

EC (Environment Canada), "Environment Canada Minimum Reporting Requirements - Inland Silverside Larval Growth and Survival Test", Conservation and Protection, Ottawa, ON, 2 p. (1998d).

EC (Environment Canada), "Environment Canada Minimum Reporting Requirements - Topsmelt

Larval Growth and Survival Test", Conservation and Protection, Ottawa, ON, 2 p. (1998e).

EC (Environment Canada), "Biological Test Method: Test for Measuring the Inhibition of Growth Using the Freshwater Macrophyte, *Lemna minor*", Conservation and Protection, Ottawa, ON, Report EPS 1/RM/37, 106 p. (1999).

Ingersoll, C.G. and M.K. Nelson, "Testing Sediment Toxicity with *Hyalella azteca* (Amphipoda) and *Chironomus riparius* (Diptera)", p. 93 - 109 in : *Aquatic Toxicology and Risk Assessment*, W.G. Landis and W.H. van der Schalle (eds). 13th Volume, ASTM SPT 1096, ASTM, Philadelphia, PA (1990).

QECD (Organization for Economic Co-Operation and Development), "The OECD Principles of Good Laboratory Practice", OECD Series on Principles of Good Laboratory Practice and Compliance Monitoring, Number 1, Environment Monograph No. 45, OECD, Paris, France, 29 p. (1992).

USEPA (United States Environmental Protection Agency), "Manual for the Evaluation of Laboratories Performing Aquatic Toxicity Tests", (Prepared by D.J. Klemm, L.B. Lobring, and W.H. Horning II), Office of Research and Development, Report EPA 600/4-90/031, USEPA, Cincinnati, Ohio, 109 p. (1990).

USEPA (United States Environmental Protection Agency), "Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Marine and Estuarine Organisms", 2nd Edition. D.J. Klemm, G.E. Morrison, T.J. Norberg-King, and W.H. Peltier (eds.), Office of Research and Development, Report EPA 600/4-91/003, USEPA, Cincinnati, OH, 439 p. (1994).

USEPA (United States Environmental Protection Agency), "Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to West Coast Marine and Estuarine Organisms", G.A. Chapman, D.L. Denton, and J.M. Lazorchak (eds.), Office of Research and Development, Report EPA 600/R-95-136, USEPA, Cincinnati, OH, 661 p. (1995).